Withania somnifera as a topical anti-melanoma agent

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DEDICATED TO MY DAUGHTER

TALIA RUTENDO MAPAMBA

"Our eyes are in front because it's more important to look ahead than to look back. Don't dwell on things in the past. Learn from them and keep MOVING forward"

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Abbreviations

2D	two dimensional
3D	three dimensional
API	active pharmaceutical ingredient
ANOVA	analysis of variance
CMM	cutaneous malignant melanoma
DLS	dynamic light scattering
DMEM	dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
ECM	extra cellular matrix
ED	epidermis-dermis
EE	encapsulation efficiency
FACS	fluorescence activated cell sorter
FBS	foetal bovine serum
FSC	forward scatter
GRAS	generally regarded as safe
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
ICH	international conference on harmonisation
KH_2PO_4	potassium orthophosphate dihydrogen
LLOQ	lower limit of quantification

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N50	50% ethanol extract
NaOH	sodium hydroxide
ND	not detected
NE	ethanol extract niosomes
NLC	nanostructured lipid carrier
NMR	nuclear magnetic resonance spectroscopy
NRF	national research foundation
NW	water extract niosomes
PBS	phosphate buffered salin
PCS	photon correlation spectroscopy
PDI	polydispersity index
PI	propidium iodide
PS	phosphatidylserine
PTFE	polytetrafluoroethylene
RSD	relative standard deviation
S50	50% ethanol extract SLNs
SANBI	South African National Biodiversity Institute
SCE	stratum corneum-epidermis
SD	standard deviation
SE	ethanol extract SLNs
SLM	solid lipid microparticles
SLN	solid lipid nanoparticle

SSC	side scatter
STD	standard
SW	water extract SLNs
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotide transferase dUTP nick end labelling
WFA	withaferin A
WNA	withanolide A
WS50	*Withania somnifera* 50% ethanol extract
WSE	*Withania somnifera* ethanol extract
WSW	*Withania somnifera* water extract
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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Abstract

In recent years people have become more attuned to the use of natural products for medicinal purposes as the belief is that natural products have fewer side effects. While it is true that natural products have medicinal value it is wise to have scientific evidence backing the suggested medicinal uses of natural products in order to ensure safe and effective use of these products. In some cases the natural product is not used in its natural form but it becomes the source of a lead compound for drug synthesis (Rishton, 2008:43D; Cragg & Newman, 2013:3671). There are numerous anti-cancer compounds of natural origin that are currently on the market or being investigated for use in different cancers. In this study the anti-melanoma activity of *Withania somnifera*, which has been reported to have anti-cancer activity was investigated.

W. somnifera is a medicinal plant commonly used in Ayurveda to treat different ailments within the home. The plant extract and compounds originating from the plant have been reported to be active against breast cancer, colon cancer, pancreatic cancer, melanoma, arthritis, hypertension and diabetes (Malik *et al.*, 2009:1508; Nagella & Murthy, 2010:6735; Samadi *et al.*, 2012; Vel Szic *et al.*, 2014:1179). It was decided in this study to investigate and compare the anti-cancer activity of crude plant extracts and that of two active metabolites present in the plant. The metabolites that were chosen were withaferin A and withanolide A. Withaferin A has been reported to be a very potent bioactive constituent of *W. somnifera* hence its use in this study (Kulkarni & Dhir, 2008:1095). The influence of solid lipid nanoparticles (SLNs) and niosomes on delivery and anti-cancer efficacy of the *W. somnifera* extracts was then investigated. Nanoformulations have been said to have the ability to increase the intracellular concentrations of active pharmaceutical ingredients (APIs) in cancer cells thus in turn enhancing the efficacy of anti-cancer compounds (Sanna *et al.*, 2013a:144).

Soxhlet extraction of *W. somnifera* leaves was done using water, ethanol and 50% ethanol as solvents to come up with three different crude extracts. Each extract contained both withaferin A and withanolide A at different percentages. The extracts were encapsulated in niosomes and SLNs then the formulations were used to determine release and skin diffusion properties. A three month stability study was conducted on the formulations at room temperature in order to determine any potential stability issues. With respect to the *in vitro* efficacy studies, both the pure compounds and crude extracts were utilised for the treatments so as to see any differences between the use of pure compounds and a blend of compounds. Cytotoxicity and apoptosis assays were conducted on human cutaneous melanoma cells (A375 cells) and keratinocytes (HaCaT cells) and the selectivity of *W. somnifera* for cancerous as opposed to normal cells was determined. Selected *in vitro* efficacy studies were also done using SLN formulations of the plant extracts in conventional two-dimensional (2D) cell culture and in three-dimensional (3D) cell

culture. For the 3D cell culture A375 cells were seeded in Matrigel® and treated with particular treatments then assessed for cytotoxicity and apoptosis. Therefore in this study the cytotoxic effects of *W. somnifera* crude extracts were compared with that of two pure metabolites in 2D and 3D cell culture. Additionally the influence of SLNs on the cytotoxicity was also investigated.

All the extracts that were prepared contained withaferin A and withanolide A with different percentage compositions and this probably influenced the differences that were seen with the Franz cell diffusion studies and efficacy results. Withaferin A and withanolide A were both released from the ethanol extract niosomes, 50% ethanol extract niosomes and all the SLN formulations. This meant that the two compounds were available for diffusing into or through the skin from the formulations in question. None of the compounds however were detected as having diffused through the skin from the formulations. Tape-stripping results revealed that withaferin A permeated into the stratum corneum-epidermis from all the formulations except the water extract niosomes. However, only the 50% ethanol extract SLNs managed to achieve deeper penetration of withaferin A to the epidermis-dermis. On the other hand all the SLN formulations resulted in withanolide A reaching both stratum corneum-epidermis and the epidermis-dermis but only the 50% ethanol extract niosomes succeeded in bringing about the permeation of withanolide A into the stratum corneum-epidermis. Stability testing of these formulations revealed that the formulations were not very stable possibly due to the presence of unidentified compounds in the crude extracts and the effects of lyophilisation without the use of a lyoprotectant (Hua *et al.*, 2010:8).

In vitro efficacy studies showed that withaferin A was toxic to melanoma cells and the presence of withanolide A enhanced the anti-melanoma effects of withaferin A. Withaferin A and withanolide A were generally more active than the crude extracts with respect to inducing apoptosis in melanoma cells. Selectivity for inducing apoptotic and necrotic cell death in melanoma cells versus keratinocytes was observed for all the treatments. The SLNs however did not have a notable influence on the apoptosis inducing effects of the plant extracts. Deoxyribonucleic acid (DNA) fragmentation, caspase 3/7 activation, increase in membrane permeability and a decrease in mitochondrial membrane potential were taken to indicate the occurrence of apoptosis in the 2D assays. For the 3D assays with the plant extracts and SLN formulations the externalisation of phosphatidylserine and reduced uptake of CellTracker™ Red dye were indicators of the occurrence of apoptosis or cell death.

In this study it was revealed that although *W. somnifera* crude extracts have activity against melanoma, withaferin A and a withaferin A/withanolide A combination had the greatest activity and maintained selectivity for melanoma cells over keratinocytes. The SLNs displayed superior ability to carry the marker compounds into the skin layers but not through the skin. This was favourable as dermal delivery was desired and not systemic delivery. The results of this study

support the further study of *W. somnifera* and its constituent compounds for use in the topical treatment of melanoma.

Keywords: *Withania somnifera*, withaferin A, withanolide A, topical delivery, apoptosis, melanoma, Matrigel®, stability, solid lipid nanoparticles, niosomes

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Uittreksel

Mense raak al hoe meer gesteld op die gebruik van natuurlike produkte vir medisinale gebruike omdat hulle glo dat hierdie produkte minder newe-effekte het. Hoewel dit waar is dat natuurlike produkte medisinale waarde het, is dit raadsaam om wetenskaplike bewyse te hê om die voorgestelde medisinale gebruike daarvan te ondersteun om so veilige en effektiewe gebruik van hierdie produkte te verseker. In sommige gevalle word die natuurlike produk nie in sy natuurlike vorm gebruik nie, maar word dit die hoofverbinding vir geneesmiddelsintese (Rishton, 2008:43D; Cragg & Newman, 2013:3671). Daar is verskeie teenkankerverbindings van natuurlike oorsprong wat tans op die mark is of ondersoek word vir gebruik teen verskillende kankers. Hierdie studie het die antimelanoomwerking van *Withania somnifera*, wat na berig word teenkankerwerking het, ondersoek. Die afsetting van whitaferien A en whitanolied A in die vel uit *W. somnifera* niosome en soliede lipiednanodeeltjies is ook ondersoek.

W. somnifera is ’n medisinale plant wat algemeen in die Ayurveda gebruik word om verskillende kwale tuis te behandel. Die plantekstrak en verbindings wat uit die plant voortkom is na wat berig word aktief teen borskanker, kolonkanker, pankreatiese kanker, melanoom, artritis, hoë bloeddruk en diabetes. Verder het *W. somnifera* teenverouderings-, antibakteriële, adaptogeniese, verjongings- en immunostimulatoriese eienskappe (Malik et al., 2009:1508; Nagella & Murthy, 2010:6735; Samadi et al., 2012; Vel Szic et al., 2014:1179). Hierdie studie ondersoek en vergelyk die teenkankerwerking van rou plantekstrakte en twee aktiewe metaboliete wat in die plant teenwoordig is. Die metaboliete wat gekies is, is whitaferien A en whitanolied A. Whitaferien A word beskou as 'n baie kragtige bioaktiewe bestanddeel van *W. somnifera*, vandaar die gebruik daarvan in die studie (Kulkarni & Dhir, 2008:1095).Die invloed van soliede lipiednanodeeltjies (SLNs) en niosome op die lewering en teenkankerdoeltreffendheid van die *W. somnifera* ekstrakte is daarna ondersoek. Nanoformulerings het na bewering die vermoë om die intrasellulêre konsentrasies van aktiewe farmaseutiese bestanddele (AFB) in kankerselle te verhoog, wat die doeltreffendheid van teenkankerverbindings verbeter (Sanna et al., 2013:144).

Soxhlet-ekstraksie van *W. somnifera* blare is gedoen met water, etanol en 50% etanol as oplosmiddels om drie verskillende rou ekstrakte te vorm. Elke ekstrak het beide whitaferien A en whitanolied A in verskillende persentasies bevat. Die ekstrakte is geënkapsuleer in niosome en SLN en daarna is die formulerings gebruik om die vrystellings- en veldiffusie-eienskappe te bepaal. ’n Drie-maande stabiliteitstudie is uitgevoer op die formulerings teen kamertemperatuur om te bepaal of daar enige potensiële stabiliteitsprobleme is. Met betrekking tot die *in vitro* doeltreffendheidstudies, is beide die suiwer verbindings en rou ekstrakte gebruik vir die behandelings om te sien of daar enige verskille is tussen die gebruik van suiwer verbindings en ’n samestelling van verbindings. Sitotoksisiteit en apoptose-essais is gedoen op menslike kutane

melanoomselle (A375 selle) en keratinosiete (HaCaT selle) en die selektiwiteit van *W. somnifera* vir kankerselle teenoor normale selle is vasgestel. Geselekteerde *in vitro* doeltreffendheidstudies is ook gedoen met die gebruik van SLN formulerings van die plantekstrakte in konvensionele twee-dimensionele (2D) selkulture en in een drie-dimensionele (3D) selkultuur. Vir die 3D-selkultuur is A375-selle in Matrigel® ge-ent en behandel met spesifieke behandelings, en daarna geassesseer vir sitotoksisiteit en apoptose. Die sitotoksiese uitwerking van *W. somnifera* rou ekstrakte is vergelyk met diè van twee suiwer metaboliete in 2D- en 3D-selkulture. Die invloed van SLN op die sitotoksisiteit is bykomend ondersoek.

Al die ekstrakte wat voorberei is, het whitaferien A en whitanolied A bevat in verskillende persentasiesamestellings, en dit het waarskynlik die verskille met die Franz-sel diffusiestudies en doeltreffendheidstudies beïnvloed. Whitaferien A en whitanolied A is beide vrygestel uit die etanolekstrak niosome, 50% etanolekstrak niosome en al die SLN formulerings. Dit beteken dat die twee verbindings beskikbaar was vir diffusie in of deur die vel vanuit die formulerings. Nie een van die verbindings het egter deur die vel gediffundeer uit die formulerings nie. Bandstropingresultate het getoon dat whitaferien A deurgedring het tot binne die stratum corneum van die epidermis uit al die formulerings behalwe die waterekstrak niosome. Slegs die 50% etanolekstrak SLN het egter dieper penetrasie van whitaferien A tot in die epidermis-dermis bereik. Daarteenoor het al die SLN formulerings tot gevolg gehad dat whitanolied A beide die stratum corneum van die epidermis en die epidermis-dermis bereik, maar net die 50% etanolekstrak niosome het die deurdringing van whitanolied A tot by die stratum corneum van die epidermis suksesvol teweeg gebring. Stabiliteitstoetse van hierdie formulerings het getoon dat hulle nie baie stabiel is nie, moontlik as gevolg van die teenwoordigheid van ongeïdentifiseerde verbindings in die rou ekstrak en die invloed van vriesdroging sonder die gebruik van ’n liobeskermer.

In vitro doeltreffendheidstudies het gewys dat whitaferien A toksies is vir melanoomselle en dat die teenwoordigheid van whitanolied A die antimelanome uitwerking van whitaferien A verhoog het. Whitaferien A en whitanolied A was oor die algemeen meer aktief as die rou ekstrakte met betrekking tot die indusering van apoptose in melanoomselle. ’n Voorkeur vir die indusering van apoptotiek en nekrotiese seldood in melanoomselle teenoor keratienosiete is in al die behandelings opgemerk. Die SLNs het egter nie ’n merkbare invloed op die apoptose-induserende uitwerking van die plantekstrak gehad nie. Deoksiribonukleïensuur fragmentasie (DNS), kaspase 3/7 aktivering, verhoogde membraandeurlaatbaarheid en ’n daling in mitochondriale membraanpotensiaal is gesien as aanduidend van apoptose in die 2D-ontledings. Vir die 3D-essais met plantekstrakte en SLN formulerings, was die eksternalisering van fosfatidielserien en verminderde opname van CellTracker™ rooi kleursel aanduiders van die voorkoms van apoptose of seldood.

Dit blyk uit die studie dat alhoewel *W. somnifera* rou ekstrakte aktief is teen melanoom, die whitaferien A en ’n whitaferien A/whitanolied A-kombinasie die meeste aktiwiteit en grootste selektiwiteit vir melanoomselle teenoor keratienosiete getoon het. Die SLN het 'n beter vermoë gehad om die merkerverbindings na binne die vellae te dra, maar nie deur die vel. Dit is verkieslik aangesien vel-lewering wenslik is, en nie sistemiese lewering nie. Die bevindinge van hierdie studie ondersteun verdere studie van *W. somnifera* en sy verbindings vir gebruik in die topikale behandeling van melanoom.

Die studie bied nuwe inligting aangesien dit volgens die literatuur die eerste keer is wat *W. somnifera* rou ekstrakte in niosome en soliede lipiednanodeeltjies geënkapsuleer is vir topikale lewering. Verder is die gekombineerde gebruik van whitaferien A en whitanolied A as anitmelanoomagente, die gebruik van *W. somnifera* rou ekstrak geënkapsuleer in SLN en die ondersoek na die antimelanoom doeltreffendheid van *W. somnifera* teen melanoomselle (A375) in Matrigel®, nuut.

Sleutelwoorde: *Withania somnifera,* whitaferien A, whitanolied A, plaaslike lewering, apoptose, melanoom, Matrigel®, stabiliteit, soliede lipied nanodeeltjies, niosome.

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Foreword

W. somnifera is a medicinal plant known to have anti-melanoma properties and that was the focus of the study. This study aimed to develop a *Withania somnifera* topical formulation with potential for use in the treatment of melanoma and to determine the *in vitro* anti-melanoma efficacy of the crude extracts and pure metabolites.

This thesis is compiled in the article format and it consists of introductory chapters, a review article (chapter two), two full length research articles (chapters three and four), a concluding chapter and appendices. The review article entitled "Review of Natural Compounds for Potential Skin Cancer Treatment" has been published in the journal "Molecules". This review article is included as chapter two and serves as the literature review chapter of this thesis. Also included are two full length articles for submission to "Pharmacognosy Magazine" and in "PLoS-ONE". Complete guidelines for submission have been included in the thesis as Appendices F to G. Moreover, detailed experimental methods and data are presented as Appendices A to E.

Throughout my PhD studies I learnt to enjoy every moment and give thanks at all times regardless of my present situation. This experience has taught me to think on my feet and think outside the box. I am very grateful to have reached this stage in my life and I look forward to the different experiences that are still to come.

Chapter 1: Introduction and problem statement

In recent years there has been renewed interest in the use of natural products such as plants in the treatment of various cancers. This study focuses on the potential of *Withania somnifera* as an anti-melanoma agent. *W. somnifera* is a plant popularly known as Ashwagandha in the Ayurveda system of traditional Indian medicine. This plant is known to have anti-cancer, anti-ageing, anti-arthritic, anti-hypertensive, anti-diabetic, adaptogenic, rejuvenating and immunostimulatory properties (Malik *et al.*, 2009:1508; Nagella & Murthy, 2010:6735; Samadi *et al.*, 2012; Vel Szic *et al.*, 2014:1179). Various studies have been conducted to investigate the medicinal properties of *W. somnifera.* This study pays particular attention to *W. somnifera* crude extracts and two metabolites of the plant. The two marker compounds that were used throughout this study are withaferin A and withanolide A. The topical delivery and anti-melanoma activity of *W. somnifera* crude extracts were also investigated in this study. Water, ethanol and 50% ethanol crude extracts were used throughout the study. Three different extracts were used in order to explore any differences in activity and skin deposition that may occur due to variations in composition of the different extracts. Most studies in literature focus on a single extract type, which results in incomplete results, whereas in this study, as the focus is on cutaneous melanoma, three extracts were considered for topical delivery, after which the superior extract formulation was investigated further for anti-melanoma efficacy.

Nanoformulations are able to increase the intracellular concentrations of active pharmaceutical ingredients (API), consequently resulting in enhanced efficacy (Sanna *et al.*, 2013:144). Withaferin A was encapsulated in niosomes for cancer treatment by Sheena *et al.* (1998:47) in a previous study. They found that niosomal encapsulation increased the *in vivo* anti-cancer efficacy of withaferin A on mice transfected with Erlich ascites. Therefore, to investigate the influence of drug delivery vesicles on drug efficacy, *W. somnifera* crude extracts were encapsulated in niosomes and SLNs for potential use in the treatment of melanoma. The current study differs from the study by Sheena *et al.* in that experiments were conducted *in vitro* on melanoma cells to avoid unnecessary animal testing.

Cutaneous malignant melanoma (CMM) is a very aggressive form of skin cancer that originates from the pigment producing cells of the skin, melanocytes (Weiner & Yoon, 2010:313). Most deaths due to skin cancer are due to melanoma as it has a high tendency to metastasise to other regions of the body (de Gruijl, 1999:2004). CMM must be treated in its early stages before it moves deeper into the skin tissues in preparation for metastasis. Skin cancers are usually treated by surgical excision, but in some instances the location of the lesion or patient health deter the performance of surgery. In such cases it may therefore be prudent to use an alternative treatment method such as topical therapy (Telfer *et al.*, 2008:36). Topical therapy is advantageous in that

it is relatively simple, painless, avoids first pass metabolism, the site of action (skin) is directly accessible and it allows for patient autonomy (Cleary, 1993; Naik *et al.*, 2000:319). Topical therapy is also advantageous in that it minimises the systemic effects of APIs, resulting in a safer side-effect profile. Imiquimod is used topically for treatment of CMM (Bichakjian *et al.*, 2011:1041), but this treatment comes with potential side effects such as inflammation, erythema, crusting, burning, pain and scaling (Narayan *et al.*, 2012:164). For this reason there is ongoing research to explore the use of alternative topical anti-melanoma agents that are safer and effective. Topical treatment of melanoma using diphencyprone was found to result in clearance of melanoma in 46% of patients while it resulted in 90% complete or partial response in those with thinner lesions (Damian *et al.*, 2014:308). This highlights that there is potential for topical treatment of melanoma. The goal of this study was to explore the topical anti-melanoma potential of *W. somnifera*. Therefore, *W. somnifera* crude extracts were assessed for their anti-melanoma efficacy and topical delivery capabilities.

In the case of CMM, topical treatment is advantageous because the condition under investigation presents on and within the skin. For this reason the medicinal compounds can easily reach their site of action and exert the anti-melanoma actions. The skin is relatively impermeable to most compounds, but techniques to enhance the penetration of medicinal substances through the skin's impervious barrier have been investigated and applied. Drug delivery vehicles are known to aid the penetration of APIs into the skin and to coat the API, protecting the API from premature degradation and the patient from any unfavourable properties of the API (e.g. taste, stinging sensation or irritation) (Honeywell-Nguyen & Bouwstra, 2005:68). Various nano-formulations have been formulated for application in transdermal drug delivery. Solid lipid nanoparticles (SLNs) and niosomes were used as drug delivery vehicles in this study.

In order to enhance the permeation of compounds through the skin barrier, the use of drug delivery vesicles as permeation enhancers was investigated. Drug delivery vesicles coat active pharmaceutical ingredients (APIs) to protect them from premature degradation. Delivery vesicles also protect the patient from any unfavourable properties of the API, such as a stinging sensation or irritation (Honeywell-Nguyen & Bouwstra, 2005:68). Solid lipid nanoparticles (SLNs) and niosomes were used as drug delivery vehicles in this study.

Niosomes are non-ionic surfactant vesicular drug delivery systems of which the main constituents are non-ionic surfactants and cholesterol (Rajera *et al.*, 2011:952). Some researchers have found niosomes to be safe, effective and permeation-enhancing drug delivery vehicles for dermal drug delivery (Paolino *et al.*, 2008:238; Junyaprasert *et al.*, 2012:309; Marianecci *et al.*, 2012:22; Yeh *et al.*, 2013:243). Other studies also reported that niosomes enhanced the cytotoxic efficacy of 5-fluorouracil (Paolino *et al.*, 2008:238) and plumbagin (Oommen *et al.*, 1999:283). Solid lipid nanoparticles, on the other hand, are oil in water emulsion systems composed of a solid oil phase.

The presence of the solid lipid results in prolonged and targeted release of encapsulated APIs into the skin (Godin & Touitou, 2012:523). Solid lipid nanoparticles have been investigated in the treatment of melanoma and it was found that the SLN encapsulated APIs were effective anti-metastatic and anti-tumour agents (Mosallaei *et al.*, 2013:1998; Athawale *et al.*, 2014:239). The use of SLNs in topical drug delivery is ideal as they have been found to increase topical drug delivery while reducing delivery of APIs to the systemic circulation (Madan *et al.*, 2014:63). Niosomes and SLNs were therefore used in this study due to their dermal permeation-enhancing and skin-deposition effects.

Though few, there are increasing reports of studies on the encapsulation of phytochemicals in drug delivery vesicles (Sanna *et al.*, 2013:142; Wang *et al.*, 2014:364; Venturini *et al.*, 2015:36). Encapsulation of crude extracts, however, is rather complex as it entails encapsulating a complex mixture of compounds (the crude extract) that cannot be entirely characterised. In this study the encapsulation of withaferin A and withanolide A in the crude extracts was taken as representative of crude extract encapsulation. This is important because the different compounds in the crude extracts have unique properties and their encapsulation subsequently differs. Encapsulation of APIs in nanovesicles has also been found to improve the efficacy of anti-cancer agents (Oommen *et al.*, 1999:283; Paolino *et al.*, 2008:238; Mosallaei *et al.*, 2013:1998; Athawale *et al.*, 2014:239) and this was also investigated in this study.

When apoptosis (programmed cell death) is not well regulated, this may result in cancer, auto-immune diseases, reperfusion injury and immunodeficiency diseases (Barisic *et al.*, 2003:151). Cells going through apoptosis have specific and characteristic metabolic, biochemical and molecular features that can be investigated using colorimetry, flow cytometry or fluorescence microscopy. Cancer cells die via different pathways, so a single *in vitro* cell culture study cannot give conclusive results and further *in vitro* or *in vivo* testing must be done to confirm any obtained results. In this project various *in vitro* apoptosis and cytotoxicity assays were done on melanoma cells and keratinocytes in two-dimensional (2D) and three-dimensional (3D) cell culture to confirm the apoptosis-inducing properties of *W. somnifera*. The 3D matrix allowed cells to interact with each other similar to how they normally interact in the human body (Li *et al.*, 2011; Mathes *et al.*, 2014:96).

This study mainly focuses on partially filling the gap in knowledge with respect to the topical delivery and anti-melanoma efficacy of *W. somnifera* extracts and metabolites. The aim of this study was to develop a potential *W. somnifera* topical formulation (based on niosomes and SLNs) for the treatment of melanoma and to determine the *in vitro* anti-melanoma efficacy of *W. somnifera* metabolites, crude extracts and formulations. There is no literature available on the SLN encapsulation and topical delivery of *W. somnifera* and therefore one of the sub-aims was the formulation of *W. somnifera* crude extract niosomes and SLNs and the topical delivery of the

formulations thereafter. Other sub-aims were assessing the anti-melanoma efficacy of the crude extracts and compounds, investigating the crude extract and metabolite specificity in 2D and 3D environment, and lastly, probing for the cytotoxic and apoptotic pathways of *W. somnifera* in melanoma cells. This is the first study to comparatively investigate the anti-melanoma efficacy of *W. somnifera* crude extracts alongside the metabolites (WFA and WNA) in A375 human melanoma cells and HaCaT human keratinocytes. The influence of nanovesicles on the anti-melanoma activity of the crude extracts in 2D and 3D was also investigated. This is the first study to assess *W. somnifera* activity in a 3D environment.

In order to achieve the above aims, the following objectives were set:

- Preparing aqueous, ethanol and 50% ethanol crude extracts of *W. somnifera* leaves using the soxhlet extraction method;
- Drying of crude extracts to result in dry extracts for use throughout the study;
- Developing a high performance liquid chromatography (HPLC) method for use in chemical finger-printing of plant extracts, skin diffusion studies and formulation stability studies;
- Using nuclear magnetic resonance (NMR) spectroscopy for chemical finger-printing of crude extracts and identification of marker molecules;
- Formulation of SLNs and niosome formulations for topical delivery of crude plant extracts;
- Assessment of physicochemical properties of crude extract formulations, namely the zeta-potential, droplet size, polydispersity index, pH and encapsulation efficiency;
- Performing a three-month stability study to determine the stability of formulated niosomes and SLNs;
- Performing membrane release studies to determine whether withaferin A and withanolide A are released from the crude extracts in the SLN and niosome formulations;
- Carrying out Franz cell diffusion studies to investigate the diffusion of withaferin A and withanolide A into and through the skin;
- Performing cell viability tests, apoptosis assays and cytotoxicity assays in conventional two-dimensional cell culture to determine the anti-cancer efficacy of the plant extracts and their action pathways;
- Performing cytotoxicity and apoptosis assays on A375 human melanoma cells in a 3D Matrigel® environment to investigate the influence of a 3D environment on the effects of the *W. somnifera* extracts;
- Investigating the influence of SLNs on the cytotoxicity and apoptosis-inducing effect of *W. somnifera* crude extract.

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Chapter 2: Review article published in Molecules

Chapter two is written in the form of a review article and it serves as the literature review chapter of the thesis. It includes a review of the use of plants in the topical treatment of melanoma. This chapter was published in the journal “Molecules” (doi:10.3390/molecules190811679) in 2014. The formatting of chapter two differs from that of the rest of the thesis as it is written in US English and according to the authors guidelines. The complete authors guide is included in the thesis as Appendix F.

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Review

Review of Natural Compounds for Potential Skin Cancer Treatment

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Abstract: Most anti-cancer drugs are derived from natural resources such as marine, microbial and botanical sources. Cutaneous malignant melanoma is the most aggressive form of skin cancer, with a high mortality rate. Various treatments for malignant melanoma are available, but due to the development of multi-drug resistance, current or emerging chemotherapies have a relatively low success rates. This emphasizes the importance of discovering new compounds that are both safe and effective against melanoma. *In vitro* testing of melanoma cell lines and murine melanoma models offers the opportunity for identifying mechanisms of action of plant derived compounds and extracts. Common anti-melanoma effects of natural compounds include potentiating apoptosis, inhibiting cell proliferation and inhibiting metastasis. There are different mechanisms and pathways responsible for anti-melanoma actions of medicinal compounds such as promotion of caspase activity, inhibition of angiogenesis and inhibition of the effects of tumor promoting proteins such as PI3-K, Bcl-2, STAT3 and MMPs. This review thus aims at providing an overview of anti-cancer compounds, derived from natural sources, that are currently used in cancer chemotherapies, or that have been reported to show anti-melanoma, or anti-skin cancer activities. Phytochemicals that are discussed in this review include flavonoids, carotenoids, terpenoids, vitamins, sulforaphane, some polyphenols and crude plant extracts.

Keywords: anti-cancer; melanoma; plant; natural; dietary; phytochemical

Abbreviations

AMPK, adenosine monophosphate activated protein kinase; Bax, Bcl-2 associated X protein; BCC, basal cell carcinoma; Bcl-2, B cell lymphoma 2; CAM, complementary and alternative medicine; CDK, cyclin dependent kinase; Cip, CDK interacting protein; CMM, cutaneous malignant melanoma; COX-2, cyclooxygenase 2; CXCR4, C-X-C receptor 4; DNA, deoxyribonucleic acid; EGCG, epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; FADD, Fas-Associated protein with Death Domain; FDA, Food and Drug Administration; GM-CSF, *granulocyte-macrophage colony-stimulating factor*; IC_{50}, *50% inhibitory concentration*; IFN, interferon; IGF-1R, Type 1 insulin like growth factor receptor; IL, interleukin; lncRNA, long non-coding RNA; MAPK, mitogen activated protein kinase; MMP, matrix metalloproteinase; mPTP, mitochondrial permeability transition pore; mRNA, m-ribonucleic acid; NCCAM, National Centre for Complementary and Alternative Medicine; NCI, National Cancer Institute; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NIH, National Institute of Health; PCNA, proliferating cell nuclear antigen; PDT, photodynamic therapy; PI3K, phosphoinositide-3 kinase; RAR, retinoic acid receptor; ROS, reactive oxygen species; SCC, squamous cell carcinoma; STAT-3, *signal transducer and activator of transcription 3*; TNF-R, tumor necrosis factor receptor; TRAIL-R, TNF-related apoptosis inducing ligand receptor; USA, United States of America; UV, ultra-violet; VEGF, vascular endothelial growth factor.

1. Introduction

Cancer is considered as a major cause of mortality worldwide, while the incidence of skin cancer is ever increasing in countries where such tumors are prevalent. Between 1970 and 2007, among all of the documented cancers in Canada, melanoma had the second highest increase in mortality rate [1] and it is estimated that skin cancer is the most common form of cancer in the United States of America (USA). According to Erb *et al.* [2], skin cancers are the most frequently diagnosed malignancies in Caucasians worldwide, whilst their incidence keeps increasing, due to increased exposure to ultra-violet (UV) radiation. Cancer of the skin is characterized by an imbalance towards too little apoptosis, or too much cell proliferation and survival in the epidermis [3]. Although UV radiation is the leading cause of skin cancer, other causative agents include viruses, mutagens in food, mutagens in chemicals and genetic susceptibility [4,5]. Skin cancer can be prevented by controlling, or eliminating these causative agents. Skin cancer can be effectively removed by hindering blood supply to the tumor (anti-angiogenesis), which curbs tumor growth and enhances patient survival. Most cancer cells develop ways to evade apoptosis, or exhibit defective apoptosis mechanisms, thus allowing uncontrollable cell development [2]. The apoptosis process is therefore the major target of anti-cancer chemotherapeutics. Currently, skin cancer is treated by surgical removal, radiation therapy, chemotherapy, or cryosurgery, amongst other techniques. Both 5-fluorouracil and imiquimod are used in topical chemotherapies of superficial, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) *in situ*, while only imiquimod is approved for topical therapy of cutaneous malignant melanoma (CMM) [6–8]. The different treatment methods have

both their advantages and disadvantages, therefore, choosing a treatment option is never easy and the preferred choice is influenced by factors, such as the site of the cancer, health status of the patient, as well as patient and doctor opinions.

The main problems that exist with chemotherapeutic agents are severe adverse effects and multi-drug resistance formation. Some of the methods by which cancer cells become resistant to therapies are drug efflux systems, amplification of drug targets, or changes in drug kinetics [9–11]. Various strategies have been attempted to overcome drug resistance, such as the use of nanoparticles, liposomes and micellar drug delivery vehicles, with some reported successes [11]. The adverse effects of cancer chemotherapy can be treated symptomatically, but in some instances such secondary treatments may be very toxic, which is unacceptable to some cancer patients [12–14].

There has been a growing interest in the use of complementary and alternative medicines (CAM), due to the disadvantages associated with conventional cancer chemotherapies and the supposed advantages of more natural treatment options [15]. Phytochemical compounds from extracts of plant roots, bulbs, barks, leaves, stems and others have shown promising potential as anti-cancer drugs, or for serving as lead compounds in the synthesis of new drugs. They are often utilized as traditional medicines in the form of home-made tinctures, teas, or crude extracts. Disadvantages of natural products and traditional medicines include variation in preparation methods and therefore also chemical composition, dosage determination and adjustment, and the suitable route of administration. Although much research on compounds of natural origin to produce new drug substances occurs, research, specifically aimed at naturally derived medicines to optimize dosages for the intended route of administration and to design the most effective dosage forms, has become essential [16]. The worldwide increase in the use of CAM is mainly due to the false perception, or belief that natural products are safe, while scientifically proven information on clinical aspects of some CAMs generally does not exist [15,17]. Figure 1 shows an overview of the anti-melanoma natural products that are discussed in this review.

2. Natural Sources of Anti-Cancer Compounds

An abundance of natural resources for medicinal use exist worldwide, of which many have not yet been exploited for possible application in the pharmaceutical industry. Over 50% of all available drugs on the market originated from natural sources, of which over 70% of anti-cancer agents have their origin in natural sources. Natural sources include plants, animals, microbes and marine life [17]. Plants are the most utilized natural resource for applications in the pharmaceutical science and still comprise the leading natural source for new drugs and lead compounds, due to their accessibility and abundance. To date, only a few naturally derived drugs exist on the market that target skin related cancers, whereas none have yet been approved for topical application. This could be attributed to the known side effects of these agents when topically applied to the skin.

The following sections offer an overview of compounds from different natural sources that have been found to exhibit activity against different types of cancer, with a specific focus on melanoma.

Figure 1. A scheme showing the anti-melanoma actions of the compounds and extracts discussed in this review.

Natural compounds active against skin cancer
(Dietary components, phytochemicals and crude extracts)

Apoptosis promoter

- Quercetin
- Kaempferol
- EGCG
- Apigenin
- β-carotene
- Fucoxanthin
- Vitamin C
- *Ganoderma lucidum* extract
- *Coriolus versicolor* extract
- Resveratrol
- Curcumin
- Sulforaphane
- *Melaleuca alternifolia* extract
- *Zingiber officinale* extract
- Withaferin A from *Withania somnifera*
- Eupatilin from *Artemisia*
- Galangin from *Alpinia officinarum*

Anti-proliferative

- Kaempferol
- EGCG
- Apigenin
- Vitamin A
- Vitamin C
- Vitamin D
- Vitamin E
- *Ganoderma lucidum* extract
- *Coriolus versicolor* extract
- *Hypericum perforatum* extract
- *Melaleuca alternifolia* extract
- *Calendula officinalis* extract
- Emodin from *Aloe*
- Eupatilin from *Artemisia*
- *Alpinia oxyphylla* extract
- *Alpinia galangal* extract

Anti-metastatic

- Amentoflavone
- Hinokiflavone
- β-carotene
- Fucoxanthin
- Vitamin A
- Vitamin C
- Resveratrol
- Sulforaphane
- *Withania somnifera* extract
- *Viscum album* extract
- *Calendula officinalis* extract
- Carnosol from *Rosmarinus officinalis*
- Ursolic acid from *Rosmarinus officinalis*

2.1. Marine Sources

In recent years, interest in the potential of marine fauna and flora as a source of novel medicinal agents has grown significantly. Substantive research, aimed at utilizing this vast natural resource, is being carried out worldwide. The high anti-tumor potency of agents, discovered from marine resources, reflects the high potential of the ocean as a possible source of anti-cancer drugs [17]. Extracts from sponges, algae and marine cyanobacteria have shown strong anti-cancer activities [18–20]. Laminarans, fucoidans, alginic acids and carrageenans are some of the compounds isolated from marine sources that have been found to exhibit effective anti-cancer activities. An assortment of polysaccharides from marine animals, bacteria and fungi have also been tested for anti-cancer activity, of which some were found promising for further drug development [18]. Although various anti-cancer compounds from marine origin have been isolated and tested *in vitro* and *in vivo* and taken through different stages of clinical testing, only four anti-cancer drugs of marine origin have reached the market so far. These anti-cancer drugs are cytarabine, trabectedin, eribulin mesylate and brentuximab vedotin, derived from *Cryptotethia crypta*, *Ecteinascidia turbinate*, *Halichondria okadai* and *Symploca hydnoides*, respectively [21,22].

Cytarabine is a pro-apoptotic compound that also acts by inhibiting cell growth in cancerous cells. In 1998, the Food and Drug Administration (FDA) had approved the first marine derived compound, cytarabine, for clinical use as an anti-cancer agent in the treatment of acute myelogenous leukemia. Trabectedin, a derivative of Caribbean tunicate, was next approved for treatment of metastatic soft tissue carcinoma in 2007 by the European Commission. In 2009, trabectedin received even further approval for the treatment of relapsed, platinum sensitive ovarian cancer. Eribulin mesylate was then approved by the FDA for clinical use as part of a third line treatment regimen for advanced, metastatic breast cancer in 2010 [22]. Brentuximab vedotin received FDA approval for treatment of systemic, anaplastic, large cell lymphoma and Hodgkin's lymphoma in 2011. These four anti-cancer drugs have been further subjected to the various stages of clinical trials for their possible use in more diverse types of cancer, either alone or as part of a treatment regimen.

Aplidin, bryostatin-1, salinosporamide and zalypsis are other examples of marine-derived compounds that are currently undergoing clinical trials for potential use as anti-cancer drugs [16]. Many more marine derived compounds with anti-cancer potential are currently undergoing pre-clinical investigation [20,23].

2.2. Microbial Sources

The tumor regression activity of bacteria was discovered and used clinically over a century ago, when Coley [24] observed that tumors in patients that had been accidentally infected with *Streptococcus pyogenes* had degenerated. Such regression was due to an immune response stimulated by the bacterial infection and it was this discovery that caused the advent of cancer immunotherapy. Ever since, much research has been performed on microbes to explore their anti-neoplastic potential. The chemical diversity and ease of access of microbes with respect to collection, culturing and fermentation make them an extremely relevant source of pharmaceutically active compounds [17]. Anthracyclins, bleomycins, staurosporins and actinomycins are groups of microbially derived anti-cancer compounds in clinical use [16,17].

Whole bacteria can be used in their live, attenuated, or genetically modified forms to stimulate immune responses, but this may potentially result in side effects that can be avoided by using bacterially derived products instead. Ongoing research is carried out on the use of bacterial toxins and spores and on the use of bacteria as vectors for gene therapy. Toxins from microorganisms can have advantageous effects in humans, such as destroying rapidly dividing cells in tumors [25].

2.3. Plant Sources

Over 50% of all drugs currently in clinical use worldwide have originated from compounds extracted from plants [26]. From 1960 to 1982, the National Cancer Institute (NCI) in the USA embarked on a plant collection program, aimed at boosting progress in the discovery of plant derived anti-cancer agents [27]. During this time, a wide range of cytotoxic agents were discovered from plant extracts, but very few of these managed to reach the market for clinical use. The development of taxanes and camptothecins as drugs for clinical use took over twenty years [17,27,28].

The vinca alkaloids, including vincristine, vinblastine and vinorelbine, were the first plant-derived anti-cancer agents to gain approval for clinical use. Thereafter came the discovery and approval of the podophyllotoxin derivatives (*i.e.*, etoposide and teniposide), taxanes (*i.e.*, paclitaxel and docetaxel) and camptothecin derivatives (*i.e.*, irinotecan and topotecan) [27,29]. The mechanism of action of the vinca alkaloids involves interaction with tubulin so as to disrupt the assembly of the mitotic spindle, which in turn leads to the demise of actively dividing cells [29]. Contrary to the vinca alkaloids, taxanes work by stabilizing the microtubule, instead of destabilizing it. The stabilization of the microtubule results in an imbalance between tubulin and microtubules, which affects normal cellular function and in turn results in cell death. Camptothecins and podophyllotoxins inhibit topoisomerase I through different mechanisms, but both cause disruption of the cell division process [17].

An example of a plant that is currently being investigated for possible use in the treatment of advanced pancreatic cancer, non-small cell lung cancer, metastatic colorectal cancer and breast cancer, is *Viscum album L*. It was found that the combination of *Viscum album L*. whole extract and gemcitabine had been relatively well tolerated [30]. A phase II clinical trial is reported to have been conducted on using a green tea extract, Polyphenon E, for the treatment of chronic lymphocytic leukemia. Shanafelt *et al.* [31] found that this green tea extract had been relatively effective and well tolerated in patients.

Berberine, a naturally occurring isoquinolone alkaloid was tested in combination with doxorubicin on human melanoma cells and *in vivo* on mice. It was found that this combination had suppressed tumor growth *in vitro* and *in vivo* [32]. Extracts of *Tilia amurensis* and *Camellia sinensis* were tested on cancer cell lines originating from the skin and they were found to have cytotoxic effects *in vitro* [33,34]. It is further reported that some phytochemicals, such as epigallocatechin-3-gallate and apigenin have demonstrated a higher inclination for cytotoxicity towards melanoma and epidermoid carcinoma cells, compared to normal cells and such chemicals are increasingly being investigated [35,36].

3. Anti-Cancer Dietary Components and Phytochemicals

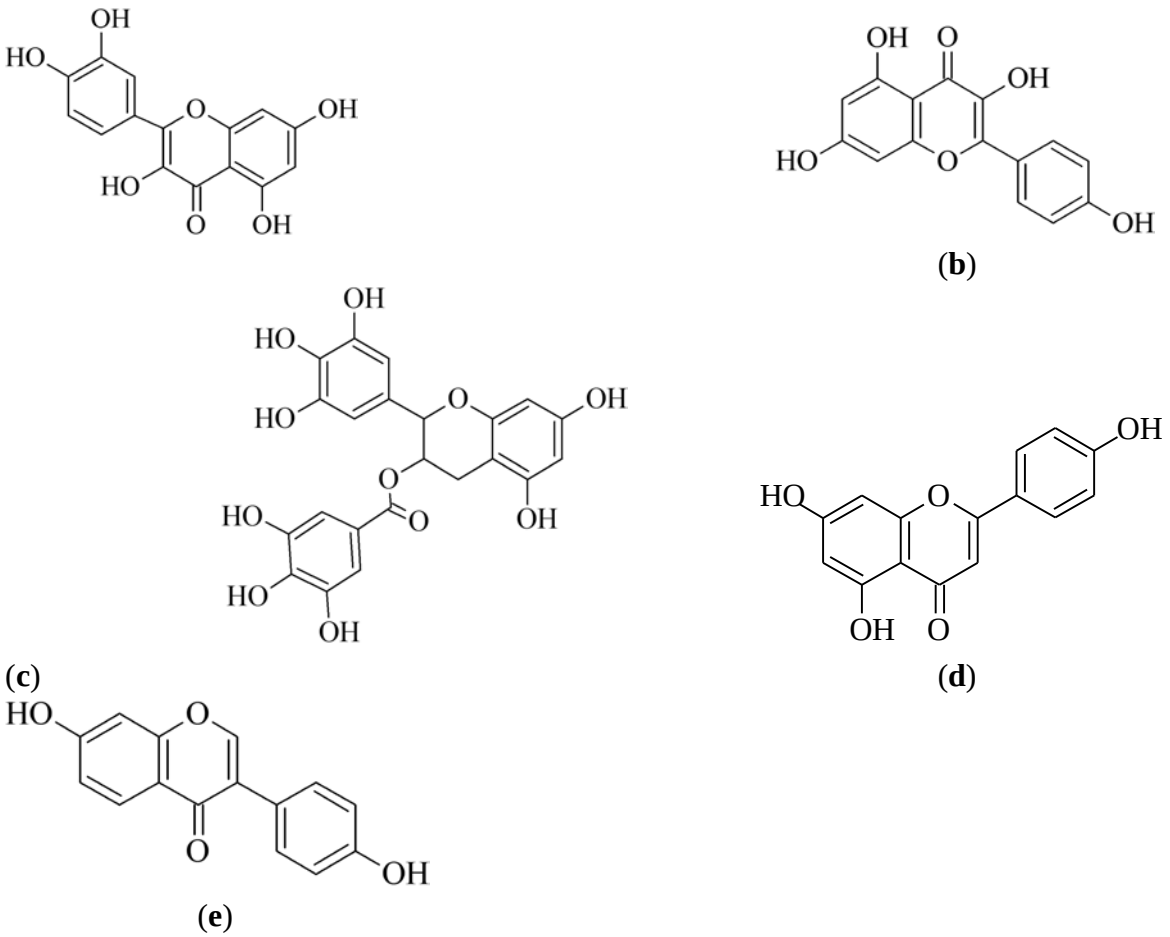
Phytochemicals having anti-inflammatory, immuno-modulatory and anti-oxidant properties, generally have the highest potential of exhibiting chemo-preventive behavior in skin cancers [37]. Numerous attempts have been made to find the correlation between antioxidant properties of

phytochemicals and their anti-cancer potential. Although no concrete evidence of such a correlation has been found yet, the anti-oxidant activity of a phytochemical is being regarded as an indication of potential anti-cancer activity [38,39]. Carotenoids, flavonoids and terpenoids are some of the groups of phytochemicals with high anti-cancer potential [40–42].

3.1. Flavonoids

Flavonoids are acetogenins from plant and flower pigments [43] and their chemical structures are characterized by two benzene rings that are connected through a linear carbon chain and an aromatic chromophore [44]. The bright colors of plant parts rich in flavonoids are due to the aromatic chromophore. The main groups of flavonoids include flavonols, flavanones, flavones, isoflavones, flavan-3-ols (catechins) and anthocyanins. Figure 2 illustrates the chemical structures of some flavonoids that have been found to exhibit anti-cancer activities and are discussed in the sections that follow.

Figure 2. Chemical structures of selected flavonoids possessing anti-cancer potential: (a) quercetin, (**b**) kaempferol, (**c**) EGCG, (**d**) apigenin and (**e**) daidzein.



Flavonoids are well known for their anti-oxidant (or free radical scavenging) and chelating properties and are continuously being investigated for application in the treatment of diseases [45]. The anti-oxidant activity of flavonoids can act as both a trigger of tumorigenesis and/or as an inhibitor of tumorigenesis, depending on other physiological factors. Not all flavonoids would therefore be useful in cancer chemotherapy or chemo-prevention [38,39]. Some flavonoids have further demonstrated to

absorb ultra-violet B (UVB) rays, hence contributing to their photoprotective effect in plants, by behaving as UV filters and protecting underlying elements [46]. This photoprotective property of flavonoids has been adapted and investigated in human cells and in mice models, to determine whether flavonoids and their derivatives could be used as photoprotective agents in humans [47].

3.1.1. Quercetin

Quercetin is a flavonol that is identified by the presence of hydroxyl groups on positions 3, 5, 7, 3' and 4' of the flavonol skeleton [48] [Figure 2a]. This flavonol is insoluble in cold water, slightly soluble in hot water and soluble in alcohol [48]. Quercetin seems to be one of the most effective flavonoids with respect to its biological activities [49]. The anti-cancer activity of quercetin is mainly attributed to its anti-oxidant and anti-inflammatory properties [50]. Research must, however, continue to determine the actual pathways through which quercetin exhibits its anti-cancer activity [51,52].

Quercetin is the most abundant flavonol in the human diet and it is found in plants in many glycosidic forms, such as galactosides, rhamnosides, arabinosides, or glucosides [53,54]. The main sources of quercetin include, but are not limited to, apples (*Malus domestica*), tomatoes (*Solanum lycoperscium*), tea (*Camellia sinensis*), grapes (*Vitis vinifera*), Ginkgo (*Ginkgo biloba*) and St John's Wort (*Hypericum perforatum*) [48]. Onions (*Allium cepa*), although generally ingested in small amounts, are also known to contain large amounts of quercetin [48,53]. Other sources are dark chocolate (*Theobroma cacao*), capers (*Capparis spinosa*), cloves (*Syzygium aromaticum*), black elderberries (*Sambucus nigra*) and oregano (*Origanum vulgare*) [55].

Since quercetin is found in many food substances, research has been conducted to determine whether these food substances offer any protective properties against cancer formation and its progression. To date, a number of different synergistic and antagonistic effects have been found through which quercetin acts biologically to contribute towards the strength of its chemo-protective and anti-metastatic actions, or lack thereof [56]. Evidence indicated that quercetin acts against melanoma by affecting cell viability at low concentrations and by inducing apoptosis at higher concentrations [57]. During a study by Zhang *et al.* [58], quercetin induced apoptosis in murine melanoma cells (B16–BL6) by attenuating the expression of B cell lymphoma 2 (Bcl-2) and potentiating caspase-3 activity. Another mechanism through which quercetin acts according to the findings by Olson *et al.* [59], is one where quercetin potentiates the c-fos gene expression induced by UVB, while at the same time inhibiting phosphoinositide-3 kinase (PI3-K), leading to both potentiation and inhibition of carcinogenesis. Most recently, quercetin has been reported to block UVB induced oxidative stress and deoxyribonucleic acid (DNA) damage, which in turn induces apoptosis in mouse epidermal cells. The same study showed that quercetin inhibited the generation of reactive oxygen species (ROS) and restored the expression of anti-oxidant enzymes in the C141 mouse epidermal cells [60]. Cao *et al.* [61] recently reported that the anti-melanoma activities of quercetin may be due to inhibitory effects on *signal transducer and activator of transcription 3* (STAT3), an oncogenic protein.

Nonionic and anionic topical formulations, containing quercetin, were applied to the dorsal skin of UVB irradiated hairless mice. The nonionic formulation was lipid rich, while the anionic formulation had low lipid content and contained the anionic hydrophilic colloid, Carboxypolymethylene-Carbopol® 940. The *in vivo* results indicated that both formulations had resulted in the inhibition of an increase in

myeloperoxidase activity and a decrease in glutathione, both associated with UVB irradiation skin damage [62]. Glycosides and polymethoxylate derivatives of quercetin have been identified as good candidates for use in the topical delivery of quercetin, as some of them have exhibited improved therapeutic indices, metabolic stability and anti-inflammatory activity *in vitro* [63]. Nanoparticle formulations and micro-emulsions containing quercetin have worked well for targeting drug delivery to the skin *in vitro* [64,65].

3.1.2. Kaempferol

Kaempferol is one of the most abundant flavonoids found in a wide variety of food components, hence the interest in its medicinal properties. Kaempferol is a poorly water soluble flavonoid, found in tea (*Camellia sinensis*), strawberries (*Fragaria ananassa*), green chilli (*Capsicum frutescens*), carrot (*Daucus carota*), pumpkin (*Cucurbita pepo*), brinjal (*Solanum melongena*), broccoli (*Brassica oleracea var. italica*), propolis, grapefruit (*Citrus paradisi*), apples (*Malus domestica*), beans (*Phaseolus vulgaris*) and onions (*Allium cepa*) [44,66–68]. The chemical structure of kaempferol is illustrated in Figure 2b. Epidemiological studies have shown a positive relationship between consumption of foods with high kaempferol content and a reduction in the incidence of cancer (lung, ovarian, gastric and pancreatic) and cardiovascular diseases [69].

There are a number of possible mechanisms through which kaempferol exerts its anti-cancer effects, such as promoting apoptosis and inhibiting cell proliferation [67]. Kaempferol has been found to block choroidal melanoma cell cycle progression in the G2/M phase [70]. Chao *et al.* [71] had developed submicron emulsion systems for the transdermal delivery of kaempferol and found that the use of an appropriate vehicle could significantly influence the flux, the deposition amount in skin and the lag time. In a skin permeation study conducted by Park *et al.* [72], kaempferol in solution (1,3-butylene glycol-ethanol) was able to permeate the skin barrier of albino mice. An investigation by Park *et al.* [72] regarding the anti-oxidant and cellular membrane protective effects of kaempferol also generated encouraging results.

3.1.3. Epigallocatechin-3-gallate

Epigallocatechin-3-gallate [EGCG, chemical structure shown in Figure 2c] is a stable and water soluble member of the group of flavonoids referred to as flavan-3-ols [73]. Flavan-3-ols are mainly found in tea (black, green, oolong) (*Camellia sinensis*), red wine (from *Vitis vinifera*), strawberry (*Fragaria ananassa*) and cocao (*Theobroma cacao*) products, with green tea being the main source [40,44]. Geographical data indicates the probability that the incidence of prostate cancer is lower in certain Japanese and Chinese populations, due to their high green tea consumption [74].

EGCG is capable of inducing apoptosis and cell cycle arrest in melanoma cells (A374 and Hs-294T), alone and in combination with vorinostat *in vitro* [75,76]. Treatment with a combination of EGCG and interferon has also shown synergistic anti-proliferative effects against human melanoma cells *in vitro* and against a mice melanoma model *in vivo* [77]. The mechanisms through which EGCG exerts these effects include down regulation of apoptosis inhibiting proteins, or cell survival promoting proteins (Bcl-2, D1 and cyclin dependent kinase 2 (cdk2), the up regulation of Bcl-2 associated X protein (Bax), a pro-apoptosis protein, the activation of caspases-3, -7 and -9, and through the induction of tumor

suppressor proteins ($p16^{INK4a}$, $p21^{WAF1/CIP1}$ and $p2^{KP1}$) [36,78]. Other suggested mechanisms include inhibiting the activation of the epidermal growth factor receptor and the downstream adapter protein Shc in human skin carcinoma cells (A431) [79]. Nandakumar *et al.* [80] investigated the anti-skin cancer effects of EGCG, focusing particularly on its effects on silenced tumor suppressor genes. Treatment of human skin carcinoma cells (A431) with EGCG had resulted in a reduction of DNA methylation, which in turn led to protein expression of the DNA hypermethylation-silenced tumor suppressor genes, $p16^{INK4a}$ and CDK interacting protein 1/p21 (*Cip1/p21*). EGCG, however had no effect on the expression of the tumor suppressor genes in normal epidermal cells [80]. These results for EGCG were confirmed by the outcomes of another study on A431 cells, namely inhibition of proliferation and the induction of apoptosis in human skin cancer cells, but through inactivation of β catenin signaling [34]. During a study by Nihal *et al.* [36], EGCG showed pro-apoptotic activity, selective towards melanoma cells and not towards the normal melanocytes. Lu *et al.* [73] very recently reported compelling evidence regarding the induction of DNA damage and high genetic mutation frequency in normal lung and skin cells by high concentrations of EGCG, which could possibly cause cancer.

Besides the possible use as an anti-carcinogenic agent, EGCG is considered a good candidate for use as a photoprotectant. Skin photoprotection is an important aspect of skin cancer prevention and studies have illustrated that EGCG shows high potential to prevent the skin from photo induced damage, the leading cause of skin cancer. Sevin *et al.* [81] found that the topical application of EGCG to rat skin, thirty minutes prior to UVA exposure, had reduced the formation of sunburn cells.

3.1.4. Apigenin

Apigenin is a flavone, chemically referred to as 4',5,7,-trihydroxyflavone [Figure 2d], which forms yellow, needle like crystals in its pure form and is commonly found in celery (*Apium graveolens*), oranges (*Citrus sinensis*), tea (*Camellia sinensis*), parsley (*Petroselinum crispum*), thyme (*Thymus vulgaris*) and onions (*Allium cepa*) [82]. Apigenin is one of the most bioactive flavones in plants and epidemiologic observations have shown that flavone rich diets are associated with a reduction in the risk of developing certain cancers [83]. Such observations have stimulated research on the anti-cancer activities of apigenin.

The anti-cancer activities of apigenin have been observed *in vitro* in various cell lines, such as head and neck squamous cell carcinoma cells, melanoma cells and liver cells. The mechanisms of action, as determined by Chan *et al.* [35], include inducing cell cycle arrest in the G_2/M phase, up regulating tumor necrosis factor receptor (TNF-R) and the TNF related apoptosis inducing ligand receptor (TRAIL-R) apoptotic pathway, down regulating Bcl-2, and activating caspase-3. The combinations of all these actions result in the chemo-protective effects of apigenin. Apigenin has been found to exhibit UVB radiation protective effects on human keratinocytes *in vitro* and on mice skin tissue *in vivo*, by interfering with cell survival and cell proliferation via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen activated protein kinase (MAPK) pathways [84]. Furthermore, an oncogenic kinase (Src) is inhibited by apigenin, resulting in an inhibition of UVB induced expression of cyclooxygenase 2 (COX-2), thus reducing the inflammatory and oncogenic effects associated with COX-2 [85].

Ethosomes have been formulated together with apigenin and it was determined that apigenin loaded ethosomes resulted in a higher skin deposition of apigenin, compared to liposomes *in vitro* and *in vivo* [86]. The influence of nano-encapsulation of apigenin was investigated by Das, *et al.* [87], who found that the encapsulated apigenin could be a more ideal formulation, compared to the free apigenin, as the encapsulated apigenin was able to penetrate the nucleus and in turn result in higher apoptotic effects. Topical application of nano-encapsulated apigenin resulted in the inhibition of tumorigenesis in UVB exposed, Swiss, albino mice. However, a combination of oral and topical apigenin intake proved a more potent inhibitor of carcinogenesis in these mice [88].

3.1.5. Daidzein

Daidzein is a soy isoflavone [chemical structure shown in Figure 2e], which is highly soluble in alkaline environments and is part of a group of compounds, called phytoestrogens [89]. It has demonstrated some chemo-protective potential in the skin, since topical application of daidzein in a study resulted in effective photo-protection [90]. *In vitro* studies showed that daidzein was able to inhibit UVB induced production of hydrogen peroxide within cells and therefore the protection of the keratinocytes. Daidzein and genistein have been investigated as synergistic cytotoxic agents in various studies and evidence showed that the two isoflavones had worked well together [89,91,92]. Franz cell based, *in vitro* diffusion studies and tape-stripping showed that minimal amounts of daidzein had managed to penetrate through the skin [89]. This unfavorable skin permeation characteristic of daidzein may be the reason for the limited research that has been carried out with regards to its potential use as a topical photo- and chemo-protectant.

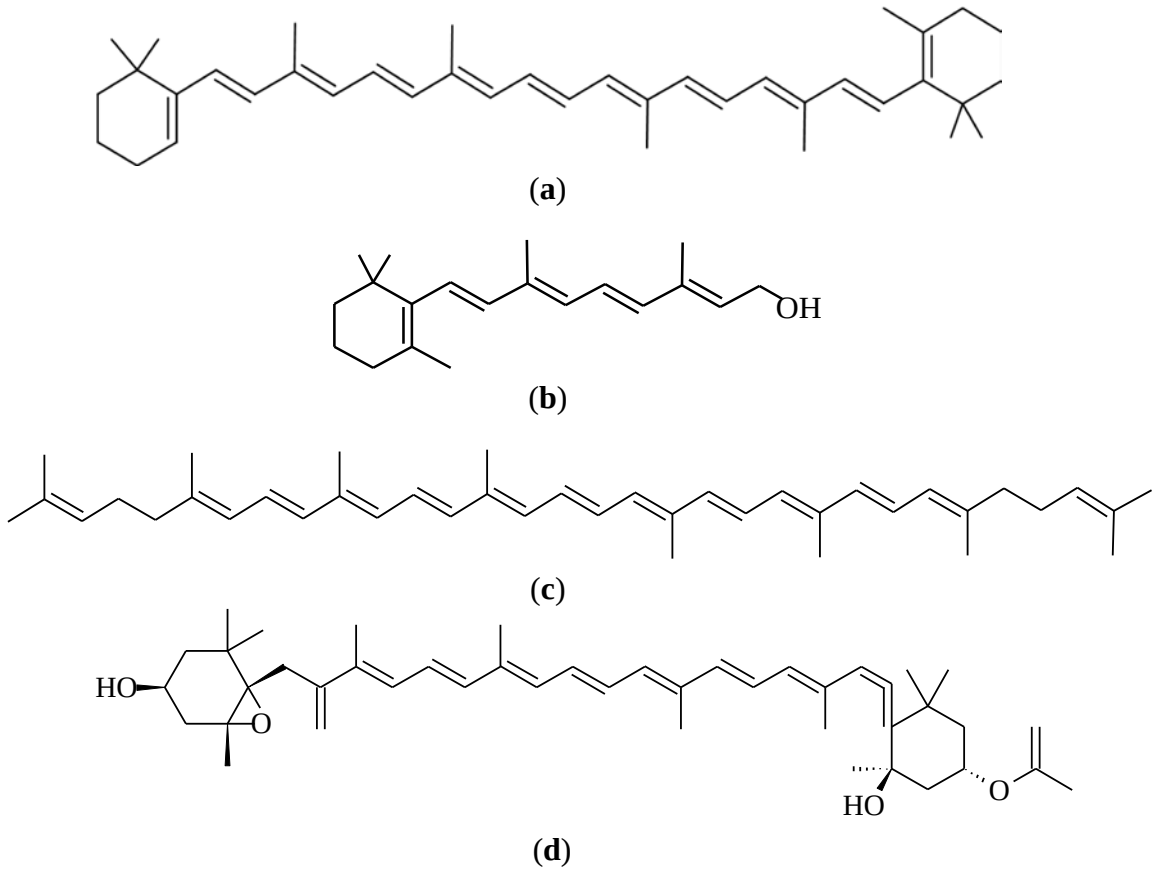
3.1.6. Biflavonoids

In recent years there has been an increased interest in the medicinal properties of biflavonoids. Biflavonoids are dimers of flavones, flavonols and flavanones [93,94], and they are known to inhibit melanogenesis [95]. Compounds that fall in this group include amentoflavone, podocarpusflavone, volkensiflavone, fukugetin, hinokiflavone. Some biflavonoids whose anti-melanoma activity has been investigated and reported are amentoflavone and hinokiflavone. Guruvayoorappan and Kuttan [96], reported that amentoflavone (from *Biophytum sensitivum*) inhibits metastasis of B16F10 melanoma cells *in vivo* by inhibiting tumor invasion, migration, proliferation and angiogenesis. These effects were found to be possibly linked to attenuation of the effects of matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF) and extracellular signal-regulated kinase (ERK). Amentoflavone has also been found to inhibit endothelial cell migration and angiogenesis which are linked to placental growth factor 1 (PlGF-1) [97]. In addition to these actions amentoflavone has been reported to prolong survival of metastatic tumor-bearing mice [98]. The potential MMP-9 inhibitory action of hinokiflavone was investigated using pharmacophore modeling and it was reported to have inhibitory effects on MMP-9 which in turn may result in anti-metastasis effects [99].

3.2. Carotenoids

Carotenoids are fat-soluble pigments that are commonly found in nature, especially in plants [100]. These compounds are made up of eight C_5 isoprenoids that are combined to form C_{40} tetraterpenoids, with various chemical modifications (e.g., hydrogenation, isomerization,dehydrogenation, presence of oxygen functions, *etc.*) to form different carotenoids. The distinctive conjugated double bond system of carotenoid structures acts as a light absorbing chromophore, which gives the yellow, orange or red colors to tomatoes, dark green vegetables, oranges and other bright colored food substances [101]. Figure 3a–d illustrate the chemical structures of carotenoids, discussed in this section. Carotenoids are divided into two main groups, namely hydrocarbons, which are highly fat soluble carotenes, and xanthophylls, which are relatively polar carotenoids that contain oxygen [102]. Pro-vitamin A carotenoids are carotenoids with an unsubstituted β ring, for example β-carotene. Pro-vitamin A carotenoids act as precursors of retinol in the body [103]. There are more than 500 carotenoids in nature, but the commonly studied carotenoids include β-carotene, α-carotene, lycopene, lutein, astaxanthin, fucoxanthin and canthaxanthin [42,104].

Figure 3. Chemical structures of selected carotenoids: (**a**) β-carotene, (**b**) retinol, (c) lycopene and (**d**) fucoxanthin.



(**a**) (**b**) (**c**) (**d**)

Much research has been undertaken with respect to the medicinal properties of carotenoids and they are believed to be potential agents for preventing cancer, diabetes and cardiovascular diseases [100]. The medicinal properties of carotenoids are assumed to be due to their anti-oxidant activities that reduce DNA damage by free radicals after exposure to UV light, but other mechanisms are also under investigation.

Pro-vitamin A carotenoids are converted into retinol and exert the effects of retinol within the body, which is essential in proliferation, maintenance and differentiation of cells within the epithelia [105].

3.2.1. β-Carotene

The most widely studied carotenoid and one of the most abundant carotenoids in the human diet is β-carotene. Some of the dietary sources of β-carotene are carrots (*Daucus carota*), spinach (*Spinacia oleracea*), kale (*Brassica oleracea* var. *acephala*), pepper (*Capsicum spp.*), pumpkin (*Cucurbita pepo*), sweet potatoes (*Ipomoea batatas*) and cantaloupe (*Cucumis melo* var. *cantalupensis*) [104]. Both melanocytes and keratinocytes are able to accumulate and store β-carotene for conversion into retinol when required [106].

β-Carotene is able to induce apoptosis in melanoma cells *in vitro* by activating caspases-3, -8 and -9 via a caspase cascade [107]. According to Guruvayoorappan and Kuttan [108], the mechanism of anti-neoplastic activity of β-carotene in murine melanoma cells may include the regulation of Bcl-2, p53 and caspase-3, which then stimulates apoptosis. In another study by Guruvayoorappan and Kuttan [109], the effect of β-carotene on tumor specific angiogenesis, which affects tumor growth, was explored. In this study, it was found that β-carotene may have inhibited tumor specific angiogenesis by inhibiting the activation or nuclear translocation of various transcription factors [109]. β-Carotene seemed to have activated Bax, a pro-apoptosis protein, in melanoma cells, although no evidence of apoptosis actually occurring in the cells have been found. In some instances, inhibition or up regulation of gene expression does not always translate into a particular protein function, as there may be other processes that could overcome the effects of the inhibition, or up regulation, of which this is one such example [110].

Initially, β-carotene was regarded in high esteem as a chemo-protectant, due to promising *in vitro* results, but a number of controlled studies in human subjects and the murine model have shown ambiguous and conflicting results with respect to the anti-cancer effects of β-carotene. It was shown that a diet high in carotenoids (β-carotene included) may be related to a decreased risk for melanoma [111,112]. In a case study, metastatic melanoma regressed in a patient after she had changed to a diet that was rich in anti-oxidants (β-carotene included). This may have been a case of spontaneous regression, but the possibility also existed that it had been as a result of her change in diet [113]. In another case, oral supplementation of a combination of β-carotene with other anti-oxidants (*i.e.*, vitamin C, vitamin E, selenium and zinc) resulted in a significant increase in the incidence of melanoma in women, but not in men [114]. This effect may have been as a result of a synergistic effect of the supplement components, or due to one particular compound. The incidence of melanoma declined after the anti-oxidant supplementation was stopped, thus supporting the idea that anti-oxidants are not necessarily beneficial to the treatment, or prevention of skin cancers [115]. Prior to these studies, it had been established that β-carotene levels in blood had no underlying influence on the incidence of melanoma [116,117]. Twelve year supplementation with β-carotene in men had shown no influence, positive or negative, on the incidence of malignant neoplasms, melanoma included [116]. Such findings have made it difficult to ascertain whether β-carotene actually is beneficial *in vivo*.

3.2.2. Lycopene

Lycopene is one of the most common acyclic carotenoids and it is the pigment found in red and orange-fleshed fruits and vegetables. Examples are watermelon (*Citrullus lanatus*), papaya (*Carica papaya*), tomato (*Solanum lycopersicum*), guava (*Psidium guajava*), grapefruit (*Citrus paradisi*), apricot (*Prunus armeniaca*) and peaches (*Prunus persica*) [101,104]. Lycopene [Figure 3b] is an unsubstituted hydrocarbon, making it a highly lipophilic carotenoid with no vitamin A activity.

As with β-carotene, lycopene blood levels of 30.9–40.8 μg/dL on average have demonstrated no influence on the incidence of melanoma [117]. The risk of melanoma in subjects with high serum levels of lycopene is not significantly different from the risk in those with middle or low levels [118]. This further illustrated that anti-cancerous effects of compounds are usually selective for a particular type of cancer, as some evidence suggests that lycopene has cancer preventative actions in models of lung, colon, liver and mammary gland carcinogenesis [102]. Lycopene acts by trapping platelet-derived growth factor-BB, which in turn inhibits migration and the signaling of fibroblasts that are induced by melanoma cells. This effect, however, does not result in a reduction in cell viability or metastatic potential, once again revealing that there are various processes at play within the complex physiology of cancer [119,120].

3.2.3. Fucoxanthin

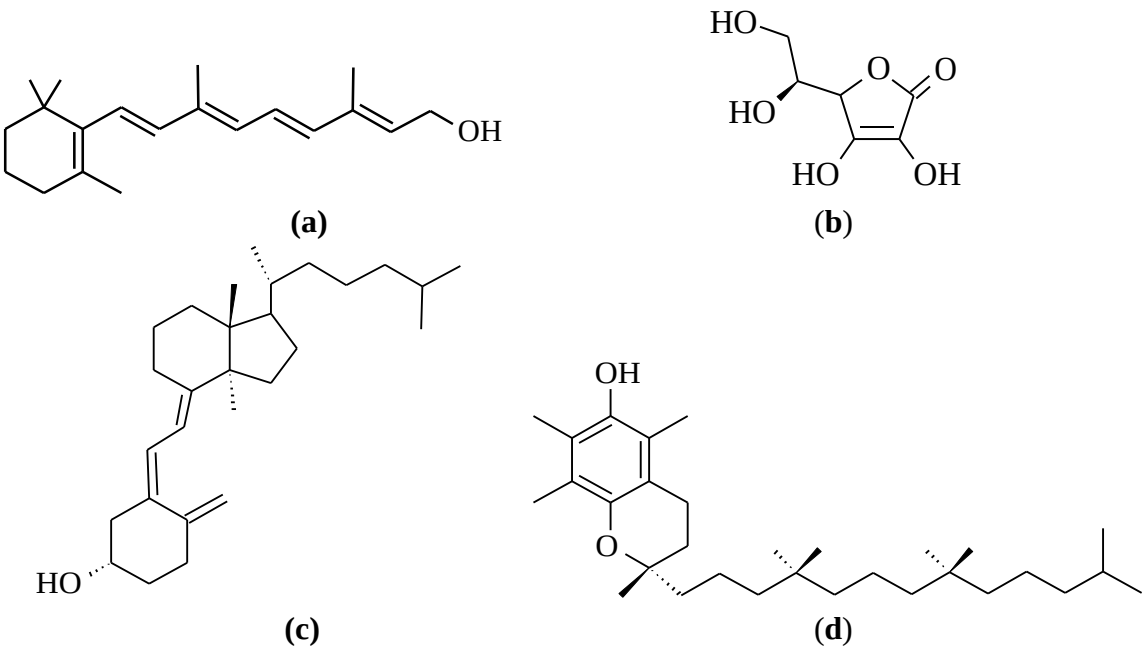
Fucoxanthin is a brown or orange marine derived carotenoid, with its main sources including edible brown seaweeds, brown algae and heterokants [104]. Examples of the sources of fucoxanthin are *Undari pinnatifida, Hijika fusiformis*, *Laminaria japonica*, *Sargassum fulvellum* [121] and *Fucus evanescens* [122].

Reduced incidences of tumors, anti-proliferation, cell cycle arrest, apoptosis induction and inhibition of metastasis are some of the anti-cancer effects that are exerted by fucoxanthin [123]. Similar to β-carotene, fucoxanthin seems to induce apoptosis via the caspase pathways [124]. Fucoxanthin inhibits growth of SK-MEL-28 and B16F10 melanoma cells in a concentration dependent manner [122,124]. A recent study has shown that fucoxanthin suppresses the metastatic potential of murine melanoma cells, by down regulating some proteins involved in cell interaction, cell migration and cell adhesion, *i.e.*, MMP 9, CD44 and C-X-C chemokine receptor 4 (CXCR4) [125]. Topical application of fucoxanthin in UV irradiated guinea-pigs resulted in the suppression of tyrosinase activity and of the melanogenesis process in melanoma. Melanogenesis suppression was achieved through inhibition of messenger ribonucleic acid (mRNA) expression of COX-2 and through the inhibition of a number of receptors (*i.e.*, melanocortin 1, prostaglandin E, endothelin A and p75 neurotrophin) [126]. Intra-peritoneal injection of fucoxanthin has been shown to inhibit the *in vivo* growth of intra-dermal B16F10 tumors in mice [124].

3.3. Vitamins

Vitamins are families of essential compounds that cannot be produced within the human body and are therefore taken in with food and as supplements. The importance of vitamins lies within the various roles they play in physiological processes. In this section reference is made to vitamins A, C, D and E, as illustrated in Figure 4.

Figure 4. Chemical structures of (**a**) vitamin A, (**b**) vitamin C, (**c**) vitamin D3 and (d)) vitamin E (α-tocopherol).



(a) (**b**) **(c)** (**d**)

3.3.1. Vitamin A (Retinol)

Vitamin A (or retinol) is well known for its roles in eye physiology, reproduction, the immune system and bone development. The main sources of retinol of importance are eggs, milk, cheese, meat and liver [127]. Retinoids are known for being key players in the processes of differentiation and cell proliferation, as they possess anti-proliferative and pro-differentiating activities [128].

Epidemiological studies have been conducted to determine whether serum levels of retinol would have any influence on the incidence of melanoma. These studies have shown that there was no direct relationship between retinol levels and the propensity to develop melanoma [117,129]. In a large cohort study conducted, the intake of retinol supplements was found to be related to a reduced risk for developing melanoma, especially in women [130]. The conflicting findings of the different studies may have been due to for example not having fully considered other risk factors, and due to possible bias in the selection of candidates. Another possible explanation could have been that some retinoic acid receptors (RAR-β2) are silenced in human melanoma and the retinol is unable to exert its full anti-proliferative effects in melanoma [128]. Another controversy, related to vitamin A, is that retinyl palmitate, the storage form of retinol in the skin and used in sunscreens, has been found to possess photo-carcinogenic potential, thus raising questions about its safety for topical applications. Retinyl palmitate has been in use for decades and no observations have been made with respect to it increasing an individual's susceptibility to develop skin cancer, hence supporting the notion that it is relatively safe for topical use [131,132].

The pharmacological effects of vitamin A are not only ascribed to retinol itself, but also to its metabolites, namely the retinoic acids. Studies regarding the anti-cancer activity of retinol sometimes include retinoic acid. *In vitro* and *in vivo* testing, aimed at determining the mechanisms of action of retinol, have found that retinol mainly acts by inhibiting angiogenesis. The anti-angiogenesis action of

retinol was observed in a murine melanoma angiogenesis model. Mice that had been treated with 13-*cis*-retinoic acid for five days showed a 50% decrease in the number of tumor directed capillaries. The anti-angiogenesis effect of 13-*cis*-retinoic acid seemed to have been as a result of inhibition of VEGF production, the inhibition of the migration of endothelial cells and the inhibition of tube formation [133]. Meyskens and Fuller [134] found that 13-*cis*-retinoic acid, retinol and β-all-*trans*-retinoic acid were able to inhibit proliferation in human melanoma cells *in vitro*, which fueled further studies on the use of retinol and related agents as possible anti-cancer compounds in melanoma. A recent study conducted by Ribeiro, *et al.* [135] showed that a combination of all-*trans*-retinoic acid and endoxifen had a high anti-proliferative and anti-migratory effect on melanoma cells, but demonstrated no toxicity towards normal endothelial cells. It has also been reported that the combination of all-*trans*-retinoic acid and EGCG had a synergistic anti-cancer effect on melanoma, as all-*trans*-retinoic acid up regulates the 67-kDa Laminin receptor, which is thought to be crucial to the action of EGCG [127].

3.3.2. Vitamin C (Ascorbic Acid)

Epidemiological studies that have investigated the possible relationship between vitamin C intake and the risk of developing melanoma, had shown no benefits of vitamin C in preventing melanoma [136]. It was found that patients who had consumed food with high vitamin C content, especially orange juice, had in fact demonstrated a higher likelihood of developing melanoma [129,136]. However, *in vitro* and *in vivo* efficacy studies have proven otherwise. The main sources of vitamin C are citrus fruits, broccoli (*Brassica oleracea var. italica*), green pepper (*Capsicum annuum*), tomatoes (*Solanum lycoperscium*), strawberries (*Fragaria ananassa*) and melons.

Vitamin C appears to act by inducing apoptosis and by inhibiting cell proliferation and cell growth. Apoptosis induction by vitamin C is thought to occur by way of pro-oxidant activities that can be blocked by N-acetyl-L-cysteine, a potent anti-oxidant. Vitamin C is generally known to have anti-oxidant activity, but contrary to expectations, its anti-cancer activity in melanoma cells has proven to be related to oxidative stress instead, due to the caspase 8 pathway [137]. According to research conducted by Neena, *et al.* [138], low concentrations of ascorbate resulted in concentration dependent melanoma cell death, but as the ascorbate concentrations were increased, a proliferative effect was observed. Ascorbic acid has been found to up regulate levels of p53 and p21 tumor suppressor proteins, resulting in cell cycle arrest in the G1 phase [139]. Another proposed mechanism for the anti-cancer activity of vitamin C includes angiogenesis. It is thought that vitamin C suppresses the expression of vascular endothelial growth factor (VEGF) in melanoma cells, thus enabling it to suppress angiogenic processes, which could result in tumor regression [140]. Type 1 insulin like growth factor receptor (IGF-1R) and COX-2 expression are also down regulated by vitamin C, resulting in anti-proliferative effects, as observed by Seung Koo, *et al.* [141].

3.3.3. Vitamin D

Vitamin D is a fat soluble vitamin that is mainly derived from 7-dehydrocholesterol, which is converted into pre-vitamin D_3 in the skin, due to sun exposure [142]. Salmon, mackerel, bluefish, cod liver oil, mushrooms, egg yolks and yeasts are reputably good dietary sources of vitamin D_3 [143].

Vitamin D exists as ergocalciferol, cholecalciferol, calcidiol and calcitriol [144]. The overall importance of vitamin D lies in its key roles in the immune system, bone development and cell proliferation [142]. Epidemiological studies have shown no relationship between vitamin D intake, nor pre-diagnostic vitamin D serum levels and a susceptibility to develop melanoma [145,146]. A direct relationship between plasma levels of vitamin D and the risk of non-melanoma skin cancers in women has, however, been reported [147]. This observation contradicted the notion that vitamin D is a chemo-protective, or tumoristatic agent. In another study performed in Italy, vitamin D intake was observed to be beneficial with respect to its hypothetical influence on melanoma risk [148].

Brozyna *et al.* [149] reported that a decrease in expression of vitamin D receptors had occurred, as skin cancers had progressed. This was indicative that vitamin D activity, or its absence, had played some role in the progression of skin cancer. The anti-carcinogenic roles of vitamin D include decreasing proliferation and increasing differentiation of keratinocytes, taking part in DNA damage repair processes, and regulating the expression of oncogenic and tumor suppression of long non-coding RNAs (lncRNAs) in keratinocytes. With a reduced expression of Vitamin D receptors it follows that the anti-carcinogenic activities of vitamin D are also reduced [149]. Paradoxically, incidences of skin cancer have been associated with genetic damage and mutation within the skin, due to excessive exposure to the sun's UVB rays, while concurrently, a relatively constant level of moderate sun exposure is believed to have protective effects against cancer, due to its role in the production of vitamin D [150].

3.3.4. Vitamin E (Tocopherol)

Vitamin E is a fat soluble, essential nutrient, having anti-oxidant activities within the body [151]. Main dietary sources include vegetable oils and margarine, while other sources are nuts, seeds, egg yolks, asparagus, lettuce and whole grains [42,105]. Tocopherols (α, β, Δ and γ) are a group of compounds, which, together with the tocotrienols (α, β, Δ and γ), form the vitamin E family [152], with the tocotrienols exhibiting a more powerful anti-tumorigenic effect [153]. This section, however, mainly focuses on α-tocopherol, as it is the most abundant and active form of vitamin E.

Treatment of murine melanoma cells with α-tocopherol acid succinate was reported by Ottino and Duncan [154] to inhibit cell growth and cell proliferation. During their study, α-tocopherol acid succinate was proposed to exert the anti-proliferation effects via a COX pathway. The anti-cancer activity of α-tocopherol acid succinate occurred due to the α-tocopherol moiety and not because of the succinate salt, as had been indicated previously by Prasad and Edwards-Prasad [155]. Ottino and Duncan [156] determined in a separate study that vitamin E supplementation in murine melanoma cells resulted in an increase of cyclic adenosine monophosphate and adenylate cyclase activity, together with an elevation in prostaglandin E_2 levels. These increases were related to the inhibition of cell growth [156]. Vitamin E succinate in a sesame oil vehicle was intra-peritoneally injected into tumor inoculated nude mice and it was observed that vitamin E had significantly inhibited melanoma tumor growth *in vivo* by inducing apoptosis [151]. Inhibition of melanoma growth and angiogenesis through the down regulation of VEGF by vitamin E succinate has also been observed in tumor inoculated mice [157].

A number of studies have been performed to determine the efficacy of vitamin E formulations as photo-protectants and chemo-protectants. Pedrelli *et al.* [158] found that a formulation, containing 10% of tocopherols and 0.3% of tocotrienols, had resulted in a photo-protective effect in humans, when

topically applied prior to UV exposure [158]. Topical vitamin E has been reported to result in an increase in tumor burden in mice. Yet, in the same report a formulation with both vitamin C and vitamin E (CE Ferulic®) had resulted in a reduced tumor burden *in vivo*. It was suggested that vitamin E was effective in late stage tumorigenesis, as it was able to affect tumor progression and not initial tumor growth [159]. Possible utilization of nanoformulation in the development of vitamin E based formulations for cancer treatment, in combination with other anti-cancer agents, has been considered and investigated [160].

3.4. Terpenoids

Terpenoids are found in higher plants, mosses, liverworts, algae and lichens. These compounds are also referred to as terpenes or isoprenoids. Structurally, terpenoids are assembled from five carbon building units and the medically important sub-classes comprise the hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}) diterpenoids (C_{20}), triterpenoids (C_{30}), tetraterpenoids (C_{40}) and polyterpenoids $(C_5)_n$ [17]. Tetraterpenoids are also referred to as carotenoids (discussed in Section 3.2). Early uses of terpenoids included perfumes, flavorants, preservatives and pigments, but their applications have long since diversified to include medicines, soaps and narcotics [161]. A commercial anti-neoplastic agent has been developed from the diterpene, paclitaxel (Taxol), which was originally extracted from *Taxus brevifolia* [17].

Ferula spp and some mushroom (*Ganoderma lucidum* and *Coriolus versicolor*) are some of the plants containing terpenoid extracts that have been tested for cytotoxic activity against melanoma cells *in vitro*. A methanol extract and a purified methanol extract (mainly containing acidic terpenoids) of *Ganoderma lucidum* were prepared and used to determine their *in vitro* and *in vivo* anti-cancer properties. It was found that both extracts had shown strong anti-melanoma activity *in vitro* and they had reduced tumor volume *in vivo*, but the purified methanol extract was less potent than the methanol extract. The mechanisms by which these *Ganoderma lucidum* extracts exerted these activities were through oxidative stress, apoptosis induction and the inhibition of cell proliferation [162]. A terpenoid and polyphenol containing methanol extract of *Coriolus versicolor* was also tested for anti-melanoma activity *in vitro* and *in vivo*. The *Coriolus versicolor* methanol extract reduced melanoma cell growth and tumor volume through the inhibition of cell proliferation and the induction of apoptotic and necrotic cell death. This methanol extract also demonstrated synergistic effects on the anti-melanoma activities of macrophages [163]. The monoterpenes, *i.e.*, stylosin and tschimgine, were extracted from *Ferula ovina* and found to have potent cytotoxic activity against melanoma [164].

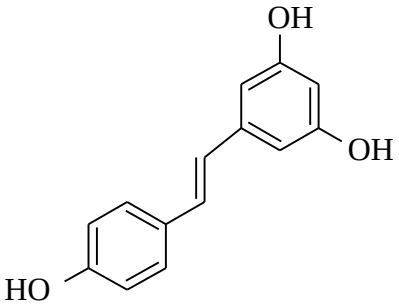
The photo-protective effect of the terpenoid β-damascenone was investigated *in vivo* and it was found that 20 μL of orally administered β-damascenone was able to protect UV light exposed mice from sunburn [165]. Tea tree oil is an example of a commercial product that contains varied terpenoids, and is extracted from *Melaleuca alternifolia.* Tea tree oil has been found to exhibit some anti-cancer activity and is relatively safe if taken in low concentrations, thus making it an ideal candidate for further investigation with respect to its anti-cancer potential [166]. Actions of home remedies of tea tree oil, as reported by the public, include being effective against actinic keratosis, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Tea tree oil in 10% dimethylsulphoxide resulted in a direct cytotoxic effect on tumor cells and induced local immune activation when applied topically. Studies performed on subcutaneous mesothelioma in mice revealed that one day after topical application of tea tree oil, the

tumor cells had shown signs of damage and death, such as compressed nuclei, contracted chromatin and swollen mitochondria. By day three the mitochondria had burst and the endoplasmic reticulum had dissolved. These results serve as an indication that topical application of tea tree oil could potentially be used in skin cancer therapy [167].

3.5. Resveratrol

Resveratrol is a plant phytoalexin that is classified as a group A stilbene. Stilbenes are part of the stilbenoid family of compounds found in nature that are characterized by two aromatic rings, being joined by a methylene bridge [168]. Resveratrol (Figure 5) is a model stilbene and possesses a number of pharmacological activities, such as cardio-protection, chemo-prevention and anti-tumor activities [169]. The main source of resveratrol is grape vine skin, where it serves to protect the plant from bacterial (*Bothrytis cinerea*) infection. Other sources are Japanese knotweed (*Polygonum cuspidatum*) and Mojave yucca plant (*Yucca schidigera*). It is believed that resveratrol is capable of affecting the carcinogenesis process in the tumor initiation, -promotion and -progression phases [170].

Figure 5. Chemical structure of resveratrol.



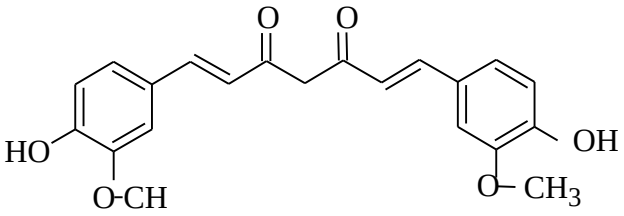
Resveratrol has been investigated as an anti-cancer agent and it has been found that it is capable of inhibiting the growth of melanotic and amelanotic cells through apoptosis induction [171]. The potency of resveratrol has been demonstrated by its ability to induce apoptosis in doxorubicin resistant murine melanoma cells and its ability to inhibit growth of doxorubicin resistant melanoma tumors in mice [172]. Resveratrol possesses some anti-metastatic potential, as it has been reported to inhibit lipopolysaccharide induced epithelial to mesenchymal transition, possibly by inhibiting NF-κB signaling [173]. There is also potential for application of resveratrol as a radiation sensitizer in melanoma treatment, as it has been observed that radio resistant melanoma cells had responded well to a treatment combination of resveratrol and radiation [174]. The combined treatment showed better results, compared to treatment with either radiation, or resveratrol alone. A combination of resveratrol and temozolomide has been found to act as an effective cytotoxic agent against melanoma cells *in vitro*, however, the *in vitro* effects were not translatable into *in vivo* effects after intra-peritoneal administration [175]. This phenomenon was confirmed by the findings of Niles *et al.* [176], who reported that orally administered resveratrol had not inhibited melanoma tumor growth in mice [176]. It has been argued that the *in vivo* anti-cancer effects of resveratrol are strongly limited by its low bioavailability [177]. It seems that resveratrol works well in combination with other treatment

modalities, as it has also been reported that resveratrol sensitizes melanoma cells to interleukin (IL) 2 immunotherapy induced cell death [178].

3.6. Curcumin

Curcumin (Figure 6) is a yellow plant polyphenol that has been used over the years as a spice and as a medical agent. The main medicinal properties being attributed to curcumin are its anti-inflammatory and anti-oxidant activities. *Curcuma longa*, better known as turmeric, is the main source of curcumin. The curcumin being used in some studies usually contains a mixture of curcuminoids in which only 2%–6% comprise of curcumin. Commercial grade turmeric contains ~80% curcumin, which may account for any inconsistencies in outcomes, as observed among different studies [179]. Although curcumin has not been approved for the treatment of any disease, it has been established that curcumin exhibits some efficacy against various diseases (epilepsy, cancer, human immunodeficiency virus, diabetes and psoriasis), whilst also having a good safety profile at gram doses [180]. The possible application of curcumin as a drug for controlling and treating cancer symptoms, such as pain, depression, fatigue and neurodegeneration, has been investigated by various researchers and seemed promising [181].

Figure 6. Chemical structure of curcumin.



In a recent study it was found that the anti-melanoma activity of curcumin was dependent upon the opening of mitochondrial permeability transition pore (mPTP), as had been accomplished by curcumin in melanoma cells *in vitro* [182]. Curcumin is able to induce apoptosis that is independent of p53 activity in melanoma cells in a time and dose dependent manner. Treatment of melanoma cells with curcumin resulted in inhibition of the NF-κB pro-survival pathway and activation of the death receptor Fas-initiated Fas-Associated protein with Death Domain (FADD) apoptotic pathway via caspase 8 [183]. Studies were performed to observe the effects of topically applied curcumin on UVB induced carcinogenesis in mice. It was observed that both pre- and post UVB exposure to topical applications of curcumin had resulted in a delay in tumor appearance, and a reduction in tumor multiplicity and tumor volume, without causing any toxic effects. Exposure to curcumin also resulted in an increase in the p53 tumor suppressor protein [184]. Oral administration of curcumin has been reported to cause down regulation of anti-apoptotic Bcl-2 and proliferating cell nuclear antigen (PCNA) in subcutaneous melanoma tumors, possibly regulated by microRNA [185]. Besides inhibiting growth of melanoma, curcumin inhibited growth of squamous cell carcinoma tumor through inhibition of ribosomal S6 phosphorylation [186].

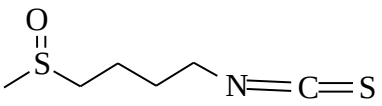
Curcumin is a hydrophobic compound with low solubility and oral bioavailability, hence presenting challenges for use in the possible treatment of illnesses. Research is being carried out regarding the incorporation of curcumin into nanoformulations, such as liposomes, cyclyodextrins, solid dispersions

and lipid nanoparticles, aimed at increasing its solubilization [187,188]. A comparison was made between the physicochemical properties of ethosomes, traditional liposomes and propylene glycol liposomes and their prospective uses as curcumin transdermal delivery vehicles. Propylene glycol liposomes showed favorable characteristics with respect to curcumin release profiles, particle size and encapsulation efficiency [189]. A microemulsion drug delivery system, comprising of limonene, has been reported to have promising properties for the transdermal delivery of curcumin. Successful formulation of a curcumin transdermal microemulsion should allow for the optimized delivery of curcumin to the skin where it would exert its anti-melanoma effects [190]. Moorthi and Kathiresan [191] have proposed the use of dual drug loaded (*i.e.*, curcumin-piperine, curcumin-quercetin, or curcumin-silibinin) nanoparticulate formulations in order to address the risk of multi-drug resistance formation, water insolubility and low bioavailability [191].

3.7. Sulforaphane

Sulforaphane is an isothiocyanate found in cruciferous vegetables, such as broccoli (*Brassica oleracea* var. *italic*), cabbage (*Brassica oleracea* var. *capitata*), radish (*Raphanus sativus*), kale (*Brassica oleracea* var. *acephala*) and cauliflower (*Brassica oleracea* var. *botryitis*) [192]. The chemical structure of sulforaphane is shown in Figure 7. The anti-cancer activities of sulforaphane being reported include apoptosis induction, inhibition of cell proliferation and the inhibition of metastasis. Studies have been performed on melanoma cells to confirm whether sulforaphane would exhibit these activities in melanoma cells.

Figure 7. Chemical structure of sulforaphane.



Sulforaphane, like other compounds, acts on various receptors and pathways in order to induce apoptosis in melanoma cells. Sulforaphane induces apoptosis by up regulating caspase 9, caspase 3, p53 protein and the Bax gene. It also down regulates Bcl-2, Bid and caspase 8, and thus inadvertently works against the apoptosis process [193,194]. *In vivo* studies on the actions of sulforaphane in a murine melanoma model have revealed that sulforaphane is anti-metastatic and has potential for application in cancer immunotherapy. Sulforaphane inhibits metastasis by stimulating the cell mediated immune response, by up regulating IL-2 and interferon gamma (IFN-γ), while down regulating IL-1β, IL-6, TNF-α and *granulocyte-macrophage colony-stimulating factor* (GM-CSF) [195,196].

Sulforaphane is reported to be unstable at temperature conditions between 60–90 °C, has a very short half-life and a low bioavailability, which makes it unsuitable for formulation into a clinically effective product [197,198]. Do *et al.* [198] investigated the potential of albumin microspheres as sulforaphane drug delivery agents in mice. Mice were subcutaneously injected with B16 melanoma cells to form tumors. The mice were then treated with sulforaphane microspheres through intra-peritoneal injection and it was observed that sulforaphane from the microspheres had a sustained inhibitory effect on tumor growth, compared to the sulforaphane solution [198]. In a later study, sulforaphane loaded magnetic microspheres

were formulated. Compared to the albumin microspheres, the magnetic microsphere increased therapeutic efficiency by 10% [199].

4. Anti-melanoma Activity of Crude Plant Extracts

4.1. Hypericum perforatum

Hypericin, a penanthroperylene quinone and known for its photo-sensitizing effects, is one of the active components of St. John's Wort (*Hypericum perforatum*). The concentration and light dose dependent photo-sensitizing effects of hypericin make it an ideal candidate for use in photodynamic therapies (PDT) of skin cancers. Davids, *et al.* [200] found that hypericin had been activated by UVA and resulted in melanoma cell death through the processes of apoptosis and necrosis [200,201]. However, the photo-sensitizing effects of clinically used aminolevulinic acid methyl ester have been found to be much superior to the effects of hypericin in photodynamic therapies [202]. This may have been due to its properties, such as low solubility and low stability in solution, which are relatively unfavorable for topical application [203]. A hydro-alcoholic extract of *H. perforatum* was tested against human malignant melanoma cells and it was observed to inhibit free radical formation, inhibit cell proliferation and to enhance UVA induced photo-toxicity [204]. According to Skalkos, *et al.* [203], a polar methanolic extract of *H. perforatum* has good physical and fluorescence properties, which make it an ideal candidate for further investigation for use as a PDT photosensitizer and thus for possible use in PDT of skin cancer [203].

4.2. Withania somnifera

Ashwaghanda or Indian ginseng (*Withania somnifera*) is a plant from the Solanaceae family and is the main source of a group of potent medicinal compounds, called withanolides. Two melanoma cell lines were treated with natural and derivative withanolides and an anti-proliferative effect was observed [205]. The most powerful and commonly studied withanolide is withaferin A (WA) and it is reported that WA could be useful in hyperthermia treatment of melanoma. Withaferin A resulted in an increased tumor response during hyperthermia treatment, whilst a decrease in the extent of thermo-tolerance was observed [206]. The combination of radiotherapy, hyperthermia treatment and a non-toxic dose of WA has been suggested as a possible alternative for melanoma therapy, following findings that such a combination had resulted in a better therapeutic outcome than radiation alone in a murine model [207]. Withaferin A alone induces apoptosis in melanoma cells by initiating production of ROS and by down regulation of Bcl-2 [208], while a methanolic extract of *Withania somnifera* has been found to inhibit metastasis in a murine melanoma model [209].

4.3. Melaleuca alternifolia

Tea tree oil, extracted from *Melaleuca alternifolia*, is well known for its various medicinal properties, especially with regards to the skin. The main active component of *M. alternifolia* is terpinen-4-ol and its anti-cancer activity has been researched. Terpinen-4-ol and tea tree oil have been reported to inhibit melanoma cell growth *in vitro* through cell cycle arrest, apoptosis, necrosis and inhibition of cell proliferation at doses that are not toxic to normal fibroblast cells [210]. The effect of topical application

of 10% tea tree oil in dimethylsulphoxide to subcutaneous melanoma tumor bearing mice was also investigated and it was reported that a significant inhibition in tumor growth had been observed [211]. Generally, oil from *M. alternifolia* and its terpene components have proven to inhibit the *in vitro* and *in vivo* growth of melanoma cells and tumors [211,212].

4.4. Zingiber officinale

Zingiber officinale (ginger) is a monocotyledonous herb, which exists as a rhizome. An active component of *Zingiber officinale*, [6]-gingerol, has been investigated for its anti-cancer potential in melanoma and epidermoid carcinomas cells (SCC). [6]-Gingerol potently inhibited VEGF induced angiogenesis, but had no direct effect on the melanoma cells [213]. The effects of [6]-gingerol on epidermoid carcinoma cells have been reported to include growth inhibition, anti-proliferative and apoptosis induction. The apoptotic action seems to be regulated by ROS [214]. According to these reports, [6]-gingerol inhibits melanoma tumor growth by affecting the venous supply to the tumor, but it is capable of causing cell death through apoptosis in SCC.

4.5. Viscum album

Viscum album (*V. album*), well known as mistletoe, has been reported to improve the quality of life of cancer patients, and have shown some anti-cancer and immuno-stimulatory activities. Most studies have been performed on European mistletoe (*Viscum album loranthaceae*), but activities of Korean mistletoe (*Viscum album coloratum*) are also reported on. Lectins, isolated from Korean mistletoe, are reported to have prophylactic and therapeutic metastasis inhibitory actions. The anti-metastatic action is thought to occur by way of natural killer cell and macrophage activation [215,216]. Anti-angiogenic activity at the primary and metastatic sites is also responsible for the anti-metastatic effect of Korean mistletoe [217]. European mistletoe lectins directly act on human melanoma cells, causing dose dependent apoptosis [218]. These lectins were found to inhibit tumor growth in mice, due to the immuno-modulatory effects of interleukin-12 that enhance the functions of T-cells and NK-cells [219]. A terpene extract of European mistletoe mainly containing oleanolic- and betulinic acid is capable of inducing early stage apoptosis and late stage necrosis in melanoma cells [220]. In a comparative, epidemiological cohort study, the influence of subcutaneous injection (2–3 times/week) of *V. album* to patients suffering from stage II–III primary malignant melanoma, who had been surgically treated, was observed. Long-term treatment with *V. album* seemed to have significant survival benefits [221]. A case study of a patient suffering from metastatic malignant melanoma has shown that subcutaneous mistletoe therapy seems safe and could possibly result in a complete remission of metastasis [222].

4.6. Calendula officinalis

Extracts from the flowers of *Calendula officinalis* (*C. officinalis*) (marigold) are well known for their anti-inflammatory and anti-cancer properties. Jimenez-Medina *et al.* [223] reported that a laser activated extract of *C. officinalis* had inhibited cell growth in murine melanoma cells and had also inhibited tumor cell proliferation in human cells [223]. Some triterpene glycosides from *C. officinalis* extract exhibited potent cytotoxicity against melanoma and further studies on the individual compounds, or combinations

thereof are recommended [224]. Marigold was reported to have an anti-cancer effect that is highly selective for human melanoma Fem-x cells, with 50% inhibitory concentration (IC_{50}) values of 0.36 ± 0.12 mg/mL, compared to those for HeLa cells (0.75 ± 0.21 mg/mL) and for human colon carcinoma cells (2.30 ± 0.08 mg/mL). Marigold tea showed higher anti-melanoma action, compared to chamomile (*Matricaria chamomilla*) tea, with an IC_{50} value above 16.67 mg/mL [225]. Orally administered *C. officinalis* extract furthermore showed anti-metastatic effects that led to an increase in the life span of metastatic tumor bearing mice [226].

4.7. Rosmarinus officinalis

Rosmarinus officinalis (*R. officinalis*) is a herbal, evergreen plant, commonly referred to as rosemary. It contains phenolic diterpene and triterpene anti-oxidants [227]. Carnosol, a phenolic diterpene, is an active constituent of *R. officinalis* extract. In a study by Huang, *et al.* [228], carnosol had inhibited the migration of metastatic murine melanoma cells into a basement membrane gel and this effect was attributed to the inhibition of MMP-9. Carnosol was also reported to decrease cell viability and cell growth at high concentrations [228]. Another active constituent of *R. officinalis* is the pentacyclic triterpene, *i.e.*, ursolic acid. Ursolic acid has been observed to stimulate expression of the p53 protein and to inhibit activation of NF-κB in human and murine melanoma cells, which in turn resulted in apoptosis [229,230]. In another study, ursolic acid had enhanced apoptosis, induced by radiotherapy, thus indicating a possible role for it as an adjuvant to radiotherapy in the treatment of melanoma [231]. The radiation therapy potentiating effects of ursolic acid were established to have been partly caused by p53 activation through adenosine monophosphate activated protein kinase/mitogen activated protein kinase (AMPK/MAPK) signaling [232]. Additionally, ursolic acid has been classified as a potent anti-angiogenic agent in melanoma, due to its inhibitory effects on the production of VEGF, MMP-2, MMP-9 and nitric oxide [233].

4.8. Aloe Species

Aloe is a plant that is well-known for its medicinal properties and specifically for applications, such as wound healing, as a laxative and for the treatment of skin irritations [26]. Murine melanoma cells were treated with saline extracts of *Aloe vera* and was it observed that such treatments had reduced cell viability in a concentration dependent manner [234]. Emodin, an active component of Aloe, is a natural hydroxyanthraquinone, which has been studied for its possible anti-melanoma properties. The anti-melanoma effects of aloe-emodin included time dependent anti-proliferation and inhibition of MMP-9. Aloe-emodin treatment had resulted in the interference of murine melanoma cell aggregation, migration, invasion and adhesion, which presented as anti-metastatic activities [235]. During a study by Radovic, *et al.* [236], they determined that aloe-emodin had reduced the growth of human and murine melanoma cells and had promoted cell differentiation. Unexpectedly, in the presence of other toxic stimuli (*i.e.*, doxorubicin and paclitaxel), aloe-emodin had antagonized the cytotoxic actions of the toxic stimuli and was it cyto-protective instead [236]. Another compound found in Aloe, namely aloin, was reported to have significantly inhibited melanoma cell growth, interfered with cell adhesion processes and had sensitized melanoma to the cytotoxic agent, cisplatin [237].

4.9. Artemisia Species

Artemisia is a plant genus consisting of over 500 different species of aromatic herbs or shrubs. Eupatilin, a flavonoid that is isolated from *Artemisia* species, has shown to inhibit cell growth, induce apoptosis and induce G2/M cell cycle arrest in human melanoma cells [238]. Other previously studied artemisin derived compounds are dehydroleucodine, dehydroparishin-B from *A. douglasiana* (California Mugwort) and ludartin from *A. amygdalina* (almond wormwood). Dehydroleucodine and dehydroparishin-B have been found to block cell proliferation and inhibit murine melanoma cell migration [239]. Ludartin is also reported to have cytotoxic activity against human epidermoid carcinoma (IC_{50} of 19.0 μM) and mouse melanoma cells (IC_{50} of 6.6 μM) [240]. Besides individual compounds, *Artemisia* essential oils have also been tested for cytotoxic activity. Essential oils extracted from *A. anomala* (diverse wormwood herb) were tested for cytotoxic activity against melanoma and were reported to have an IC_{50} value of 0.2 μL of oil per mL [241].

4.10. Alpinia Species

Extracts from various *Alpinia* species have been tested for possible anti-cancer activities. Extracts from *A. oxyphylla* (sharp leaf galangal), *A. galangal* (greater galangal) and *A. officinarum* (lesser galangal) have in particular been tested for possible anti-melanoma activities. An extract of *A. oxyphylla* from supercritical fluid carbon dioxide was found to act as an anti-proliferation agent in human melanoma cells [242]. Two compounds extracted from *A. galangal*, *i.e.*, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one and bisdemethoxycurcumin, are reported to have significantly inhibited melanoma cell proliferation [243]. Galangin, a compound from *A. officinarum*, had suppressed cell proliferation and induced apoptosis via the mitochondrial pathway and through activation of p38 MAPK [244]. In another study it was further determined that galangin had inhibited cell adhesion, spreading, motility and lamellipodia formation *in vitro*. *In vivo*, galangin was reported to have inhibited lung metastasis in a mouse melanoma model [245].

5. Conclusions

From this review, is has become clear that naturally derived compounds could very likely become key role players in future melanoma treatments. This article has summarized some of the compounds and plants that have been studied to date for their possible anti-cancer properties. Many more untapped resources, however, remain in nature. Phytochemicals have been reported to possess numerous health benefits and ongoing research is conducted to determine their physiological effects. Anti-cancer activities of plants can be ascribed to a distinct compound, or to a combination of the effects of different compounds in the crude extract and/or in the human body. Although all of the compounds and crude extracts, discussed in this review, are reported to have demonstrated some form of anti-cancer activity *in vitro*, most of these identified actions must still be clinically proven for indeed causing favorable clinical effects in humans.

Traditional use of natural compounds in cancer treatment is relatively cheap due to the availability of plants and the simple methods used in product preparation. However, commercialization of natural compounds for cancer treatment may result in dwindling of natural resources and problems with

producing a consistent quality of adulteration. Due to this, most naturally derived medicinal compounds are eventually manufactured semi-synthetically, through *in vitro* cultivation or fermentation technology for commercial use then formulated into an appropriate dosage form which increases costs. Similar to conventional drugs herbal medicinal products can be toxic if they accumulate beyond the acceptable level in the human body. Another safety issue which exists is misidentification of a plant coupled with unavailability of analytical methods to confirm plant species which may lead to adverse effects [17]. There are various advantages to traditional use of herbal remedies but it is vital for toxicology data to be available in order to avoid toxicity issues. Scientific research is needed in order to optimize the herbal medicinal products for safe human use.

Since some natural products have shown the potential for use in the symptomatic treatment of cancer, or to treat the adverse effects associated with cancer therapies, this has led to an increase in self-medication by cancer patients, seeking safer and more effective products. In certain cultures, traditional healers have been reported to formulate herbal mixtures of unpurified and non-quantified extracts, which are given as tinctures for the treatment of diseases, such as cancer, without any scientific evidence of the efficacy and safety of these natural medicines. A need for sound scientific research to bridge the gap between certain medicines from natural origin and conventional prescription medicines therefore exists. Patients often associate natural products with safety, but many herbal or natural products are not necessarily safe, as they can cause adverse effects, either alone, or due to an interaction with other substances/drugs in the body. Practitioners must therefore be educated in the science of herbal medicines, so that they can appropriately advise their patients regarding the safe and suitable use of complementary and alternative medicines [246,247].

Assessing the quality of herbal or supplemental therapies offered by complementary and alternative medicines is quite a challenge, as some therapies are individually tailored. Concern also exists with respect to the concentrations used and the standardization of formulations, as most natural products available on the market are not classified as medicines and are thus not regulated by any monitoring body. Due to the lack of regulation, large variations in formulations and bioactivity among batches are expected, since the active components, minimum effective concentrations and maximum safe concentrations are usually unknown [248]. Healthcare professionals are advised to practice pharmaco-vigilance in order to detect possible adverse interactions between prescription medication and non-conventional medicine. It is also suggested that large, randomized trials should be conducted that would assist in determining the effectiveness of herbal medicines [249]. The US National Institute of Health (NIH), National Centre for Complementary and Alternative Medicine (NCCAM) is making strides to determine the safety and effectiveness of various CAM botanical therapies through funding of researchers [250]. There has been an increase in research on the safety and effectiveness of CAM and the expectation is that an increase in the availability of accurate information on the web will result in informed decisions and good outcomes for patients interested in CAM [251–253]. In light of this, online news sites have been urged to ensure that they report accurate news with respect to CAM, as many people seek health advice on the internet [254].

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Author Contributions

All the named authors played an active role in the planning and writing of this publication. TN Chinembiri is the student working on this project. She performed the literature search and analysis. She wrote this manuscript in order to form part of her thesis. J du Plessis has 30 years' experience in topical and transdermal drug delivery which includes a vast amount of experience in the field of delivery of drugs through the skin and she is the supervisor of the study. M Gerber has expertise in transdermal drug delivery kinetics as well as an in depth knowledge of pharmaceutical chemistry and synthesis of drug analogues. LH du Plessis is a molecular biologist. She has vast experience in *in vitro* efficacy testing of compounds. JH Hamman has extensive experience in natural medicines. All the named authors contributed to the final manuscript as it was submitted to the journal be it by analysis of information, organizing of data and construction of text.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3: Research article for publication in Pharmacognosy Magazine

This thesis is written in the article format and Chapter 3 is a manuscript that has been submitted to the “Pharmacognosy Magazine” for publication. The guidelines for authors are included in this thesis as Appendix G. The writing style and formatting of this chapter is therefore different from the rest of the thesis as it follows the journal guidelines.

Topical Delivery Of *Withania Somnifera* Crude Extracts In Niosomes And Solid Lipid Nanoparticles

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ABSTRACT

Background: *Withania somnifera* is a medicinal plant native to India and is known to have anti-cancer properties. It has been investigated for its anti-melanoma properties and since melanoma presents on the skin it is prudent to probe the use of *W. somnifera* in topical formulations. In order to enhance topical drug delivery and to allow for controlled release, the use of niosomes and solid lipid nanoparticles (SLNs) as delivery vesicles was explored. **Objective:** To determine the stability and topical delivery of *W. somnifera* crude extracts encapsulated in niosomes and solid lipid nanoparticles. **Materials and Methods:** Water, ethanol and 50% ethanol crude extracts of *W. somnifera* were prepared using 24 h soxhlet extraction which were each encapsulated in niosomes and SLNs. Franz cell diffusion studies were conducted with the encapsulated extracts to determine the release and skin penetration of the phytomolecules, withaferin A and withanolide A. **Results:** The niosome and SLN formulations had average sizes ranging from 165.9 ± 9.4 to 304.6 ± 52.4 nm with the 50% ethanol extract formulations having the largest size. A small particle size seemed to have correlated with a low encapsulation efficiency of withaferin A, but a high encapsulation efficiency of withanolide A. There was a significant difference ($p<0.05$) between the amount of withaferin A and withanolide A that was released from each of the formulations, but only the SLN formulations managed to deliver withaferin A to the stratum corneum-epidermis and epidermis-dermis layers of the skin. **Conclusion:** SLNs and niosomes were able to encapsulate crude extracts of *W. somnifera* and release the marker compounds, withaferin A and withanolide A, for delivery to certain layers in the skin.

KEYWORDS: Ashwagandha; niosomes; skin diffusion; solid lipid nanoparticles; stability; tape-stripping, *Withania somnifera*.

INTRODUCTION

Withania somnifera (also known as Ashwagandha, Indian ginseng or winter cherry) is a plant well-known for its diverse medicinal properties in the Ayurveda system of natural medicine. Extracts from the plant leaves have a high anti-oxidant potential and they contain a high concentration of bioactive compounds.(Kulkarni & Dhir, 2008; Fernando *et al.*, 2013) Therefore, the plant leaves were used in this study as the aim was to prepare formulations that contain different *W. somnifera* extracts for potential use in the treatment of skin conditions such as skin cancer (melanoma) and ageing. The main bioactive compounds in *W. somnifera* are steroidal lactones collectively known as withanolides.(Ganzera *et al.*, 2003; Kulkarni & Dhir, 2008) Throughout this study, the main focus was on withaferin A and withanolide A as bioactive marker molecules, which are known to be present in the leaves of *W. somnifera*.(Malik *et al.*, 2009)

Some of the medicinal properties of *W. somnifera* that have been identified to date include anti-diabetic, anti-hypertensive, anti-bacterial, anti-ageing and anti-cancer properties.(Winters, 2006; Nagella & Murthy, 2010) The plant extract is currently available on the market as a powder, tonic and as capsules.(Kulkarni & Dhir, 2008) In this study, it was decided to encapsulate different *W. somnifera* crude extracts in niosomes and solid lipid nanoparticles (SLN) for topical delivery to the skin.

The skin is the body's first line of defence and is thus rather impermeable to any foreign substances.(Prow *et al.*, 2011; Lam & Gambari, 2014) Nanovesicles such as niosomes, SLNs, liposomes, ethosomes and ufosomes are being investigated for use in the delivery of medicinal compounds to and through the skin.(Manosroi *et al.*, 2013; Yeh *et al.*, 2013) Nanoparticles are advantageous in cancer therapy because they can aid in the transport of therapeutic agents through

barriers such as the skin, improve the pharmacokinetic profile of medicinal agents and they can be used for targeted drug delivery (e.g. dermal vs transdermal delivery).(Sanna *et al.*, 2013a; Estanqueiro *et al.*, 2015) Niosomes are known to enhance the absorption of compounds through the skin, increase physico-chemical stability of compounds and protect the skin from the potential irritating effects of medicinal compounds.(Paolino *et al.*, 2008; Manosroi *et al.*, 2012) Various plant extracts have been successfully encapsulated in niosomes and delivered to the skin,(Manosroi *et al.*, 2013; Yeh *et al.*, 2013) hence the use of niosomes in the topical delivery of *W. somnifera* crude extract. Solid lipid nanoparticles have been reported to be suitable for topical drug delivery, resulting in reduced systemic delivery of medicinal compounds due to controlled and targeted drug delivery.(Jain *et al.*, 2010; Musicanti & Gasco, 2012)

The aim of this study was to prepare three different *W. somnifera* crude extracts and encapsulate these extracts in niosomes and SLNs for use in Franz cell diffusion studies. A stability assessment of the formulations was conducted in order to determine if certain marker molecules in the extracts remained stable in the niosomes and SLN.

MATERIALS AND METHODS

Materials

The withaferin A and withanolide A USP analytical standard compounds were purchased from ChromaDex (Irvine, California, USA). Ethanol and methanol for plant extractions and analytical standard preparation were purchased from Associated Chemical Enterprises (Johannesburg, South Africa). High Performance Liquid Chromatography (HPLC) grade acetonitrile and deuterated chloroform ($CDCl_3$) were obtained from Merck Chemicals (Johannesburg, South Africa). The

Compritol 888 ATO (glyceryl dibehenate) that was used for formulation of the SLN was a generous gift from Gattefossè (Lyon, France).

Preparation of plant extracts

W. somnifera leaves were purchased from Mountain Herb Estate Nursery (Kameeldrift-West, Pretoria, South Africa) and authenticated at the South African National Biodiversity Institute (SANBI) National Herbarium (Pretoria, South Africa). Plant leaves were cleaned, air-dried then crushed to a fine powder upon receipt. A 24 h soxhlet extraction was used to prepare three separate crude extracts from the leaf powder using water, ethanol and ethanol/water (50:50) as the solvents. After the soxhlet extraction the ethanol was evaporated using a rotary evaporator and the water was removed by using a freeze dryer (VirTis, Gardiner, NY, USA). The dry end-products were stored in glass containers, protected from light at -20 °C.

Chemical characterisation of Withania somnifera extracts with nuclear magnetic resonance spectroscopy

For each individual extract, approximately 50 mg of plant extract was weighed out and dissolved in 1.5 ml deuterated chloroform then filtered into a nuclear magnetic resonance (NMR) tube to remove any undissolved residue. Both ^{1}H-NMR and C^{13}-NMR spectra were obtained using an Avance III 600 Hz NMR Spectrometer (Bruker, Rheinstetten, Germany).

Chemical characterisation of Withania somnifera extracts with High Performance Liquid Chromatography

The high performance liquid chromatography (HPLC) analytical method was developed in the Analytical Technology Laboratory (ATL) of the North-West University, Potchefstroom, South

Africa. This method was used for chemical finger-printing of the plant extracts and for the detection of the marker compounds (withaferin A and withanolide A) throughout the study. The separation was carried out on an Agilent 1100 series HPLC equipped with a quarternary gradient pump, autosampler, diode array detector andChemstation A.10.01 data acquisition and analysis software (Agilent, Palo Alto, CA) on a Venusil XBP C18(2), 150 x 4.6 mm, 5 µm column (Agela Technologies, Newark, DE). A gradient elution method was used in which mobile phase A was HPLC-grade water and mobile phase B was 100% acetonitrile. The run was started at 10% acetonitrile with a linear gradient to reach 100% acetonitrile after 10 minutes and holding to 20 minutes before re-equilibrating at the start conditions. The flow rate, injection volume, detection wavelength and stop time were set to 1 ml/min, 50 µl, 210 nm and 22 min respectively. The standard solutions and samples for chemical finger-printing were all prepared using analytical grade methanol and HPLC-grade water.

In order to obtain a chemical finger-print of *W. somnifera* crude extracts 10 mg of plant extract was dissolved in 1 ml of methanol with the aid of sonication and topped to 10 ml using Milli-Q water. The resulting solution was filtered then analysed using HPLC.

Formulation of niosomes and solid lipid nanoparticles

The solvent injection method was utilised for formulation of both the niosomes and SLN. Pando *et al.* reported that the solvent injection method for niosome formulation resulted in a higher resveratrol encapsulation efficiency and higher stability. Pre-formulation studies confirmed that this method of preparation of the nanoformulations was acceptable for encapsulation of the *W. somnifera* crude extracts.(Pando *et al.*, 2015)

For the niosomes, a 2:1 mixture of surfactant (tween 80/span 60) and cholesterol (w/w) was dissolved in diethyl ether, while the aqueous phase was heated to 60 ± 2 °C. The diethyl ether solution was slowly injected using a hypodermic needle into the pre-heated aqueous phase. When it came to the SLNs, a 2:1:1 mixture of surfactant, compritol 888ATO and L-α-phosphatidylcholine (w/w) was weighed and dissolved in the organic solvent. This organic phase was then slowly injected into a pre-heated (60 ± 2 °C) aqueous phase. The organic and aqueous phases were continuously magnetically stirred and the temperature maintained at 60 ± 2 °C until the organic solvent was driven off. The resulting formulation was cooled and sonicated on ice using a Hielscher UP 200ST sonicator (Hielscher Ultrasound Technology, Teltow, Germany). The ethanol and 50% ethanol extracts (2.0% w/w) were added to the organic phase, while the water extract was added to the aqueous phase prior to the injection step. Zorzi *et al.*, advise that a maximum of 2.0% crude extract should be incorporated into nanoformulations.(Zorzi *et al.*, 2015) In total six formulations were prepared as one niosome and one SLN formulation was prepared for each extract.

Physico-chemical characterisation of formulations

The physico-chemical characteristics of the niosomes and SLN formulations that were assessed in this study include morphology, particle size, zeta-potential, polydispersity index (PDI), pH and encapsulation efficiency (withaferin A and withanolide A). Transmission electron microscopy (TEM) was used to visualise the morphology of the formulations. Zeta-potential, size and PDI were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Approximately 1 ml of each formulation was injected respectively into a disposable folded capillary cell for zeta potential measurement and the reading was taken using the Zetasizer Nano ZS. Freshly prepared formulations had their pH measured at 25 °C using a Mettler Toledo pH

meter (Mettler Toledo, Columbus, OH, USA). Encapsulation efficiency of the formulations was determined according to the method as described by Junyaprasert *et al.*(Junyaprasert *et al.*, 2008) Briefly, the formulations were centrifuged in an Optima L-100XP ultra centrifuge (Beckman Coulter, Brea, California, USA) for 30 min at a speed of 30 000 rpm and temperature of 4 °C. The supernatant was then diluted and analysed using the HPLC analytical method for withaferin A and withanolide A. The percentage encapsulation efficiency (%EE) was then calculated as follows

$$\%EE=\left(\frac{\text{Total amount of compound added - Free amount of compound}}{\text{Total amount of compound}}\right)x100\%$$

Stability testing of formulations

A three month temperature stability assessment was conducted on lyophilised niosomes and SLNs. Niosomes and SLNs were formulated according to the described method, lyophilised using a VirTis freeze-dryer (Gardiner, NY, USA) and stored at room temperature for three months. The formulations were kept in temperature controlled laboratories at a temperature of 22 °C. The formulations were re-suspended in Milli-Q water then particle size, zeta-potential, pH and percentage encapsulation efficiency were measured after 0; 7; 14; 28; 56 and 84 days.

Skin preparation for skin diffusion studies

Caucasian, female, abdominal skin obtained from abdominoplasty patients was used for the skin diffusion studies. Informed consent was obtained from each patient and the NWU Research Ethics Committee gave approval for obtaining, preparing and using human excised skin for research purposes (Ethical approval number – NWU-00114-11-A5). The collected skin was inspected for imperfections such as holes and stretch marks so that such areas would be excluded from the experimental skin samples. Split-thickness skin at a thickness of 400 μm was prepared using a

Zimmer® dermatome (Warsaw, IN, USA). The skin was placed on Whatmann® filter paper, wrapped in foil, placed in Ziploc® bags then frozen at -20 °C for not more than 3 months.

Franz cell diffusion studies

Franz cell membrane diffusion studies were done in order to determine the withaferin A and withanolide A release characteristics of the niosomes and SLNs. Subsequent to the membrane diffusion studies, skin diffusion studies were performed in order to assess the diffusion of withaferin A and withanolide A into and through the skin. Static Franz diffusion cells with a diffusion area of 1.075 cm^2 and receptor capacity of at least 2 ml were used for the membrane diffusion studies and the skin diffusion studies.

The formulations were pre-warmed to 32 °C (temperature at the surface of the skin)(Williams, 2003) and phosphate buffer solution (0.06 M NaOH and 0.08 M KH_2PO_4, pH 7.4) was pre-warmed to 37 °C (physiological temperature) in appropriately set water baths. This was done in order to mimic *in vivo* conditions.(Williams, 2003; Clares *et al.*, 2014) The donor and receptor compartments were greased with Dow Corning® vacuum grease and a magnetic stirring rod was placed inside the receptor compartment. A 0.45 µm polytetrafluoroethylene membrane filter (Whatmann plc, Maidstone, UK) or piece of skin (stratum corneum facing the donor compartment) was placed between the donor compartment and receptor phase. To avoid leaks, the two compartments were sealed and fastened together using vacuum grease and a horseshoe clamp. Two millilitres of buffer solution were added to the receptor compartment and 1.0 ml of formulation was added to the donor compartment. Ten samples from the same skin donor (n=10) were setup and two Franz cells were setup with placebo formulations as the controls. The Franz cells were placed on a Franz cell stand in a 37 °C water bath with a Variomag® magnetic stirrer to

stir the receptor phase and keep it homogenous. The receptor phase was extracted at predetermined time intervals and replaced with fresh buffer. The extracted receptor phase was then analysed using HPLC. Extractions for membrane diffusion studies were done every hour up to 6 h while a single extraction after 12 h was done for the skin diffusion studies.

Tape-stripping studies

The tape-stripping technique was used to determine the amounts of withaferin A and withanolide A that had permeated into the different skin layers. This technique works by selectively removing the upper skin layer and analysing for the amount of compound within the stripped layer.(Surber *et al.*, 1999; Brain *et al.*, 2002) The method as described by Pellet *et al.*(Pellett *et al.*, 1997) was followed for the tape-stripping study. After the skin diffusion study was completed, the skin was cleaned using a paper towel to remove any unabsorbed drug. Thereafter a piece of 3M Scotch® Magic tape was applied to the diffusion area, removed and discarded to strip off any unabsorbed compound on the skin surface. The stripping process was repeated with 15 pieces of tape and these tape-strips were all placed into a polytop containing 5 ml of phosphate buffer solution. The remaining piece of skin was cut into small pieces to increase surface area and placed into a polytop containing 5 ml of phosphate buffer solution. This process was repeated for each Franz cell and the polytops were stored at 4 °C overnight. On the following morning the buffer solution was filtered into appropriately labelled HPLC vials and the samples were analysed using the HPLC analytical method.

Statistical analysis

Statistical analysis of the Franz cell diffusion data was done using Statistica data analysis software system (StatSoft, Inc. (2015), version 12). The mean and median flux values were calculated for

each experiment. A one-way and a two-way analysis of variance (ANOVA) were done together with t-tests in order to determine any significant differences within and between the different experiments.

RESULTS AND DISCUSSION

Chemical characterisation of Withania somnifera extracts

The HPLC analytical method was robust and suitable for use in the analysis of both withaferin A and withanolide A. Withaferin A eluted at approximately 7.5 min and withanolide A at 8.5 min. Figure I shows the chromatograms of the individual standard compounds and those of the crude extracts. Analysis of the crude plant extracts revealed that the withaferin A (w/w) content of the extracts was 0.98% w/w (water extract), 1.76% w/w (ethanol extract) and 4.55% w/w (50% ethanol extract), respectively. The withanolide A content of the three extracts was 5.04% w/w (water extract), 1.21% w/w (ethanol extract) and 3.04% w/w (50% ethanol extract), respectively.

Figure I: HPLC chromatograms of withaferin A and withanolide A standards (a), ethanol extract (b), 50% ethanol extract (c) and water extract (d) for HPLC fingerprinting

The ^{1}H-NMR and C^{13}-NMR spectra of the different *Withania somnifera* crude extracts are shown in Figure II and Figure III, respectively.

Figure II: ^{1}H-NMR spectra of water (a), 50% ethanol (b) and ethanol (c) crude extracts for NMR finger-printing

Figure III: C^{13}-NMR spectra of water (a), 50% ethanol (b) and ethanol (c) crude extracts for NMR finger-printing

Physico-chemical characterisation of formulations

The physico-chemical properties of all the formulations are summarised in Table I. The mean of three independent experiments is shown ± standard deviation (SD). Figure IV shows the TEM micrographs of the formulated placebo niosomes and SLNs.

Table I: Average values for the physicochemical properties of freshly prepared formulations ± SD (n=3)

Figure IV: Transmission electron micrographs of placebo niosomes (A, B) and placebo SLNs (C, D)

The 50% ethanol formulations displayed with relatively larger average particle sizes as compared to the other formulations. The different chemical compositions of the crude extracts possibly played a role as the 50% ethanol extract was expected to contain both polar and non-polar compounds due to the presence of both an aqueous solvent and an organic solvent during the extraction process. All the freshly prepared formulations had pH values that were between 5.017 and 5.709 which is considered safe for topical application as the skin's pH lies between 4 and 6.(Ali & Yosipovitch, 2013; Clares *et al.*, 2014) Solid lipid nanoparticles were generally the least homogenous and this was probably due to the high lipid content of the SLNs. It is possible that the lipids were affected by the energy released during the sonication process resulting in aggregation of some particles thus resulting in relatively higher PDI values. Peres *et al.*, found that a long sonication time (90 sec) resulted in an increase in particle size. This was possibly due to slight destabilisation which resulted in very small droplets that could not be completely covered by the surfactant in the formulation.(Becker Peres *et al.*, 2016) The presence of water-soluble compounds in the water extracts possibly contributed to the low absolute zeta-potential values of the water extract formulations by reducing the cohesive properties of the formulations. Use of a higher concentration of a high HLB surfactant may be able to improve the issues to do with the

stability of the water extract formulations. These water extract formulations had a very low percentage encapsulation of withaferin A but a high withanolide A percentage encapsulation. It is apparent that a change in formulation (SLN vs niosome) did not cause any major changes in particle size and encapsulation efficiency of withaferin A. The SLNs exhibited a slightly higher encapsulation of both withaferin A and withanolide A than the respective niosome formulations. This effect of the SLNs was more apparent for the withanolide A as compared to the withaferin A and it may have been due to the different physico-chemical properties of the two compounds. Both vesicle types managed to encapsulate withaferin A and withanolide A from all the extracts. The highest percentage encapsulation (95.3%) that was obtained was for withanolide A from the water extract SLNs. It is however possible that the non-encapsulated extract compounds could have been solubilised in the external aqueous phase or adsorbed on the surface of the carrier vesicles instead of being encapsulated in the vesicles.(Zorzi *et al.*, 2015)

Stability testing of formulations

The changes that transpired over the 84 day test period have been summarised in Table II. The changes in the pH values of the formulations ranged from 0.089 (50% ethanol extract SLNs) to 0.890 (ethanol extract niosomes) over the three month period. The final values were still within an acceptable range for topical application. Zeta potential measurements of some of the formulations fluctuated over the three month period with the changes per formulation ranging from 0.78 mV (ethanol extract niosomes) to 13.12 mV (water extract niosomes). The formulations which had the most electronegative initial zeta-potential values (ethanol extract niosomes and SLNs) exhibited the smallest fluctuations with respect to zeta potential measurements thus implying that these formulations were relatively stable colloidal systems. At the end of the three-month testing period all the average particle sizes were above 300 nm with the ethanol extract

niosomes having the smallest change (137.73 nm) and the 50% ethanol extract SLN having the largest size increase (1454.33 nm). The changes in the PDI values ranged from 0.001 (water extract SLN) to 0.185 (50% ethanol extract SLN). Changes in percentage encapsulation of withaferin A ranged from 2.03% (50% ethanol extract niosomes) to 26.00% (ethanol extract SLN) while the changes for withanolide A ranged from 0.72% (water extract SLN) to 37.61% (50% ethanol extract SLN). Percentage encapsulation efficiencies of both withaferin A and withanolide A generally varied the most with the SLN formulations as compared to the niosome formulations. SLNs stored at 4 °C are said to have better stability as compared to SLNs stored at room temperature, therefore the higher storage temperature may have been responsible for the instability that was observed.(Geszke-Moritz & Moritz, 2016) Stability problems of nanovesicles are usually due to post-formulation expulsion of the API and particle aggregation. To increase the stability of nanoformulations it may be necessary to increase surfactant content as this increases the physical stability of the nanoparticles and also results in a high concentration of smaller nanoparticles.(Shah & Khan, 2009:629) The relative instability of the formulations that was observed may have been due to the presence of sodium cholate which is capable of accelerating degradation of formulations in the long-term.(Mehnert & Mäder, 2012)

Table II: Mean initial (day 0) and final (day 84) physico-chemical values recorded for the stability study with an indication of percentage change over the period. A negative percentage indicates that absolute value dropped over the test period.

Initial physico-chemical characterisation was done on freshly prepared samples while the stability experiments were done after freeze-drying. The formulations in this study were freeze-dried in the absence of a lyoprotectant such as sucrose, mannitol or trehalose and this may have been responsible for some of the instability issues encountered.(Hua *et al.*, 2010:8) Reports have been

made that lyophilisation of nanoformulations can cause instability with respect to particle aggregation, physical properties, osmolarity, pH and drug loading.(Majuru & Oyewumi, 2009:614) The absence of lyoprotectants during freeze-drying can also affect the encapsulation efficiency of compounds in liposomes and a similar phenomenon may also occur with encapsulation in niosomes and SLNs.(Hua *et al.*, 2010:213) The presence of many unidentified compounds in the crude extract may have also contributed to the physico-chemical changes that were detected.

Franz cell diffusion studies

The average percentage release of withaferin A and that of withanolide A after the 6 h membrane diffusion was calculated after the membrane release experiment and is represented in Table III. Average cumulative amount of withaferin A released per unit area is also shown as is that of withanolide A.

Table III: Total amount of marker compound released as a percentage of initial amount in donor formulation and average cumulative amount of marker compound released after the 6 h membrane diffusion studies ± SD (n=10). A superscript letter signifies a significant difference between comparisons.

After the twelve hour skin diffusion study neither withaferin A nor withanolide A were detected in the receptor phase. This led to the assumption that the compounds had only been retained within the skin and had not permeated through the skin to reach receptor phase so the tape-stripping study was conducted to determine the quantities of these marker molecules in the distinctive layers of the skin.

Tape-stripping studies

The average concentration of each compound that was detected in the stratum corneum-epidermis and in the epidermis-dermis was calculated and tabulated in Table IV. A comparison was made between the amount of marker compound that reached the two skin layers and a statistically significant difference was detected. This implied that the difference was due to effect of the physical, biological and chemical differences between the stratum corneum-epidermis and epidermis-dermis. The extent of the skin penetration of the marker compounds was different for each formulation. Only the 50% ethanol extract SLNs managed to deliver both withaferin A and withanolide A to both the epidermis and dermis. Permeation to the epidermis-dermis level was only achieved by the SLN formulations. It is thus conceivable that the SLNs had a greater ability to deliver withaferin A and withanolide A to the deeper skin layers as compared to the niosomes. In any study it is imperative to select the most appropriate nanocarrier as it will result in the required amounts of API reaching the desired skin layers (Clares *et al.*, 2014).

The 50% ethanol SLNs were the only formulation which was able to deliver both withaferin A and withanolide A to the stratum corneum–epidermis and the epidermis–dermis. These results suggested that the 50% ethanol SLN formulation was the optimum formulation as it was capable of delivering the two marker compounds to the target skin layers for topical cancer chemotherapy. Melanoma penetrates vertically into the dermis prior to metastasising therefore delivery of an API into the dermis is ideal for potential skin cancer treatment. The 50% ethanol niosomes, however, depicted the highest average concentration of withaferin A in the stratum corneum-epidermis (1.364 µg/ml), followed by 50% ethanol SLNs (0.489 µg/ml), water SLNs (0.299 µg/ml), ethanol niosomes (0.298 µg/ml) and lastly the ethanol SLNs (0.061 µg/ml). The withaferin A content of the extracts influenced the permeation of withaferin A into the skin as is reflected by the 50% ethanol SLNs and niosomes resulting in the highest concentrations of withaferin A in the stratum

corneum-epidermis. The 50% ethanol extract contained 4.55% withaferin A which was considerably higher than the withaferin A content of the water (0.98%) and ethanol (1.76%) extracts.

With respect to withanolide A all the SLN formulations resulted in withanolide A reaching the stratum corneum-epidermis and the epidermis-dermis while the 50% ethanol extract niosomes only managed to result in withanolide A reaching the stratum corneum-epidermis. The SLNs were clearly superior to niosomes in terms of the ability to deliver withaferin A and withanolide A to the deeper skin layer (epidermis-dermis). This is similar to what was observed by Dwivedi *et al.*, in the topical delivery of artemisone whereby SLNs delivered artemisone to the stratum corneum-epidermis and epidermis-dermis while the niosomes only delivered artemisone to the stratum corneum-epidermis.(Dwivedi *et al.*, 2016) The lack of ability to penetrate right through the skin barrier may be the reason why niosomes have been conventionally used for topical delivery of APIs to the stratum corneum versus transdermal delivery of APIs.(Manosroi *et al.*, 2013:474) The occlusive effect of SLNs which inhibits *trans*-epidermal water loss may have influenced this observed result of the SLNs.(Majuru & Oyewumi, 2009:610)

None of the marker compounds were detected in stratum corneum-epidermis or the epidermis-dermis after the water extract niosome diffusion study, which was consistent with the membrane release and skin diffusion results. The lack of information with respect to all the phytocompounds in all the crude extracts makes it difficult to account for all the differences that were observed. This, however, reflects that relative high variation can be expected when it comes to the medicinal use of plant extracts as there is no set standard for composition and expected effects or outcomes. There is a need for standardised plant extracts or methods for extract preparation so as to ensure that the expected treatment outcomes are achieved.(Aqil *et al.*, 2013) The use of pure compounds

in order to avoid issues due to complex mixtures may be tempting, but it is has been found that isolation of pure compounds at times resulted in loss of activity, chemical instability and eliminates possible synergism.(Zorzi *et al.*, 2015)

Table IV: Average concentrations of withaferin A and withanolide A that remained in the epidermis and dermis after the 12 h skin diffusion studies (n=10). Showing comparison between marker compound in stratum corneum-epidermis (SCE) and epidermis-dermis (ED) with a superscript letter signifying a significant difference.

CONCLUSION

The results of the membrane release studies showed that withaferin A and withanolide A were released from the niosome and SLN formulations to varying extents. Therefore these compounds would be available for diffusion into and through the skin. The different release characteristics of the formulations and the differences in skin samples were partly responsible for the differences that were observed for the skin diffusion studies (Flaten *et al.*, 2015).

During the 12 h skin diffusion study, relatively low concentrations of withaferin A and withanolide A diffused into the skin. Possibly a longer time frame would have resulted in higher concentrations of compound being detected; since SLNs are said to allow for sustained release of encapsulated APIs into the skin as the API must firstly diffuse through the solid lipid matrix.(Godin & Touitou, 2012:523) It has been suggested that nanovesicles above 20 nm in diameter do not permeate the skin but rather accumulate in hair follicles where they act as API reservoirs. It is possible that the marker compounds in this study were slowly being released from the nanovesicle reservoirs and penetrating the skin barrier to reach the stratum corneum-epidermis and epidermis-dermis.(Prow *et al.*, 2011) Withaferin A and withanolide A being fairly lipophilic compounds could easily overcome the stratum corneum barrier but the aqueous layer beneath the horny layer

was possibly the biggest deterrent when it came to reaching the deeper layers (dermis).(Flaten *et al.*, 2015) In this study, it was also revealed that a high encapsulation efficiency does not necessarily correlate with a high drug release and skin permeation as other researchers have also reported.(Pando *et al.*, 2015)

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Tables and Figures

Table I: Average values for the physicochemical properties of freshly prepared formulations ± SD (n=3).

Sample	pH	Zeta potential (-mV)	Size - D (nm)	PDI	EE WFA (%)	EE WNA (%)
Placebo niosomes	7.7 ± 0.1	34.2 ± 0.1	131.7 ± 0.3	0.27 ± 0.01	-	-
Placebo SLN	7.0 ± 0.1	38.5 ± 0.1	180.2 ± 3.6	0.27 ± 0.01	-	-
Water extract niosomes	5.6 ± 0.02	10.5 ± 4.1	165.9 ± 9.4	0.17 ± 0.02	32.0 ± 5.9	93.2 ± 0.4
Water extract SLN	5.2 ± 0.03	16.7 ± 1.5	186.8 ± 4.6	0.48 ± 0.02	35.4 ± 1.7	95.3 ± 0.4
Ethanol extract niosomes	5.4 ± 0.1	35.0 ± 0.8	173.7 ± 52.6	0.23 ± 0.12	69.1 ± 1.0	41.8 ± 4.7
Ethanol extract SLN	5.0 ± 0.5	27.3 ± 2.2	172.3 ± 44.5	0.74 ± 0.22	70.0 ± 5.7	81.5 ± 2.5
50% Ethanol extract niosomes	5.7 ± 0.1	25.8 ± 2.1	304.6 ± 52.4	0.26 ± 0.03	65.8 ± 1.0	70.2 ± 0.8
50% Ethanol extract SLN	5.3 ± 0.04	25.0 ± 3.0	260.8 ± 51.4	0.74 ± 0.22	67.7 ± 3.7	81.6 ± 0.9

D – diameter;
PDI – polydispersity index;
EE WFA - encapsulation efficiency of withaferin A;
EE WNA – encapsulation efficiency of withanolide A

Table II: Mean initial (day 0) and final (day 84) physico-chemical values recorded for the stability study with an indication of percentage change over the period. A negative percentage indicates that absolute value dropped over the test period.

Formulation	pH			zeta-potential			size - D (nm)			EE WFA (%)			EE WNA (%)		
	Initial	Final	%	Initial	Final	%	Initial	Final	%	Initial	Final	%	Initial	Final	%
NW	5,59	5,26	-5,92	-17,1	-4,0	-76,6	166,0	405,4	144,3	32,0	24,6	-23,0	93,2	92,2	-1,1
NE	5,40	4,51	-16,48	-35,0	-34,2	-2,2	173,7	311,5	79,3	69,1	58,9	-14,8	41,8	32,8	-21,6
N50	5,71	5,16	-9,67	-25,8	-24,7	-4,4	145,4	418,4	187,8	65,8	65,7	-0,2	70,7	65,4	-7,5
SW	5,19	5,30	2,08	-16,7	-14,0	-15,9	186,8	495,5	165,3	35,4	17,1	-51,8	95,3	96,0	0,8
SE	5,71	4,90	-14,11	-27,3	-20,6	-24,5	172,3	476,8	176,7	70,0	44,0	-37,1	81,5	52,0	-36,2
S50	5,31	5,40	1,68	-25,0	-13,0	-48,1	260,8	1715,2	557,6	67,7	47,4	-30,0	81,6	44,0	-46,1

D – diameter;
EE WFA - encapsulation efficiency of withaferin A;
EE WNA – encapsulation efficiency of withanolide A
NW – water extract niosomes
SW – water extract solid lipid nanoparticles
NE - ethanol extract niosomes
SE – ethanol extract solid lipid nanoparticles
N50 - 50% ethanol extract niosomes
S50 – 50% ethanol extract solid lipid nanoparticles

Table III: Total amount of marker compound released as a percentage of initial amount in donor formulation and average cumulative amount of marker compound released after the 6 h membrane diffusion studies ± SD (n=10). A superscript letter signifies a significant difference between comparisons.

Formulation	Average % withaferin A diffused after 6 hours	Average cumulative withaferin A /area (µg/cm^2)	Average % withanolide A diffused after 6 hours	Average cumulative withanolide A /area (µg/cm^2)
NW	ND*	ND*	ND*	ND*
SW	12.308	44.856±3.579^a	0.578	10.835±0.936^d
NE	54.379	355.933±70.491^b	33.370	150.164±27.355^e
SE	10.048	65.771±7.586^c	4.741	21.332±1.197^f
N50	1.250	21.151±0.703$_b$	1.941	21.943±0.633^e
S50	2.842	48.097±2.825^{a,c}	5.398	61.034±2.521^{d,f}

*-Not detected; a: p=0.000132, b: p<0.000000, c: p=0.000132, d: p=0.000132, e: p<0.000000, f: p=0.000132
NW – water extract niosomes
SW – water extract solid lipid nanoparticles
NE - ethanol extract niosomes
SE – ethanol extract solid lipid nanoparticles
N50 - 50% ethanol extract niosomes
S50 – 50% ethanol extract solid lipid nanoparticles

Table IV: Average concentrations of withaferin A and withanolide A that remained in the epidermis and dermis after the 12 h skin diffusion studies (n=10). Showing comparison between marker compound in stratum corneum-epidermis (SCE) and epidermis-dermis (ED) with a superscript letter signifying a significant difference.

Formulation	Marker compound	Average concentration in SCE ± SD (µg/ml)	Average concentration in ED ± SD (µg/ml)
NW	WITHAFERIN A	ND*	ND*
SW		0.299±0.077	ND*
NE		0.298±0.044	ND*
SE		0.061±0.012	ND*
N50		1.364±0.256	ND*
S50		0.489±0.079[a]	0.129±0.013[a]
NW	WITHANOLIDE A	ND*	ND*
SW		0.837±0.160[b]	0.642±0.161[b]
NE		ND*	ND*
SE		0.828±0.098[c]	0.970±0.011[c]
N50		0.360±0.153	ND*
S50		0.311±0.046[d]	0.579±0.046[d]

*-Not detected; a: p=0.000001, b: p<0.018820, c: p=0.000429, d: p=0.000204.
NW – water extract niosomes
SW – water extract solid lipid nanoparticles
NE - ethanol extract niosomes
SE – ethanol extract solid lipid nanoparticles
N50 - 50% ethanol extract niosomes
S50 – 50% ethanol extract solid lipid nanoparticles

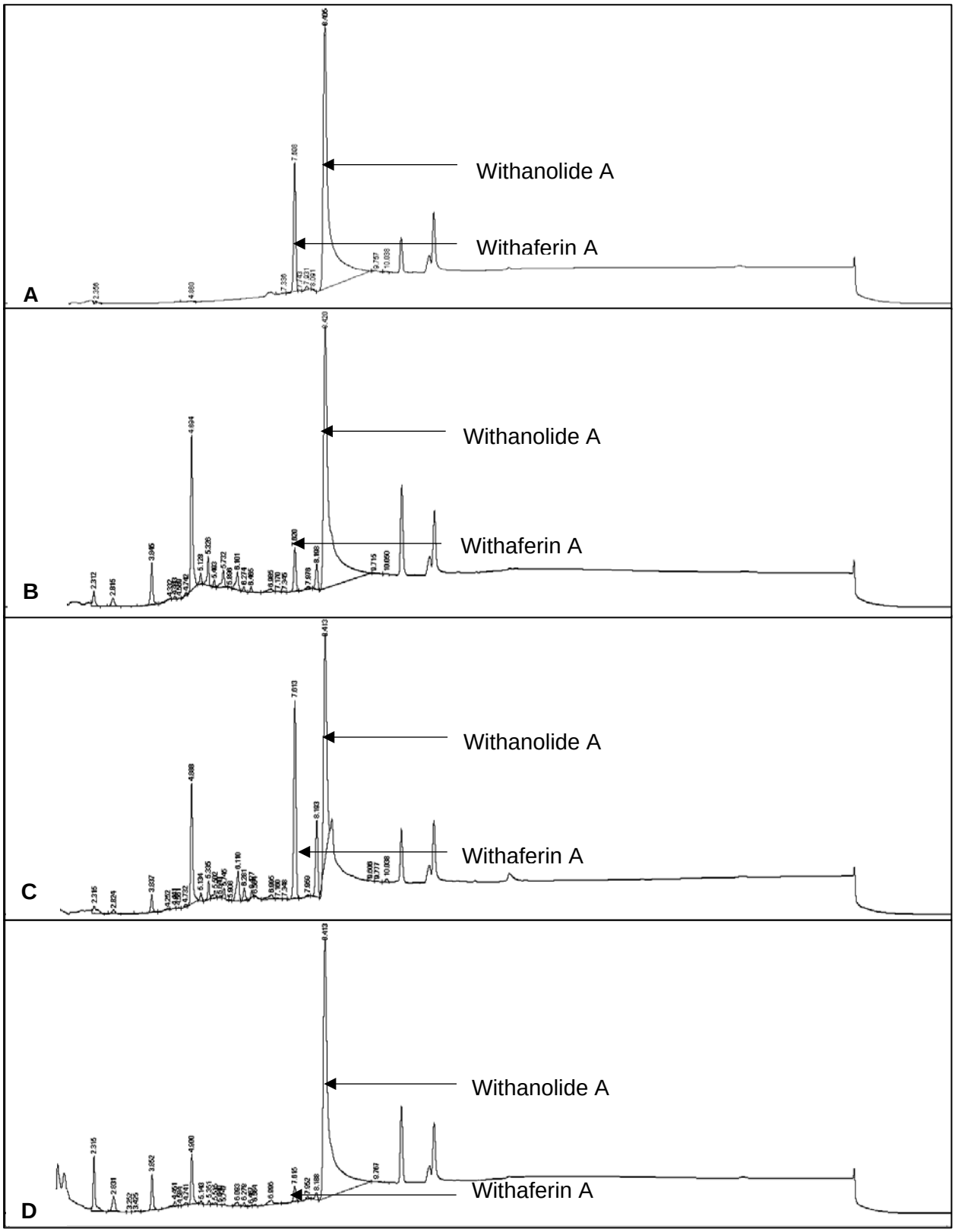

Figure I: HPLC chromatograms of withaferin A and withanolide A standards (A), ethanol extract (B), 50% ethanol extract (C) and water extract (D) for HPLC finger-printing

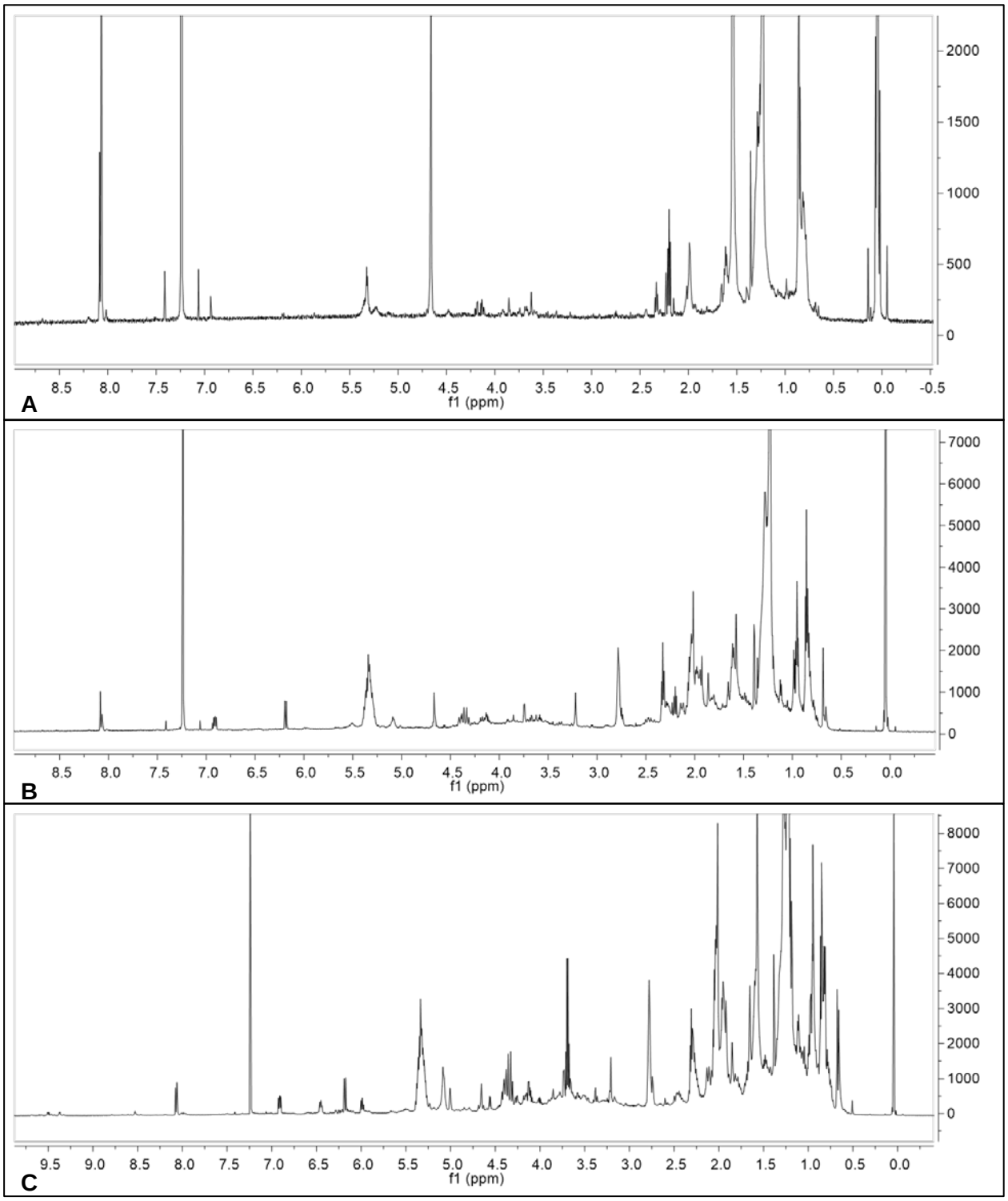

Figure II: ^{1}H-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR fingerprinting

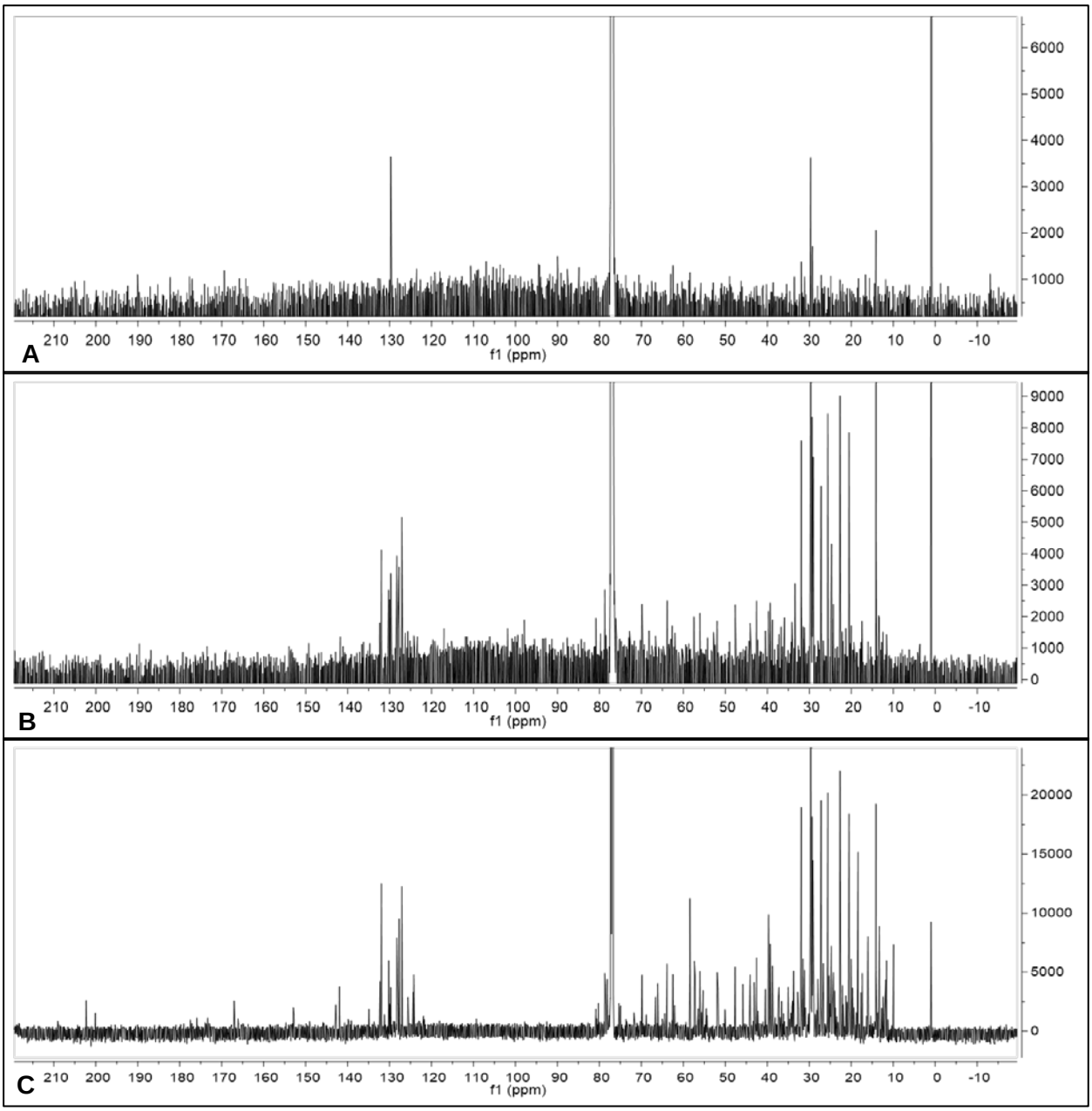

Figure III: C^{13}-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR fingerprinting

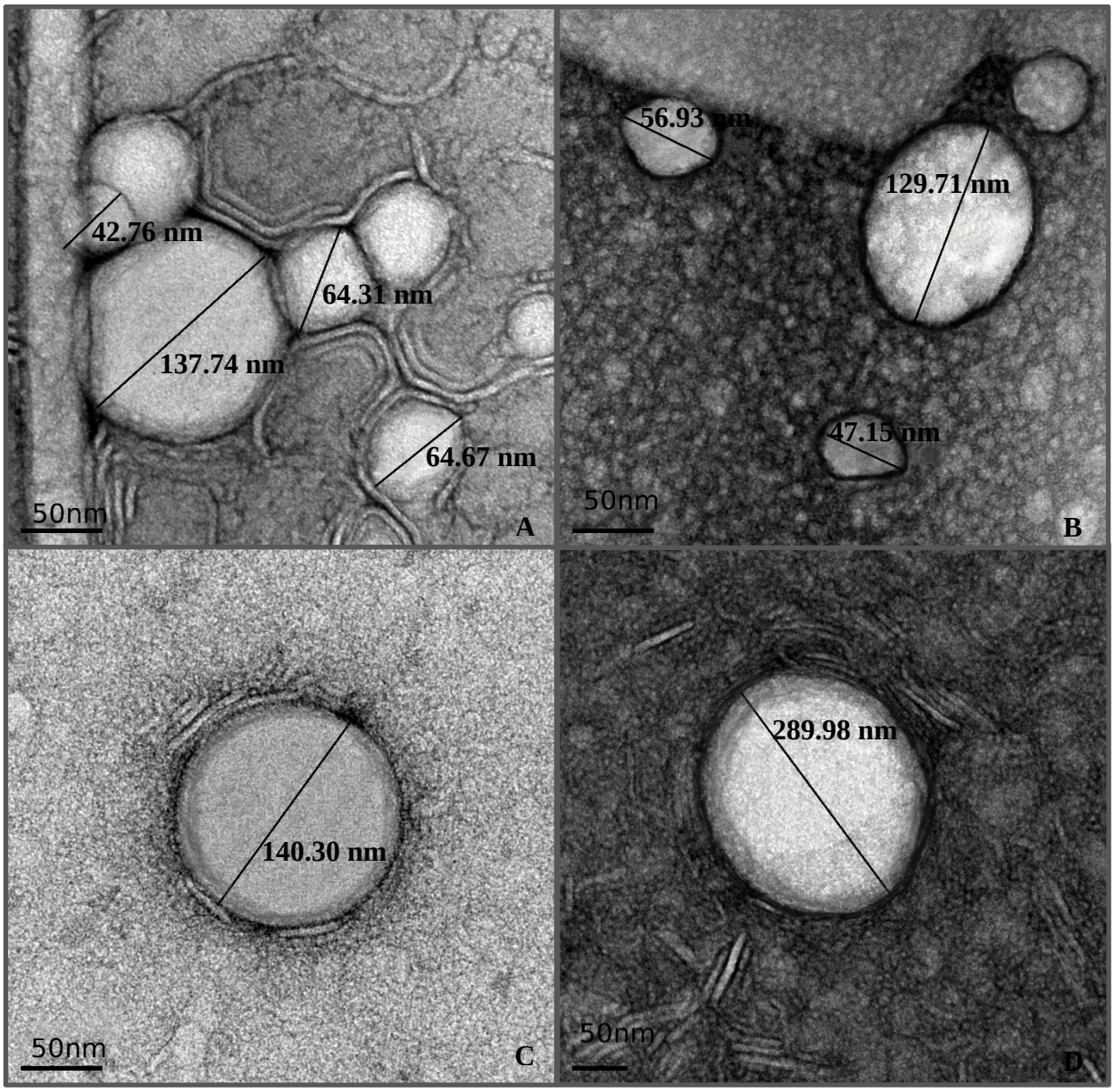

Figure IV: Transmision electron micrographs of placebo niosomes (A, B) and placebo SLNs (C, D)

Abbreviations

API, active pharmaceutical ingredient; ANOVA, analysis of variance; ED, epidermis-dermis; HPLC, high performance liquid chromatography; HLB, hydrophilic-lipophilic balance; NMR, nuclear magnetic resonance spectroscopy; PDI, polydispersity index; SLN, solid lipid nanoparticle; SD, standard deviation; SCE, stratum corneum-epidermis; TEM, transmission electron microscopy.

Table legend

Table I: Average values for the physicochemical properties of freshly prepared formulations ± SD (n=3)

Table II: Mean initial (day 0) and final (day 84) physico-chemical values recorded for the stability study with an indication of percentage change over the period. A negative percentage indicates that absolute value dropped over the test period.

Table III: Total amount of marker compound released as a percentage of initial amount in donor formulation and average cumulative amount of marker compound released after the 6 h membrane diffusion studies ± SD (n=10). A superscript letter signifies a significant difference between comparisons.

Table IV: Average concentrations of withaferin A and withanolide A that remained in the epidermis and dermis after the 12 h skin diffusion studies (n=10). Showing comparison between marker compound in stratum corneum-epidermis (SCE) and epidermis-dermis (ED) with a superscript letter signifying a significant difference.

Figure legend

Figure I: HPLC chromatograms of withaferin A and withanolide A standards (A), ethanol extract (B), 50% ethanol extract (C) and water extract (D) for HPLC finger-printing

Figure II: ^{1}H-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR fingerprinting

Figure III: C^{13}-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR finger-printing

Figure IV: Transmision electron micrographs of placebo niosomes (A, B) and placebo SLNs (C, D)

Chapter 4: Article for publication in PLoS – ONE journal

This dissertation is written in the article format hence Chapter 4 is presented in the form of a research article for publication in the journal PLoS-ONE. The formatting of this chapter differs from the rest of the thesis as it follows the guidelines as stipulated in the authors' guide. The complete authors' guide is included in the thesis as Appendix H.

In vitro Anti-melanoma Efficacy and Selectivity of *Withania Somnifera* Crude Extracts and Marker Compounds

Short title: Anti-melanoma efficacy of *Withania somnifera*

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Abstract

The anti-melanoma efficacy of *Withania somnifera* was investigated in this study. *W. somnifera* is a shrub which is known to have anti-cancer, anti-hypertensive, anti-inflammatory and immunostimulatory properties to name a few. The main bioactive compounds present in this plant are steroidal lactones known as withanolides. In this study the main aim was to develop potential topical anti-melanoma treatment with *W. somnifera* in solid lipid nanoparticles (SLNs). The anti-melanoma efficacy of *W. somnifera* extracts and bioactive compounds and any selectivity for melanoma cells versus keratinocytes was investigated. Withaferin A (WFA) and withanolide A (WNA) were chosen to be used as marker compounds in this study. Three crude extracts were prepared using water, ethanol and 50% ethanol as the solvents. Cytotoxicity and apoptosis assays were carried out on melanoma cells (A375) and keratinocytes (HaCaT) in order to determine the effects of the marker compounds and crude *W. somnifera* extracts. Investigations into the roles of deoxyribonucleic acid (DNA) fragmentation, caspase activity, mitochondrial membrane potential and membrane permeability were carried out. Further investigations were carried out to determine the influence of crude extract encapsulation in solid lipid nanoparticles (SLNs) and the presence of an extracellular matrix (Matrigel®). Both WFA and WNA presented with cytotoxic and apoptotic properties which were relatively selective for melanoma cells over keratinocytes. Selectivity indices (HaCaT IC_{50}/A375 IC_{50}) that were calculated for the water extract, ethanol extract, 50% ethanol extract, WFA and WFA+WNA are 3.362, 1.035, 1.731, 3.844 and 3.545, respectively. The combination of WFA and WNA resulted in a synergistic effect as shown by the WFA (26.25 µg/ml), WNA (80.61 µg/ml) and WFA+WNA (13.87 µg/ml) IC_{50} values in A375 cells. Results obtained revealed that the pure compounds were selective for melanoma and more active than the crude extracts in both 2D and 3D environments.

Introduction

Withania somnifera L. Dunal is a medicinal plant that is native to India, but is used worldwide due to its vast medicinal potential. This plant is also referred to as Ashwagandha, poisonous gooseberry, Indian ginseng or winter cherry, depending on the part of the world. The medicinal properties of *W. somnifera* include anti-cancer, immunostimulatory, anti-arthritic, anti-ageing, anti-bacterial, anti-diabetic, anti-hypertensive, rejuvenating and adaptogenic properties (Winters, 2006; Malik *et al.*, 2009:1508; Nagella & Murthy, 2010; Vel Szic *et al.*, 2014). These medicinal properties are due to a high concentration of anti-oxidants, phenols, fatty acids, carotenoids and other bioactive compounds (Kulkarni & Dhir, 2008; Chatterjee *et al.*, 2010; Fernando *et al.*, 2013). The main bioactive compounds present in *W. somnifera* are withanolides, which are steroidal lactones with an ergostane backbone (Ganzera *et al.*, 2003; Kulkarni & Dhir, 2008). Withaferin A (WFA) has been repeatedly stated to be one of the most potent and abundant withanolides

(Ganzera *et al.*, 2003; Jayaprakasam *et al.*, 2003; Kulkarni & Dhir, 2008). The anti-cancer activity of *W. somnifera* has mainly been ascribed to the potent WFA together with other compounds present in the plant (Sheena *et al.*, 1998; Kalthur *et al.*, 2009; Kalthur & Pathirissery, 2010; Mayola *et al.*, 2011; Huaping *et al.*, 2012; Samadi *et al.*, 2012; Vel Szic *et al.*, 2014). Withaferin A and withanolide A (WNA) are both present in the plant leaves so they were selected as the marker compounds throughout this study, as the plant leaves are commonly used in households that use *W. somnifera* for its medicinal properties (Malik *et al.*, 2009).

It is crucial to determine *in vitro* efficacy prior to doing *in vivo* testing, thus the *in vitro* depigmenting (Nakajima *et al.*, 2011:1405), antiproliferative (Jayaprakasam *et al.*, 2003:130; Zhang *et al.*, 2012:1360), cytotoxic (Al-Fatimi *et al.*, 2005:356), pro-apoptotic and anti-cancer (lung, neuroblastoma, prostate and colon) (Malik *et al.*, 2009:1498; Mayola *et al.*, 2011:1017) efficacy of *W. somnifera* and WFA have been investigated in the past. In this study the anti-melanoma efficacy and selectivity of *W. somnifera* was determined using a human depigmented melanoma cell line (A375) and a human keratinocyte cell line (HaCaT). The crude extracts were encapsulated in solid lipid nanoparticles (SLNs) as SLNs have the potential for use as *W. somnifera* delivery vesicles and nanoparticulate formulations have been found to improve the delivery and efficacy of active pharmaceutical ingredients (Sanna *et al.*, 2013b; Wang *et al.*, 2014; Venturini *et al.*, 2015). This is the first study to investigate the use of SLN encapsulated *W. somnifera* crude extracts as potential anti-melanoma agents.

It is wise to verify *in vitro* results obtained from conventional cell culture using three dimensional (3D) cell culture because conventional cell culture studies do not take into account the influence of cell to cell communication and effects of the extracellular matrix (ECM) (Freshney, 2005:360). Therefore, in order to close the gap between *in vitro* and *in vivo* studies, it is recommended to carry out *in vitro* studies using 3D models (Li *et al.*, 2011; Mathes *et al.*, 2014:96). Matrigel™ (Corning Inc., Corning, New York) is a basement membrane extract that is used in *in vitro* and *in vivo* studies as it is able to mimic the ECM and thus give results that are a better representation of what occurs within the body (Amatangelo *et al.*, 2013; Benton *et al.*, 2014). When skin cells are grown in the presence of ECM proteins they may behave differently from cells growing directly on plastic flasks in normal cell culture (Mathes *et al.*, 2014:96). Cells in 3D culture are said to mimic critical features of healthy or diseased human skin. Tumour spheroid models (Thoma *et al.*, 2014:33) and 3D skin models (Mathes *et al.*, 2014:81) for psoriasis, melanoma and wound healing have been used in order to investigate disease etiology, drug development and screening (Groeber *et al.*, 2011:353). Various *in vivo* studies have been conducted to further investigate some of the anti-cancer properties of *W. somnifera* since *in vitro* studies provide insufficient information for making a conclusion (Diwanay *et al.*, 2004:53; Leyon & Kuttan, 2004:119; Malik *et al.*, 2009:1504).

The novelty of this study lies in that a comparison was made between the effects of WFA, WNA and *W. somnifera* crude extracts on A375 cells and HaCaT cells and the melanoma selectivity of *W. somnifera* crude extracts and bioactive compounds was determined. A375 cells were also treated with *W. somnifera* SLNs and the effects of *W. somnifera* on A375 cells in Matrigel® were investigated, which has never been done to date. Matrigel® is generally used *in vitro* for invasion and metastasis assays (Liu *et al.*, 2008) but in this study it was used for cytotoxicity, apoptosis and imaging assays. The purpose of this part of the study was to compare between the effects of *W. somnifera* on A375 cells in 2D and 3D environments. *W. somnifera* crude extracts have never been encapsulated in SLNs as was done in this study, so this study is the first to highlight the effects or lack thereof of SLN encapsulation on *W. somnifera* anti-melanoma efficacy.

Reports in literature show that WFA is a potent anti-melanoma compound and that crude extracts are also potent against melanoma (Mathur *et al.*, 2006; Mayola *et al.*, 2011; Halder *et al.*, 2015). Due to the lack of standardisation with crude plant extracts it is however challenging to accurately compare the effects of crude extracts from different studies. With respect to anti-cancer mechanisms of action, WFA is said to increase generation of reactive oxygen species, increase caspase 3 and 9 activity and reduce Bcl-2/Bax and Bcl-2/Bim ratios (Lee, 2016). It has also been reported that WFA induces apoptosis in uveal melanoma cells (Samadi *et al.*, 2012) while a leaf aqueous extract has been reported to suppress cell proliferation and induce apoptosis via the PARP and Bcl-xl pathways (Gupta & Kaur, 2016). An aqueous alcoholic crude extract of both roots and leaves was found to result in phosphatidylserine translocation, lowering of mitochondrial membrane potential and activation of caspases 3,8 and 9 of human leukaemia cells (HL-60) (Malik *et al.*, 2009). The effects observed in the different studies may differ due to variations in extraction method, extraction solvent (water, ethanol, hexane etc.), plant part used (leaf, root, bark or whole plant), type of cancer investigated or particular cell line used in study. In order to account for some of the potential differences this study focused on the anti-cancer efficacy of three different crude extracts on A375 cells with keratinocytes acting as a reference.

This study had three main aims which included testing for the anti-melanoma effects of the different extracts and compounds; evaluating the specificity of the crude extracts and compounds in 2D and 3D; and lastly determining the mode of cytotoxic and apoptotic action of *W. somnifera* crude extracts and bioactive compounds in A375 melanoma cells. *In vitro* cell culture studies were utilised to investigate the induction of apoptosis inducing effects of the crude plant extracts and marker compounds in human melanoma cells. The specificity towards melanoma cells was determined by comparing the cytotoxicity against normal human keratinocytes. An investigation into possible mechanisms of action was done. The roles of caspases, mitochondrial membrane potential and deoxyribonucleic acid (DNA) damage were investigated during the mechanistic studies.

Materials and Methods

Materials

The acetonitrile, deuterated chloroform, dimethyl sulfoxide, Tween® 80 and Span® 60 were purchased from Merck Chemicals (Darmstadt, Germany). Phosphatidylcholine and sodium cholate were obtained from Sigma Aldrich (St Louis, MO, USA) while Compritol® 888 ATO for the SLNs was a generous gift from Gattefossé (Lyon, France). The A375 (ATCC # - CRL1619) melanoma cell line (ATCC, Manassas, VA) and HaCaT human keratinocyte cell line (University of Witwatersrand) were used throughout the study. The A375 cell line was purchased directly from ATCC, while the HaCaT cell line was received as a gift from the University of Witwatersrand, Johannesburg, RSA. Withaferin A and withanolide A USP standards were obtained from ChromaDex (Irvine, CA, USA).

Preparation and characterisation of *W. somnifera* leaf extracts

W. somnifera leaves were purchased from Mountain Herb Estate in Kameeldrift-West, South Africa. The leaves were cleaned, air dried and ground into a fine powder upon receipt. Thereafter, soxhlet extraction (24 h) was used to prepare crude extracts from the powdered leaves. Ethanol, 50% ethanol and water were used as the extraction solvents with dried extract yields of 9.2%, 8.3% and 6.7%, respectively. The three crude extracts were characterised using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. An Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) with a quartenary pump, autosampler and a diode array detector was used for the analyses and ChemStation Rev. A.10.01 software was used for data analysis and acquisition. A gradient elution method was used with acetonitrile (mobile phase B) and HPLC-grade water (mobile phase A) as the mobile phases. Acetonitrile was initially set at 10% with a linear gradient to reach 100% acetonitrile after 10 min and holding to 20 min before re-equilibrating. The flow rate, injection volume, detection wavelength and stop time were set at 1 ml/min, 50 µl, 210 nm and 22 min, respectively. A chemical finger-print was obtained for each *W. somnifera* crude extract using the described HPLC method. The lower limits of quantification for WFA and WNA were 0.05 µg/ml and 0.25 µg/ml, respectively.

^{1}H-NMR and C^{13}-NMR fingerprint spectra were obtained using an Avance III 600 Hz NMR Spectrometer (Bruker, Billerica, MA, USA). Approximately 10 mg or 50 mg of marker compound or crude extract respectively were weighed and dissolved in 1.5 ml deuterated chloroform ($CDCl_3$; Merck, South Africa). The filtered solutions were thereafter analysed using NMR spectroscopy.

Formulation of solid lipid nanoparticles

The SLNs were formulated using the solvent injection method with diethyl ether as the solvent. A ratio of 2:1:1 was used for the surfactant, Compritol® 888ATO and L-α-phosphatidylcholine. Surfactant, extract and solid lipid were weighed and dissolved in diethyl ether. The ethanol and 50% ethanol extracts were incorporated in the organic phase, while the water extract was incorporated in the aqueous phase. The solution was then slowly injected into a pre-heated (60 °C) aqueous phase, which was continuously magnetically stirred at 60 °C to drive off the organic solvent. The formulation was left to cool down to room temperature then sonicated on ice using a Hielscher UP 200ST sonicator (Hielscher Ultrasound Technology, Teltow, Germany) to homogenise the particles.

Cell propagation and assay preparation

Conventional cell culture

The human melanoma cells (A375) and keratinocytes (HaCaT) were grown in complete media, which consisted of 10% foetal bovine serum (FBS) and 1% PenStrep in DMEM (Dulbecco's Modified Eagle Medium). Both cell types were cultured in an incubator set at 37 °C and 5% CO_2. Cells were then used in assays when they were approximately 80% confluent. Cell viability of both cell types was determined using the trypan blue dye exclusion test prior to conducting any assays.

Cell culture in Matrigel®

Matrigel® gelatinises at temperatures above15 °C so the Matrigel® together with pipette tips, tubes, cell culture plates and complete media were kept on ice or in the refrigerator prior to use. A pre-chilled 96-well plate was coated with 10 µl of Matrigel® and placed in a 37 °C incubator for 20 min. Melanoma cells or keratinocytes were suspended in Matrigel® to give a final cell concentration of approximately $1.0x10^6$ cells/ml (for XTT) or $1.5x10^6$ cells/ml (for FITC Annexin V). The resulting cell suspensions were added to each coated well (70 µl) and the plate placed back in a 37 °C incubator for 30 min. Thereafter, 60 µl of complete media were added to each well and left to acclimatise in the incubator overnight prior to treating the cells for XTT and FITC Annexin V assays.

Cytotoxicity assays

MTT and XTT assays

In order to determine the A375 and HaCaT IC_{50} values of WFA, WNA, WFA+WNA, water extract, ethanol extract and 50% ethanol extract the MTT assay was conducted. The 50% ethanol extract was encapsulated in SLNs and was used as a sample treatment in order to investigate the potential influence of drug delivery vesicles on the cytotoxicity of the extract. Cells (50 000) were seeded on the first day and treated with the treatment dispersions after 24 h. The treatments that were used were WFA, WNA, WFA+WNA, water extract, ethanol extract, 50% ethanol extract, placebo SLNs and 50% ethanol extract SLNs at concentrations of 5, 25, 50, 75, 100, 150, 200 and 250 µg/ml. Complete media and methanol treatments were used as the negative and positive controls, respectively. After a further 24 h the Vybrant® MTT cell proliferation assay kit (Thermo Fisher Scientific, Waltham, MA) or the XTT TOX 2 kit (Sigma Aldrich, St Louis, MO) were used to investigate the cytotoxicity of the treatments. The absorbance for the MTT assay was measured at 540 nm and the negative control was used as a reference for the data analysis. For the XTT assay absorbance was measured at 450 nm.

The cytotoxicity of the water, ethanol and 50% ethanol extracts on A375 cells in Matrigel® was investigated using the XTT assay. Treatment dispersions at concentrations of 16.7, 33.3, 66.7 and 670.0 µg/ml were added 24 h after seeding cells in Matrigel®. Matrigel® alone and Matrigel® with XTT solution were used as blanks so as to indicate if the Matrigel® distorted the results in any way.

Apoptosis assays

DNA fragmentation, caspase 3/7 activity, membrane permeability and mitochondrial membrane potential

Melanoma or HaCaT cells ($1x10^6$) were seeded in 6 well plates. After 24 h the cells were treated with 26.25 µg/ml WFA, 80.61 µg/ml WNA, 13.87 µg/ml WFA+WNA, water extract equivalent to 13.87 µg/ml WFA, ethanol extract equivalent to 13.87 µg/ml WFA and 50% ethanol extract equivalent to 13.87 µg/ml WFA. These treatment concentrations were deduced from the IC_{50} values. Methanol was used as a positive control, while complete media was used as a negative control. Treatment was for 24 h and subsequently the different assays were carried out as described on the manufacturers' manuals.

Flow cytometric assays were carried out using a BD FACSVerse™ (BD Bioscience, Mountain View, CA) flow cytometer. The flow cytometer is equipped with a 2-laser, 6 colour configuration with a 488 nm laser and a 633 nm laser. Untreated, single-stained cells and unstained cells were used to set up gates and compensation parameters for cell analysis of each cell type prior to analysing any of the experimental samples.

With the APO BrdU™ TUNEL assay (Thermo Fisher Scientific, Waltham, MA) the cells were fixed using paraformaldehyde and with 70% ice cold ethanol prior to staining as instructed by manufacturer. In order to assess changes in membrane permeability, cells were stained with YO-PRO®-1 dye (Thermo Fisher Scientific, Waltham, MA) and propidium iodide (PI). YO-PRO®-1 enters and stains highly permeable apoptotic cells while PI can only penetrate and stain dead cells (Haughland, 2005:749; Wlodkowic *et al.*, 2011:12). The influence of caspase activity was probed using the CellEvent® Caspase-3/7 green flow cytometry kit (Thermo Fisher Scientific, Waltham, MA). Keratinocytes and melanoma cells ($1x10^6$ cells) were only treated with WFA, WNA and WFA+WNA for this assay. The CellEvent® Caspase-3/7 Green Detection Reagent detected the presence of activated caspases, while the SYTOX® AADvanced™ dead cell stain detected the presence of dead cells.

An absorbance assay was carried out to investigate the role of mitochondrion membrane potential in cells after the treatments. During apoptosis, mitochondrial membrane potential falls causing mitochondrial permeability transition pores to open and thus mitochondrial potential dye does not accumulate in the mitochondria of cells. In order to assess changes in mitochondrial membrane potential, 80 000 cells were seeded in black 96-well plates with a clear bottom and treated with WFA, WNA, WFA+WNA, water extract, ethanol extract or 50% ethanol extract after 24 h (Wlodkowic *et al.*, 2011:5). After a further 24 h, the mitochondrion membrane potential assay (Sigma Aldrich, St Louis, Missouri) was carried as described by manufacturer. Fluorescence intensity was measured using a SpectraMax® microplate reader (Molecular Devices, Sunnyvale, CA).

Annexin V-FITC apoptosis assay of cells in Matrigel®

This assay makes use of the translocation of phosphatidylserine from the inner cell membrane to the outer surface that occurs during apoptosis (Haughland, 2005:753; Wlodkowic *et al.*, 2011:10). This makes phosphatidylserine a suitable apoptosis marker which can be probed using flow cytometry. In this study the Annexin V-FITC apoptosis assay was carried out on cells in Matrigel®; this assay was chosen as it is a fairly simple first-line apoptosis assay, which does not carry the risk of losing cells due to numerous rinse steps. Apoptotic cells fluoresced green due to fluorescein and dead cells fluoresced red due to PI. Melanoma cells ($1.5x10^6$ cells/ml) were seeded in Matrigel® and treated with the crude extracts (equivalent to 13.87 µg/ml) after a 24 h acclimatisation period. After 24 h of treatment the Matrigel® was lysed using 5 mg/ml dispase for 20 min. The resulting cell suspension was centrifuged for 10 min at 1000 rpm and the supernatant was discarded. The cell pellet was rinsed with phosphate buffered saline 0.0067 M (PBS) then 300 µl of Annexin V-FITC binding buffer, Annexin working solution (4 µl) and PI (10 µl) were added to each sample. After mixing, the samples were left to stand at room temperature for 15 min then analysed using flow cytometry.

Confocal microscopy assessment of cells in Matrigel®

Confocal microscopy was used as a tool for assessing the occurrence of apoptosis in A375 cells in Matrigel®. Cells were stained using 2 µM CellTracker™ red then harvested using 0.25% trypsin-EDTA. Approximately $2x10^5$ cells were suspended in 1000 µl of Matrigel®. The chambers of a 4-chamber cell culture dish were coated with 100 µl of Matrigel® then 250 µl of the stained cells in Matrigel® were added to each chamber. After 20 min of incubation 150 µl of complete media were added to each well. Every second day the media was replaced with either 150 µl of media or 150 µl of 50% ethanol extract. Cells were viewed using confocal microscopy on day one and on day ten. On the tenth day the live cells in Matrigel® were labelled using CellTracker™ red prior to viewing them under the confocal microscope.

Statistical analysis

The data obtained was analysed using IBM SPSS Statistics 23, (IBM Corporation, Armonk, New York, USA) to test for between and within group differences. A one-way analysis of variance (ANOVA) was used to test for differences between groups and a Games-Howell post-hoc test for within group differences. A p value below 0.05 was taken to indicate that a significant difference between the means existed.

Results and Discussion

Characterisation of plant extracts

The chemical fingerprint HPLC chromatograms (Figure) and NMR spectra (Figure and Figure 3) of the crude extracts confirmed that WFA and WNA were present in the extracts. The WFA content of the water, ethanol and 50% ethanol extract was 0.98%, 1.76% and 4.55%, respectively; while the WNA content was 5.04%, 1.21% and 3.04%, respectively.

Figure 1: HPLC chromatograms of withaferin A and withanolide A standards (A), ethanol extract (B), 50% ethanol extract (C) and water extract (D) for HPLC finger-printing

Figure 2: ^{1}H-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR finger-printing

Figure 3: C^{13}-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR finger-printing

Cytotoxicity assays

The normalised and log transformed data of the cytotoxicity assays were processed using GraphPad Prism 7 (GraphPad Software, San Diego, CA) to give the 50% inhibitory concentrations (IC_{50}). The IC_{50} values have been tabulated in Table to highlight differences between A375 and HaCaT cells; cells in complete media and those in Matrigel®; and between plant extract and plant extract SLNs. The WNA treatment had a minimal effect on HaCaT cells in 2D and in Matrigel®, thus the IC_{50} values could not be computed. With respect to the crude plant extracts the IC_{50} values were calculated to give the WFA concentrations represented.

Table 1: IC_{50} values of the pure compounds, plant extracts and SLN formulation (calculated using withaferin A content in extracts). Values are shown as mean±SD of experiments done on cells grown in complete media only (n=9).

The IC_{50} values reflected that there were significant differences ($p<0.05$) between the effects of the different treatments on melanoma cells and keratinocytes. It is evident that WFA had greater cytotoxic activity than WNA both in 2D cell culture and in Matrigel®. The combination of WFA+WNA resulted in increased cytotoxicity to both A375 and HaCaT cells, suggesting the occurrence of synergism. This notion is further supported by the low concentrations of WFA required for the crude extracts to inhibit 50% growth of the cell populations. This may have been due to the presence of numerous unidentified compounds in the crude extracts, which may have acted synergistically with the WFA and WNA. The synergistic effect was most apparent at the low concentrations. All of the pure compound treatments (2D and 3D) were found to be selective for melanoma cells versus keratinocytes, which is ideal in the treatment of cancer as you want to avoid damaging the healthy tissue. There was a statistically significant difference ($p<0.05$) between the IC_{50} of A375 and HaCaT cells for all the treatments. The ethanol extract treatment was the only exception, whereby there was no significant difference between the A375 and HaCaT cells $IC_{50.}$ On comparison, it was observed that the difference between cells in media only and cells in Matrigel® was not significant for all the pure compound treatments.

With respect to the crude plant extracts, only the 50% ethanol extract treatments (2D, 3D and SLNs) presented with relative selectivity for melanoma cells over keratinocytes. The ethanol extract was the most potent extract, but it showed no selectivity for a particular cell type. The high potency may be ascribed to the extract comprising of a combination of highly toxic organic compounds. Melanoma cells and keratinocytes were both more resistant to the cytotoxic effects of the 50% ethanol extract when encapsulated in SLNs, suggesting that the SLNs somehow protected the cells from the cytotoxic effects. This observed effect diverges from what has been reported in literature. In previous studies it was observed that encapsulation of phytochemicals in SLNs resulted in a greater cytotoxic effect than that of the phytochemicals alone (Sunil Kumar *et al.*, 2011; Zorzi *et al.*, 2015). In this study, it seems the SLNs resulted in a protective effect on both the melanoma cells and keratinocytes.

These results confirm what has been stated in literature with respect to the anti-cancer activity of *W. somnifera*. A study was conducted in the past on M14, Mel501, SK28 and Lu1205 melanoma cell lines. In this study they found that WFA was toxic to melanoma cells with the 24 h IC_{50} values ranging from 1.8 – 6.1 µM (Mayola *et al.*, 2011). The WFA IC_{50} that we obtained is 55.8 µM, which is much higher than the values in the previous study. These differences may be accounted for by differences in the aggression of the different melanoma cell lines. In another study, a crude aqueous extract was prepared from *W. somnifera* roots and the IC_{50} was 350 µg/ml (Halder *et al.*, 2015) in comparison to the crude aqueous extract IC_{50} of 633 µg/ml in this study. The effect of differences in plant parts and extraction methods may be responsible for these observed deviations.

Apoptosis assays

DNA fragmentation

The APO BrdU™ TUNEL assay works by detecting DNA strand breaks and changes in the light scattering properties of cells. Apoptotic cells present with reduced forward light scatter due to cell shrinkage caused by dehydration. During early apoptosis, the nucleus and cytoplasm of the cell condense; leading to increased cell complexity, which are detected as increased side scatter. As cells go into late apoptosis; they reduce in size and in turn have a lower complexity, which is then detected as reduced side scatter. Nonetheless, light scattering properties can only be used to interpret apoptosis occurrence with the aid of other apoptosis assays (Wlodkowic *et al.*, 2011:4). In this study, both forward scatter and side scatter were reduced after treatment in comparison to the control, which implied that the cells were in the late stages of apoptosis.

The plant extract treatments resulted in minimal DNA fragmentation in both cell types as in comparison to WFA and WNA. Results are illustrated in Figure A. The plant extracts had relatively low IC_{50} values (WFA equivalent) as compared to the pure compounds, but they presented with a lower occurrence of apoptosis at concentrations higher than the IC_{50}. There was a statistically significant difference between the A375 negative control and the WFA ($p=0.023$) and WFA+WNA ($p=0.011$) treatments. This highlights that WFA (13.87 µg/ml) alone and in combination with WNA (13.87 µg/ml) was more active than the crude extracts containing 13.87 µg/ml WFA. The plant extracts may have induced cell death via a mechanism other than apoptosis, thus minimal DNA fragmentation was detected. It is also possible that the crude extracts contained other unidentified compounds, which at the higher concentrations resulted in the cells resisting apoptosis. In the study by Malik *et al.* a crude *W. somnifera* root extract induced apoptosis in A37 cells which was detected as DNA fragmentation after 48 h and 72 h treatments. There was no DNA fragmentation that had occurred after 24 h (Halder *et al.*, 2015). A similar phenomenon may have been responsible for the minimal apoptosis that was observed in this

study after treating the A375 cells for 24 h with the crude extracts. Further treatment for up to 72 h may have yielded different results.

Figure 4: Percentage apoptotic cells of A375 and HaCaT cells with respect to DNA fragmentation (A), membrane permeability (B), Caspase 3/7 activity (C), membrane permeability in 3D (D) and mitochondrial membrane potential (E). Values are presented as mean±SD.

The keratinocytes were barely affected by the plant extracts; although the pure compounds did result in some DNA fragmentation. A similar trend was observed for the melanoma cells, whereby the pure compounds caused more DNA damage in contrast to the crude extracts. As shown in Figure; the pure compound treatments were however selective for inducing DNA damage in melanoma cells and not keratinocytes, which is ideal in the use of any anti-cancer compound. A significant difference was detected between the two cell types for the WFA ($p=0.013$) and WFA+WNA ($p=0.007$).

Figure 5: Overlay histogram showing selectivity of the pure compounds to induce apoptosis in A375 cells vs. HaCaT cells.

Membrane permeability

To the best of our knowledge there are no other reports in literature whereby the effects of *W. somnifera* (or metabolites) on precisely A375 membrane permeability were investigated, thus making this the first study highlighting the influence of *W. somnifera* on the permeability of A375 cell membranes. Studies such as the annexin V FITC assay have however been conducted which indirectly measure membrane permeability. With respect to changes in membrane permeability, the pure compounds had a higher apoptosis inducing capability. It is important to note that the plant extract treatments resulted in a high percentage of necrotic cell death, while the pure compounds induced apoptosis in both cell types. As shown in Figure B apoptosis selectively occurred in melanoma cells over the keratinocytes. The WFA treatment resulted in the highest percentage apoptosis, which reiterates that WFA is a highly potent compound. The WFA+WNA treatment resulted in the detection of a high percentage of necrotic cells, which may in fact have been late apoptosis. In order to overcome the challenge of failing to distinguish between late apoptosis and necrosis; it may be necessary to use shorter treatment durations for all the experiments.

Caspase-3/7 activity

All the treatments (pure compounds only) resulted in minimal detection of apoptosis or necrotic cell death in the HaCaT cells (Figure C). An average of at least 75% keratinocytes was still alive after all the treatments. With respect to the melanoma cells, higher levels of caspase activity

together with cell death were detected after all the pure compound treatments. This implies that the pure compounds induced apoptosis, which presented as an increase in caspase activity. There was a significant difference between the A375 negative control and the WNA treatment (p=0.046). The WFA and the WFA+WNA treatments resulted in the highest percentage of combined apoptotic and necrotic cells. It is however possible that the necrotic cells were cells that died via caspase activated apoptosis and were in the late stages of apoptosis. In a previous study it was revealed that a *W. somnifera* crude extract activated caspases 3, 8 and 9 of HL-60 cells in a time-dependant and concentration dependant manner (Malik *et al.*, 2009). However, in the current study only WFA, WNA and WFA+WNA were used as treatments. It is plausible that WFA and WNA do not fall under the *W. somnifera* metabolites that activate caspase activity hence minimal caspase activation was observed in this study. The crude plant extracts may have had an effect on the caspase activity had they been used in the caspase 3/7 assay.

Mitochondrion membrane potential

Mitochondrion membrane potential is said to decline during apoptosis and this results in the JC-1 dye failing to accumulate within the cells. If exposed to the JC-1 dye; live cells will express high fluorescence of the dye, while apoptotic cells present with reduced fluorescence. In this assay, a high mean fluorescence (MFI) was taken to signify that the cells were alive, while a low MFI implied that the cells were apoptotic. Percentage MFI of treated cells was calculated as a percentage of live cells and presented in Figure E. In a past study *W. somnifera* root+leaf crude extract resulted in lowering of the mitochondrion membrane potential of HL-60 cells after a 12 h treatment (Malik *et al.*, 2009). This is consistent with what we observed in this study.

Significant differences were also found between the A375 live control and the WFA (p=0.024), WFA+WNA (p=0.009) and the ethanol extract (p=0.010) treatments on A375 cells, respectively. The WNA treatment had a significant selectivity for HaCaT cells versus A375 cells with a p-value of 0.023. On the other hand, the water and ethanol extract treatments caused a significant lowering of the mitochondrion membrane potential in A375 cells as compared to HaCaT cells (p=0.009 and 0.040, respectively); thus showing desired selectivity for A375 cells.

The plant extract treatments had a minimal effect on the mitochondrial membrane potential of the keratinocytes. The pure compounds however resulted in reduced mitochondrial membrane potentials for both cell types. The p-values for WFA versus control and WFA+WNA versus control were 0.024 and 0.009, respectively. This further highlights that the pure compounds were more active than the crude extracts. Only the WFA+WNA, water extract (p=0.009) and ethanol extract (p=0.040) treatments showed selectivity for lowering the mitochondrion membrane potential of melanoma cells over that of keratinocytes.

Annexin V-FITC apoptosis assay of cells in Matrigel®

Prior to analysing the treated samples, the negative control was analysed. It was observed that there was a high percentage of dead and apoptotic cells (31.5% and 25.3%) in the untreated cells after spending 24 h in Matrigel®. The cells may have failed to thrive as they had to be seeded at a high density in the Matrigel® in order to obtain sufficient cell numbers for this experiment. The cell densities that were used in this study are higher than A375 cell densities that have been used in past studies (Wu *et al.*, 2008; Kamran & Gude, 2012). An alternative possibility is that the dispase used to lyse the Matrigel® may have resulted in toxicity due to the relatively high concentrations and contact time that was used. After the lysis, the cells did not have any time to recover as they immediately had to be prepared for analysis.

On analysis it was found that the treatments resulted in increased cell death in comparison to the negative control. Necrosis was more rampant than apoptosis with all the treatments and this again may have been due to the high density of cells within the Matrigel®. Upon comparing the percentage of live cells, it was found that all the SLN treatments had a higher percentage of live cells remaining versus the respective crude extract alone. This brings up the issue of a protective effect being proffered by the SLNs to the melanoma cells, as was observed with the cytotoxicity assay. Statistical analysis showed that there was a significant difference between the percentage live cells of water extract versus water extract SLNs ($p=0.019969$) and ethanol extract versus ethanol extract SLNs ($p=0.000527$. Regarding apoptosis, the crude extracts alone excluding the water extract, resulted in a greater occurrence of apoptosis as compared to their encapsulated counter-parts (Figure D). In a study by Sheen *et al.* (Sheena *et al.*, 1998), *W. somnifera* extract was encapsulated in niosomes for cancer treatment and they reported that the anti-cancer efficiency was increased. This is contrary to what was found in this study; whereby encapsulation in SLNs resulted in reduced efficacy. It is proposed that the lipids of the vesicles antagonised the entry of the cytotoxic molecules into the melanoma cells and in turn reduced the observed efficacy.

Confocal microscopy assessment of cells in Matrigel®

Images were taken one day and ten days after seeding melanoma cells in Matrigel® (Figure). Untreated cells were expected to have high levels of fluorescence as compared to treated cells, since CellTracker™ Red diffuses into live cells and is converted into fluorescent membrane impermeable dye adducts by glutathione-*S*-transferase (Plaetse & Schoeters, 1995:1907; Haughland, 2005:644). The treated cells were expected to present with reduced fluorescence as the metabolic activity of these cells was expected to be reduced. The results obtained showed that the treated cells had lower fluorescence than the untreated cells at the same time-point leading to the conclusion that the 50% ethanol extract led to death of the treated cells in Matrigel™.

As cells die, their membranes become more porous so the dye adduct may have exited the cells due to this increased porosity and presented as reduced cell fluorescence. The images obtained illustrate that the melanoma cells were able to thrive in the Matrigel™ for up to ten days as shown by the high CellTracker ™ Red fluorescence.

Figure 6: Confocal images of A375 cells; untreated on day 1 (A), treated on day 1 (B), untreated on day 10 (C) and treated on day 10 (D). Treated cells were treated with 50% ethanol extract every 2nd day.

Conclusion

This study revealed that the use of pure potent plant-derived compounds can differ considerably from the use of crude plant extracts for their medicinal properties. The crude extracts were found to be rather cytotoxic to both melanoma cells and keratinocytes, which may have been caused by a combination of synergistic and antagonistic activities of all the compounds present in the extracts. The use of SLNs resulted in a diminished anti-cancer effect which was contrary to the expected outcome. The 50% ethanol extract however maintained slight selectivity for A375 cells regardless of the perceived loss in activity. On the other hand, the pure compounds were found to be relatively selective for inducing cell death in melanoma cells over keratinocytes. The selectivity of the pure compounds was present both in the 2D and in 3D experiments. Both the WFA and WFA+WNA treatments were the most promising treatments for the purpose of destroying melanoma cells, while preserving the normal skin keratinocytes. The occurrence of DNA fragmentation and lowering of mitochondrial membrane potential were significantly rampant in melanoma cells versus keratinocytes after treatment with WFA and WFA+WNA. This shows that there is greater potential for use when these two compounds are combined or WFA is used alone. This study brought new knowledge with respect to revealing the selectivity of WFA and WNA for A375 cells over keratinocytes, the synergistic relationship between WFA and WNA, the relative consistency in results obtained from 2D and 3D studies and the mechanisms of action of the different treatments. Therefore this laid the groundwork for further studies on the potential use of *W. somnifera* as a topical anti-melanoma agent.

In conclusion, the pure compound treatments reflected a higher level of anti-cancer activity than the crude extracts. Generally an effect was observed with all the treatments, but the most significant effects were the melanoma selectivity of majority of the treatments and the ability to induce DNA damage seen as DNA fragmentation and a lowering of mitochondrial membrane potential. It is prudent to bear in mind that fundamental apoptosis characteristics may not be observed *in vitro* owing to loss of signal transduction pathways or metabolic components (Bortner *et al.*, 1995:25)

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Competing Interests

The authors would like to declare that no competing interests exist with the contents of this manuscript.

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Table 1: IC_{50} values of the pure compounds, plant extracts and SLN formulation (calculated using withaferin A content in extracts). Values are shown as mean±SD of experiments done on cells grown in complete media only (n=9).

Treatment	Cell type	
	A375	**HaCaT**
WFA	26.25±1.16*	100.9±1.09
WFA (Matrigel®)	29.14±1.61*	72.64±1.09
WNA	80.61±1.12	-
WNA (Matrigel®)	78.57±1.22	-
WFA+WNA	13.87±1.13*	49.17±1.06
WFA+WNA (Matrigel®)	18.87±1.13*	39.47±1.06
Water extract	6.20±0.09*	1.85±0.03
Water extract (Matrigel®)	12.18±0.10*	6.81±0.02
Ethanol extract	0.51±0.02	0.52±0.02
Ethanol extract (Matrigel®)	21.88±0.17*	12.23±0.05
50% Ethanol extract	0.75±0.05*#	1.30±0.05#
50% Ethanol extract (Matrigel®)	3.82±0.05*	5.13±0.05
50% Ethanol extract SLNs	2.99±0.02#	3.15±0.02#

* – A375 differed significantly from HaCaT
# – 50% ethanol extract differed significantly from 50% ethanol extract SLNs

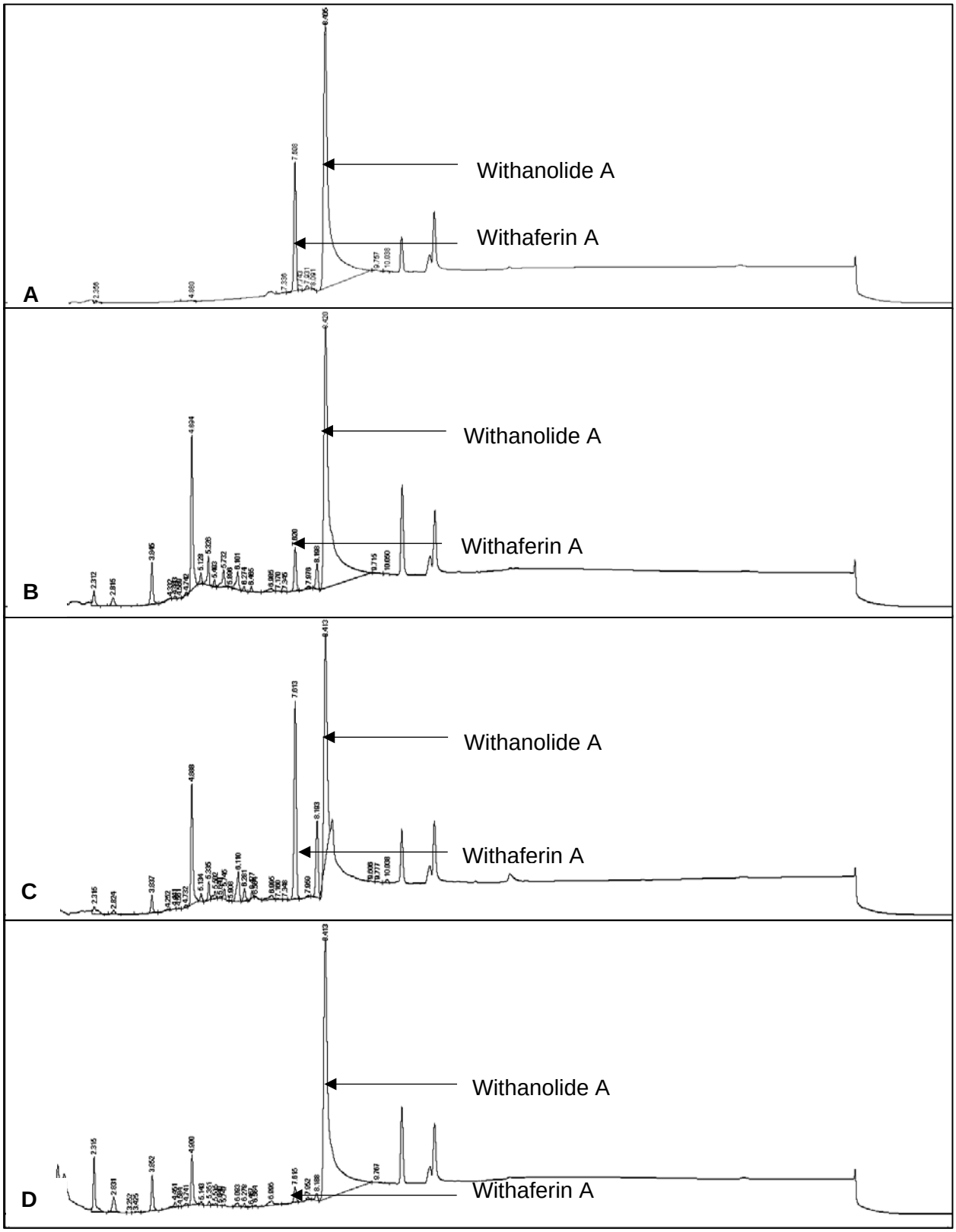

Figure 1: HPLC chromatograms of withaferin A and withanolide A standards (A), ethanol extract (B), 50% ethanol extract (C) and water extract (D) for HPLC finger-printing

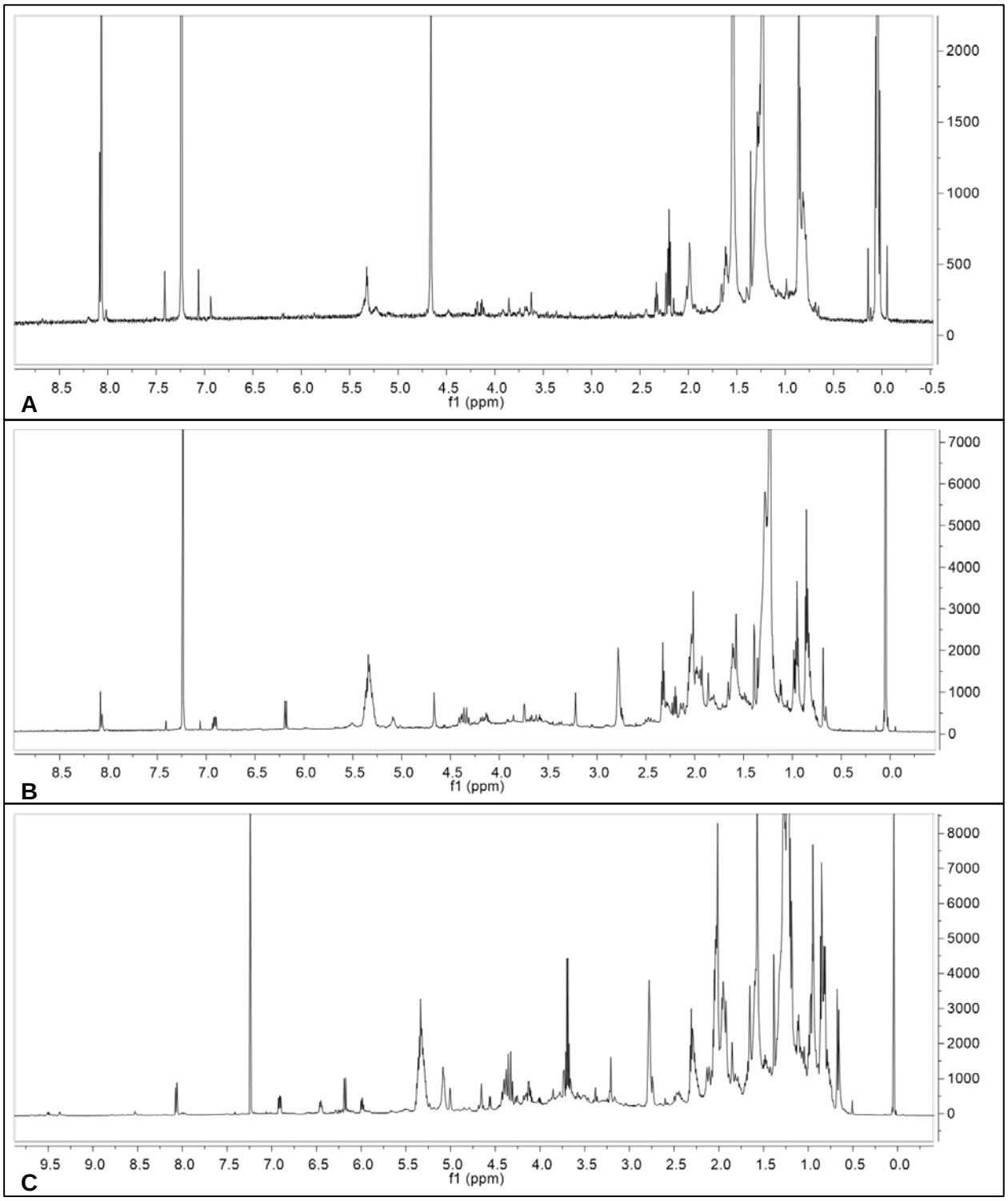

Figure 2: ^{1}H-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR finger-printing

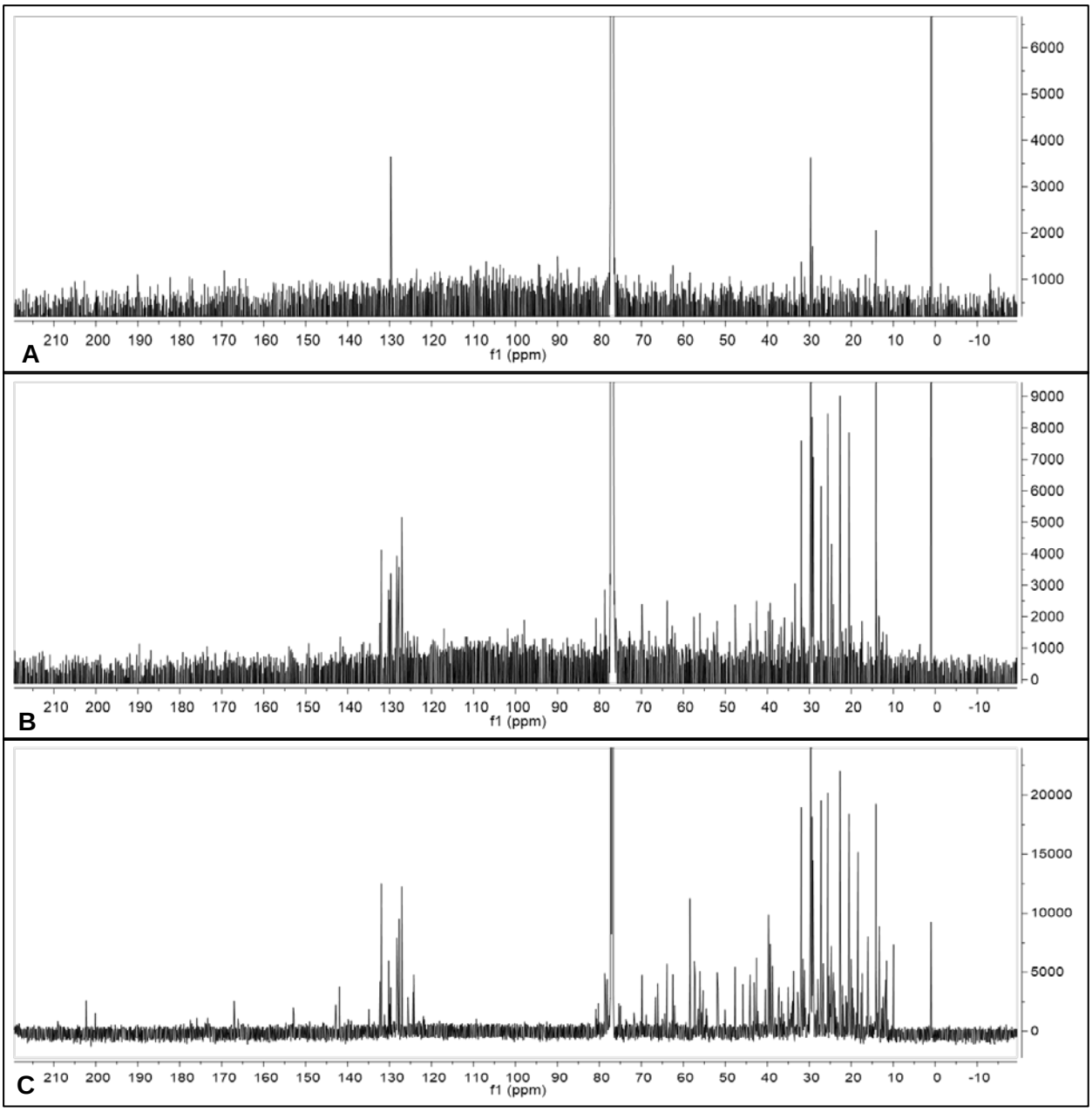

Figure 3: C^{13}-NMR spectra of water (A), 50% ethanol (B) and ethanol (C) crude extracts for NMR finger-printing

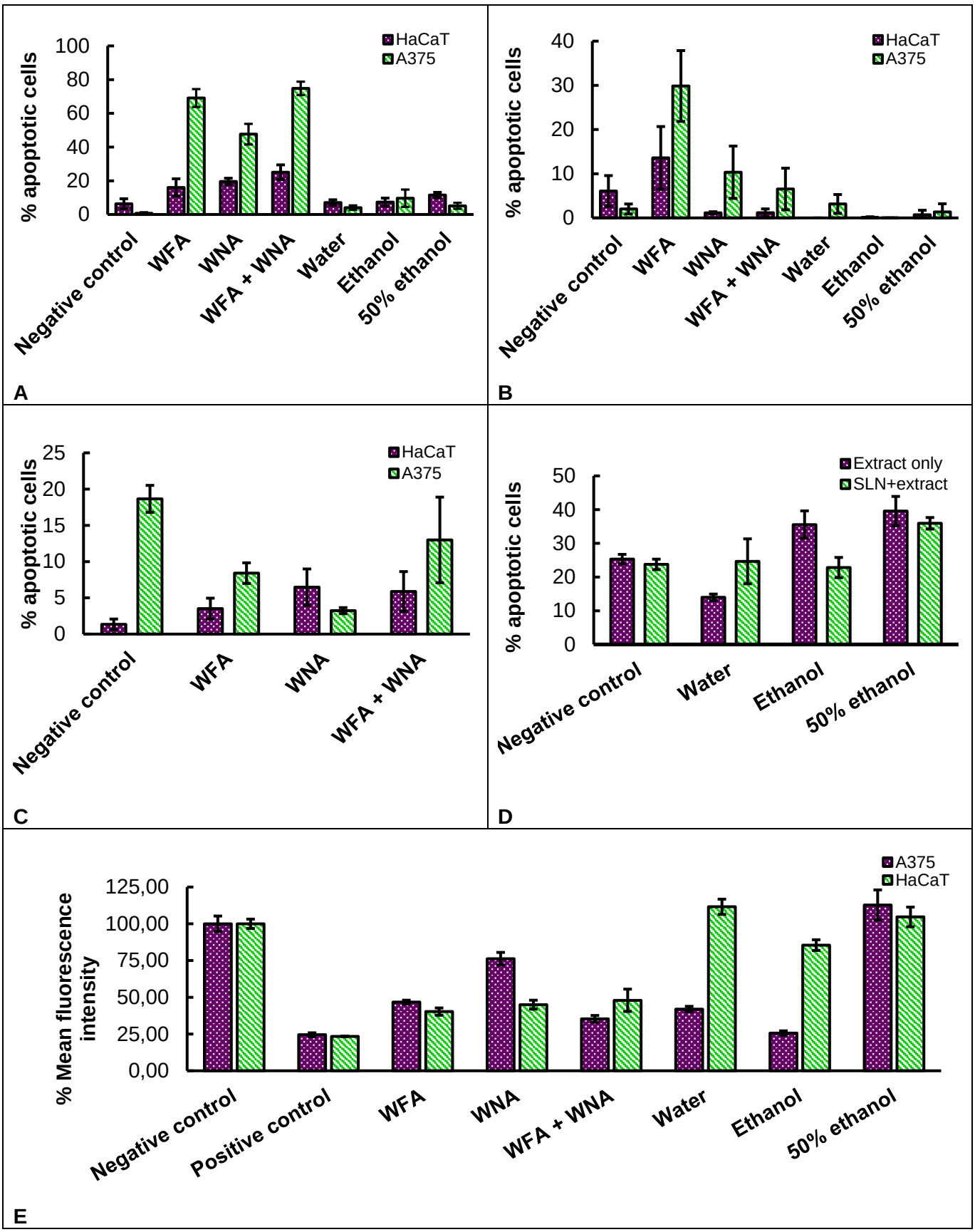

Figure 4: Percentage apoptotic cells of A375 and HaCaT cells with respect to DNA fragmentation (A), membrane permeability (B), Caspase 3/7 activity (C), membrane permeability in 3D (D) and mitochondrial membrane potential (E). Values are presented as mean±SD.

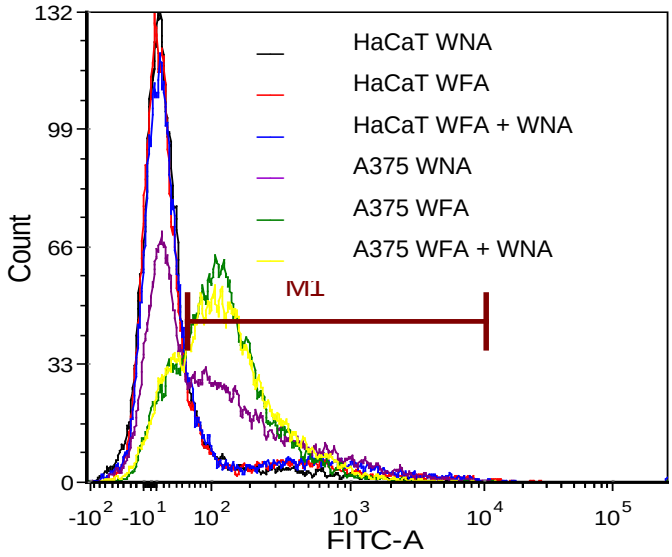

Figure 5: Overlay histogram showing selectivity of the pure compounds to induce apoptosis in A375 cells vs. HaCaT cells

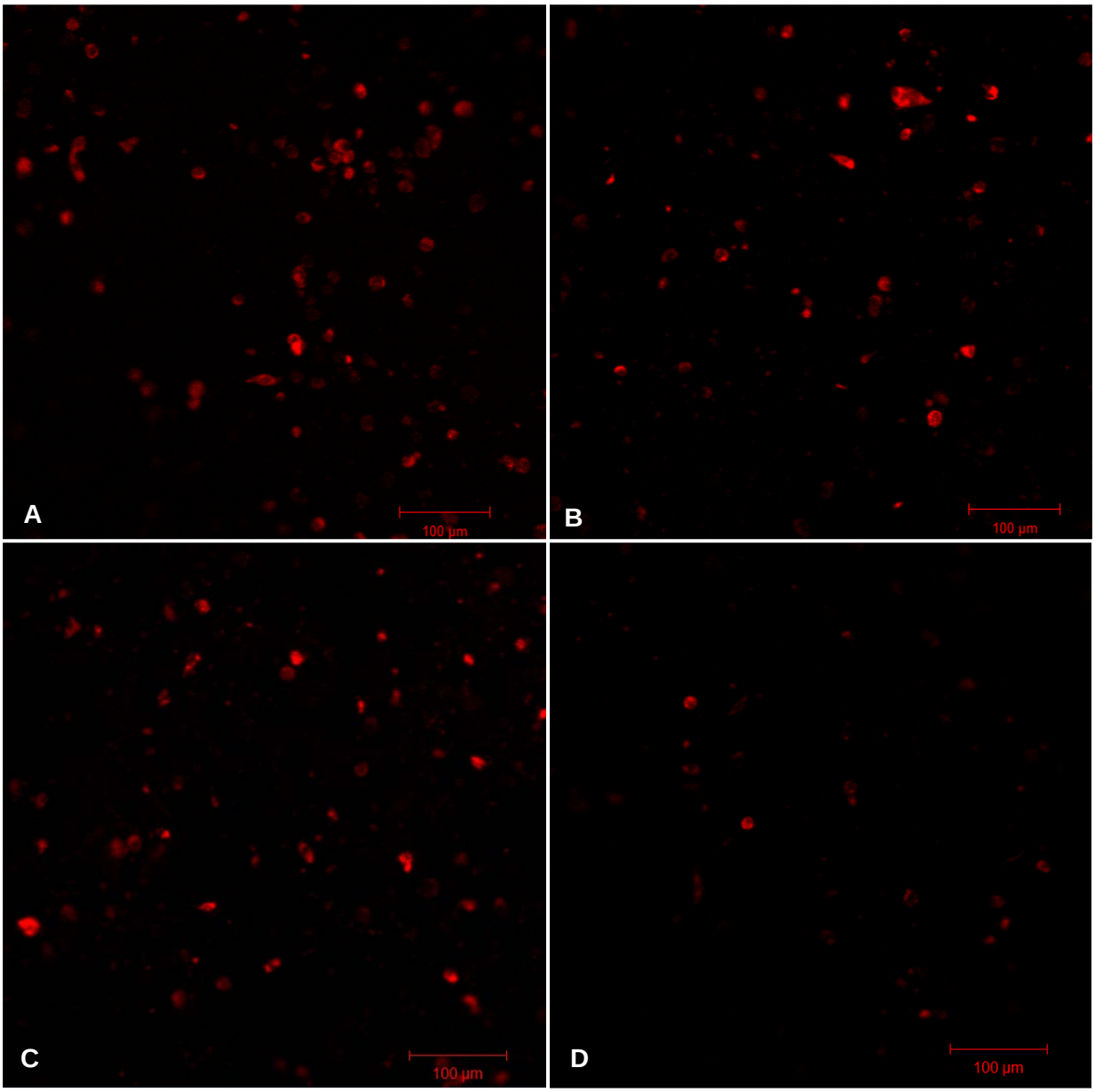

Figure 6: Confocal images of A375 cells; untreated on day 1 (A), treated on day 1 (B), untreated on day 10 (C) and treated on day 10 (D). Treated cells were treated with 50% ethanol extract every 2nd day.

Chapter 5: Final conclusion and future recommendations

This study focused on the topical delivery of *Withania somnifera* and its anti-melanoma efficacy. An investigation was made into the effects of niosomes and solid lipid nanoparticles (SLNs) on the topical delivery of *W. somnifera* extracts. The three-month stability of these formulations under room conditions (22 °C) was also investigated. In order to study the anti-melanoma efficacy, *in vitro* cytotoxicity and apoptosis assays were carried out using pure marker compounds and crude extracts. The marker compounds that were selected for use in this study are withaferin A and withanolide A. Withaferin A has been reported to be a very potent metabolite of *W. somnifera* possessing some anti-cancer activity (Jayaprakasam *et al.*, 2003:131).

Aqueous, ethanol and 50% ethanol crude extracts were prepared and finger-printed using HPLC, ^{1}H-NMR and C^{13}-NMR. The HPLC analytical method was able to detect withaferin A and withanolide A in the crude extracts. The 50% ethanol extract had the highest withaferin A content (4.55%) followed by the ethanol extract (1.76%) and lastly the water extract (0.98%). On the other hand the water extract had the highest withanolide A content (5.04%) followed by the 50% ethanol extract (3.04%) and lastly the ethanol extract (1.21%). The differences in percentage content were attributed to the effects of the solvents. Each extraction solvent had different affinities for the compounds in the leaves and therefore had distinct percentage withaferin A and withanolide A contents.

Niosomes and SLNs were formulated with the prepared crude extracts and characterised. The niosomes had a size range of 165.95 ± 9.44 nm to 304.57 ± 52.36 nm while the SLNs ranged from 172.27 ± 44.50 nm to 260.83 ± 51.37 nm. All the formulations were fairly homogenous with polydispersity indices (PDIs) below 0.5 except for the 50% ethanol SLNs which had a PDI of 0.75. The zeta potentials of the formulations ranged from -10.45 ± 4.10 mV to -34.98 ± 0.80 mV reflecting that some of the formulations had higher stability potential as compared to the low zeta potential formulations. A pH between 4 and 6 is considered as suitable for topical formulations (Ali & Yosipovitch, 2013:261) and all the formulations in this study were found to have average pH values between 5.183 and 5.709. The water extract formulations both had low withaferin A encapsulation but high withanolide A encapsulation efficiency. It was observed that the SLNs were able to encapsulate more withaferin A and withanolide A than their respective niosome formulations. This shows that SLNs were the superior formulation with respect to their ability to encapsulate the marker compounds in the extracts.

All the formulations were relatively unstable with respect to PDI, particle size and zeta-potential. The pH and encapsulation efficiencies remained fairly stable throughout the three month testing

period. Niosomes were found to be comparatively more stable than the SLNs. The stability of the formulations could have been enhanced by the use of a lyoprotectant such as sucrose during the freeze drying process (Majuru & Oyewumi, 2009:614; Hua *et al.*, 2010:213). An assessment would however need to be done to determine the compatibility of a particular lyoprotectant with the formulation excipients and required properties of the final formulation or dosage form. Nonetheless stability assessment of crude plant extracts may continue to be problematic due to them being a complex mixture of compounds.

Results of the membrane release studies revealed that after a 6 h Franz diffusion experiment withanolide A and withaferin A were not released from the water extract niosome formulation. This implied that the water extract niosomes either had a slow or negligible release of withaferin A and withanolide A. All the other formulations managed to release both withaferin and A withanolide A. There was a greater percentage and cumulative release of withaferin A as compared to withanolide A for all the formulations except the 50% ethanol extract formulations. The high withaferin A content of the 50% ethanol extract did not result in a higher release as would be expected by a steeper concentration gradient (Lu & Gao, 2010:74).

Both withaferin A and withanolide A were unable to permeate through the skin after the 12 h diffusion period. The skin deposition effects of SLNs may have been responsible for this observation (Madan *et al.*, 2014:63). This was the ideal effect as it is preferable for topical anti-cancer compounds to be deposited within the skin where they are to exert their effects on the melanoma cells. Tape-stripping results revealed that only the 50% ethanol extract SLNs were able to get withaferin A and withanolide A into the stratum corneum-epidermis (SCE) and the deeper epidermis-dermis (ED) skin layers. Withaferin A was detected in the SCE after diffusion from all the formulations except the water extract niosomes. While withanolide A only reached the SCE from the ethanol extract SLN, 50% ethanol extract niosome and 50% ethanol extract SLN formulations. Withaferin A only went deeper to the ED from the water extract SLNs and 50% ethanol extract SLNs. All the SLN formulations managed to result in withanolide A reaching the ED. The initial amounts of withaferin A and withanolide A in the different extracts probably influenced the movement of the compounds into and within the skin.

In vitro efficacy tests were conducted on A375 and HaCaT cells and it was concluded that the pure compounds had higher apoptotic activity than the crude extracts in both cell types. The results mainly reflected that the treatments had selectivity for inducing apoptosis in the melanoma cells as compared to the keratinocytes. After treatment on the A375 cells withaferin A (IC_{50} – 26.25 μg/ml) was found to be more potent than withanolide A (IC_{50} – 80.61 μg/ml) and the combination of withaferin A with withanolide A (IC_{50} – 13.87 μg/ml) had a synergistic effect. Seeing as the 50% ethanol extract SLNs were able to get withaferin A, the potent marker

compound, into the SCE and ED a cytotoxicity assay was done using it as a treatment. There was a significant difference between the results of the 50% ethanol extract SLN treatment and the crude extract treatment. Contrary to expected results, the SLNs resulted in a less toxic effect than the crude extracts thus alluding to a protective effect of SLNs.

Cytotoxicity and apoptosis assays were conducted on both cell types in two dimensional (2D) and three dimensional (3D) cell culture. The 3D cytotoxicity results were in line with what was obtained for the 2D results, thus confirming that the withaferin A and withaferin A + withanolide A combination were the most active. Statistical analysis also revealed that withaferin A and the combination treatments had the highest apoptosis inducing potential on the melanoma cells. It was found that withaferin A and the combination treatment induced a statistically significant occurrence of apoptosis as detected by DNA fragmentation and lowering of the mitochondrial membrane potential. Withanolide A induced apoptosis in melanoma cells as detected by the caspase pathway and the ethanol extract induced apoptosis which was detected by the lowering of the mitochondrial membrane potential. As an overall observation all the treatments selectively induced either apoptotic or necrotic cell death in the melanoma cells versus the keratinocytes after 24 h of treatment. Matrigel® was found to cause no significant change in the results as compared to using complete media thus implying that the effects of *W. somnifera* in a 2D environment were similar to the effects in a 3D environment.

In conclusion, the main findings of this study included:

- It is improbable to deliver clinically sufficient concentrations of withaferin A and withanolide A from the crude extracts to the systemic circulation via transdermal delivery thus making these crude extracts ideal for topical application as there will be no unwanted systemic effects.
- Solid lipid nanoparticles were found to be superior to niosomes with respect to delivering the crude extract marker compounds into the skin layers.
- Niosomes were comparatively better than SLNs regarding stability.
- Withaferin A is a more potent anti-melanoma agent than withanolide A, furthermore, the combination of withaferin A and withanolide A results in enhanced activity (synergism).
- The pure compounds used in this study were found to be more active than the crude plant extracts thus implying that although synergism may exist amongst the *W. somnifera* metabolites, antagonism within the crude extracts is also a possibility.
- The use of SLNs does not necessarily result in enhanced anti-melanoma efficacy but can instead lower the cytotoxic effect of the *W. somnifera* crude extracts as was observed in this study.

- DNA fragmentation and lowering of the mitochondrial membrane potential of the cells were the main modes of withaferin A and withanolide A induced apoptosis.

Through this study new knowledge was gained regarding; i) successfully encapsulating *W. somnifera* crude extracts in SLNs, ii) topical delivery of *W. somnifera* crude extracts using SLNs and niosomes, iii) revelation of the synergistic relationship between withaferin A and withanolide A, iii) confirmation of the apoptosis inducing ability of both withaferin A and withanolide A on A375 melanoma cells, iv) the ability to grow A375 melanoma cells in a 3D environment and conduct the XTT cytotoxic assay on the cells while in Matrigel® and v) the possibility that SLNs have a protective effect on melanoma cells against the cytotoxic effects of *W. somnifera* crude extracts. The contribution to science is very clear in that this study has revealed that withaferin A is an ideal candidate for treatment of melanoma as it is a potent anti-melanoma compound especially when combined with withanolide A. An additional contribution is the demonstration of relative selectivity of withaferin A for melanoma cells over keratinocytes.

In future it is recommended that further characterisation of the crude extracts be done in order to identify more of the compounds present in the extracts. This will allow the use of better formulation strategies as it will be possible to predict potential effects of the crude extract components on the final formulation. It will in turn be possible to improve on the stability of the formulations. Different types of drug delivery vesicles may need to be investigated so as to improve the dermal delivery of the marker compounds. With respect to the efficacy testing it may be advisable to carry out more testing with varied time-frames and concentrations so as to ensure that what has been identified as necrosis in this study is not late apoptosis as it cannot be easily distinguished from necrosis. It is also advisable to carry out further tests *in vivo* using xenografts in order to assess topical delivery and anti-melanoma efficacy. Three dimensional full thickness skin models can also be utilised for *in vitro* efficacy testing in order to minimise the use of animals. Further research on this plant is recommended as it has potential for use in the topical treatment of melanoma.

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Appendix A: Preparation of Withania somnifera crude extracts and extract characterisation

A.1 Introduction

In this study *Withania somnifera* (*W. somnifera*) crude extracts were prepared then assessed for their ability to penetrate the skin barrier and their anti-melanoma activity. The focus was on the plant leaves as the leaves are abundant, easily accessible and there is documented evidence of the leaves containing potent medicinal compounds. The plant leaves were purchased from Mountain Estate Herb Garden in Haartebeespoort, South Africa and the crude extracts for further experiments were prepared at the North-West University, Potchefstroom Campus.

A.2 *Withania somnifera*

W. somnifera is a medicinal herb that is commonly used in Ayurveda, a system of traditional medicine native to India (shown in Figure A.1). This plant is also known as Ashwagandha, Indian ginseng, poisonous gooseberry or the winter cherry. Various plant parts are used for their medicinal properties, these include the leaves, roots, bark, stems and fruits (Kulkarni & Dhir, 2008:1094). Studies have been conducted to investigate the medicinal potential of *W. somnifera* and it has been found that this plant has anti-diabetic, anti-hypertensive, anti-bacterial, adaptogenic, rejuvenation and anti-cancer properties (Nagella & Murthy, 2010:6735). In this study an investigation into the anti-melanoma potential of *W. somnifera* was carried out.

Figure A.1 *W. somnifera* whole plant (Planet Ayurveda, 2016)

Fernando *et al.* (2013:539) determined that the leaves of *W. somnifera* compared to other plant parts have the highest percentage of bioactive compounds such as polyphenols. In this same study it was also determined that the leaves have the highest anti-oxidant activity compared to the roots, stems, flowers and pods. The polyphenol content and anti-oxidant activity may be some

key contributors to the medicinal properties of *W. somnifera*. This project focused on the medicinal properties of the leaves which contain our compounds of interest, withaferin A and withanolide A whose chemical structures are shown in Figure A.2 (Malik *et al.*, 2009:1498).

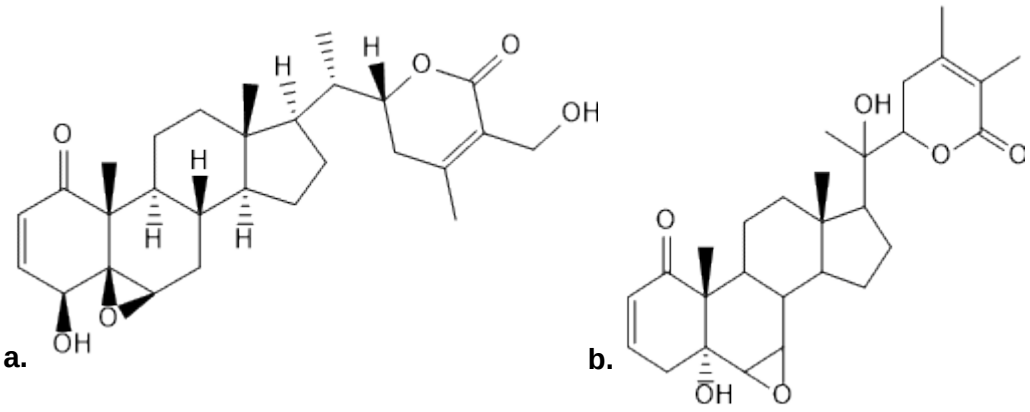

Figure A.2 Chemical structures of withaferin A (a) and withanolide A (b)

The main bioactive compounds present in *W. somnifera* are steroidal lactones with an ergostane backbone that are collectively known as withanolides (Ganzera *et al.*, 2003:69). Withaferin A and withanolide A were selected as our marker compounds for use throughout the project. The most potent bioactive compound present in *W. somnifera* has been reported to be withaferin A and there are various studies in which withaferin A was found to exhibit some anti-cancer properties (Jayaprakasam *et al.*, 2003:131).

Both withaferin A and withanolide A have $C_{28}H_{38}O_6$ as their molecular formulas. Their chemical structures have the same basic structure with a few differences in the positioning of functional groups. These differences however are the reason why the two compounds will display different activity against different conditions. In this study we assessed the differences in the anti-cancer activity of withaferin A and withanolide A.

A.3 Materials and Methods

Milli-Q water was used for the extractions and for preparation of samples and standards for HPLC analysis. Macherey-Nagel extraction thimbles with an inner diameter of 400 mm and a height of 1230 mm were used in all the extractions. The organic solvents that were used for the extractions, HPLC analysis and NMR analysis were ethanol, acetonitrile and deuterated chloroform ($CDCl_3$).

A.4 Preparation of *W. somnifera* crude extracts

W. somnifera leaves that were purchased from Mountain Herb Estate were harvested in the summer after at least two growth cycles. The plant specimen were dried and deposited at the

South African National Biodiversity Institute, National Herbarium in Pretoria for identification. The plant was identified by L.E. Makwarela as *Withania somnifera (L.) Dunal* (Figure A.3).

Upon receipt, the leaves were cleaned by manually separating them from dirt and other unwanted plant parts (fruits and stems) then air-dried for at least 7 days. Dried leaves were crushed to a fine powder by a ball mill (Retsch, Dusseldorf, Germany) in order to increase the surface area for extraction and stored at 4 °C until the time of extraction.

Initially, extractions were attempted with a Dionex accelerated solvent extractor (Sunnyvale, CA, USA) but due to the very fine powdered leaf particles the instrument kept clogging. It was therefore decided to use the conventional soxhlet apparatus for further extractions. Water, ethanol and 50% ethanol are the three solvents that were used for the extractions thus resulting in the three different extracts that were used throughout the project.

Soxhlet extraction is described as a continuous discrete extraction method since it occurs in a stepwise repetitive manner as described by Luque de Castro and Priego-Capote (2010). The following method was followed for the soxhlet extractions,

- Dry powdered leaf was measured (40g) and packed into a soxhlet thimble.
- The soxhlet, round-bottomed flask and glass beads were rinsed thoroughly with the solvent of interest then left to dry.
- Glass beads were added to the round-bottomed flask to allow for even distribution of heat, 250 ml of solvent was added to the flask then it was placed on the soxhlet extraction heating plate.
- The thimble was placed inside the soxhlet which was attached to the round-bottomed flask and connected to the cooling system.
- The heating plate was switched on and the temperature of the solvent was maintained at a steady “pre-boiling” stage.
- Extractions were left to continue for at least six cycles, over 24 h.

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Ref:15093 **Gift Dispatch List** **25 June 2015**

Client: Mrs. Chinenbiri
Address: North West University
Internal Box 36
Private bag X 6001
Potchefstroom
2531

Tel: 0182852485
Cell: 0729294101
Email: 20945175@nwu.ac.za

ID CODES:
1 = Specimen too poor to ID
2 = Label information inadequate
3 = Cannot match specimen in herbarium
4 = Specialist not available to do ID
5 = Genus requiring/under revision
6 = Specimen closest to name listed (cf)
7 = Please send more material
8 = Please refer to attached note/letter
9 = New record
K = specimen kept for herbarium
R = specimen returned
S = specimen scrapped

Collector	No.	Plant Name	Det. By	Det. Notes	ID Code	Fate
	1	*Withania somnifera (L.) Dunal*	Makwarela, L.E. 30/6/2015			K

Curator

Please note a handling fee is charged for each specimen received for identification. 1

Figure A.3 Plant specimen identification

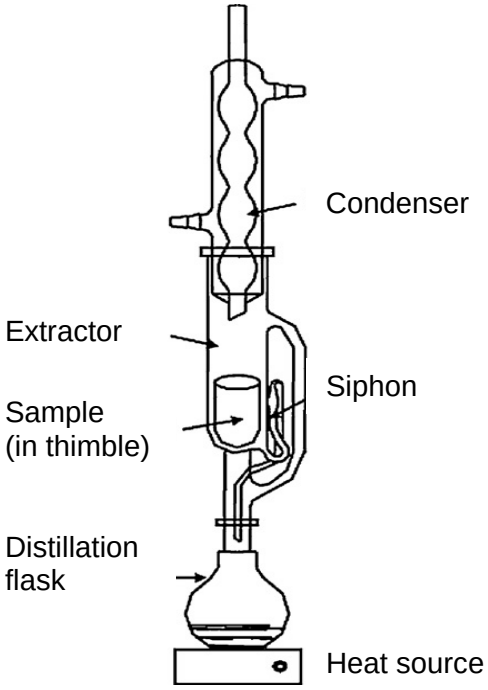

Figure A.4 Conventional soxhlet extraction setup (Luque de Castro & Priego-Capote, 2010)

Subsequent to soxhlet extraction the ethanol was removed from the extracts using a rotary evaporator. Care was taken to ensure that extract did not burn or overheat. The water was removed from extracts using a freeze-drier (VirTis, Gardiner, NY, USA).

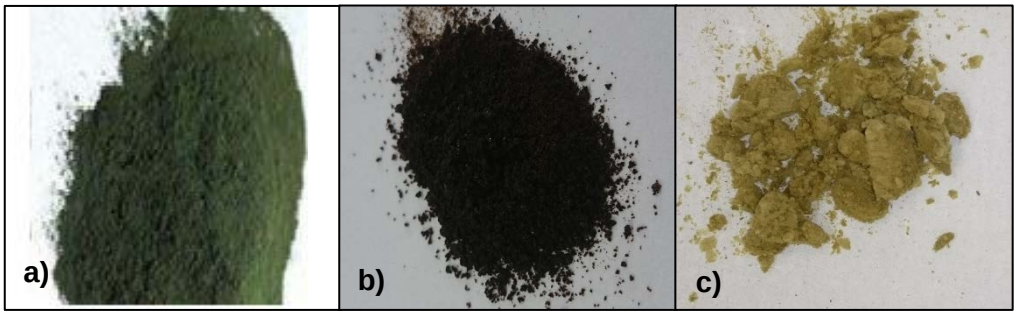

Figure A.5: Dried ethanol extract (a), 50% ethanol extract (b) and water extract (c)

The end products were dry powdered extracts (Figure A.5) and they were kept in glass containers at -20 °C for the duration of the study. The extraction yields for the ethanol, 50% ethanol and water extractions respectively were 9.2%, 8.3% and 6.7%.

A.5 HPLC method for *W. somnifera* extracts and standard compounds

A.5.1 Chromatographic conditions

The HPLC analytical method that was used in this study was developed with the assistance and knowledge of Professor Jan Du Preez of the North-West University (NWU), Potchefstroom. There are various methods in literature for detecting withaferin A and withanolide A, in this case it was ensured that the method was suitable for all the analyses that would be done (i.e. HPLC finger-printing of extracts, Franz cell diffusion studies and encapsulation efficiency). All the HPLC analyses were done in the Analytical Technology Laboratory (ATL) of the NWU. The parameters that were used in this HPLC analytical method are:

Analytical instrument: An Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) was used for the analyses. The machine consists of a quaternary pump, a degasser, an auto-sampler injection mechanism and a diode array detector. Chemstation Rev. A.10.02 software was used for the acquisition of data and analysis.

Column: A Phenomenex® Synergi Fusion Reversed Phase column (4.6 mm x 250.0 mm), with a 4 µm particle size was used (Phenomenex®, Torrance, CA).

Mobile phase A: Milli-Q water

Mobile phase B: 100% Acetonitrile

Flow rate: 1 ml/min

Injection volume: 50 µl

Detection wavelength: 210 nm

Stop time: 22 min

Solvent: Methanol and Milli-Q water were used as the solvents for standard preparation

Table A.1 HPLC timetable for mobile phases A and B

Time (minutes)	Mobile phase A % v/v	Mobile phase B % v/v	Comment
0.00-10.00	90.0→0.0	10.0→100.0	Linear gradient

10.00-15.00	0.0	100.0	Isocratic
15.00-15.10	0.0→90.0	100.0→10.0	Linear gradient
15.10-20.00	90.0	10.0	Re-equilibration

A.5.2 Preparation of standard

The standard was prepared by dissolving 0.50 mg of compound (withaferin A or Withanolide A) in 1 ml of methanol then filling to 10 ml with Milli-Q water. This gave a standard with a concentration of 50 μg/ml.

A.5.3 Linearity

In order for an analytical method to be considered accurate within a particular concentration range the peak areas detected should be directly proportional to the analyte concentration in the sample (ICH, 2005:5). Therefore, the linearity of an analytical method must be determined beforehand so as to ensure that all analyses are done within the appropriate concentration range. If the analytical method is stable and reliable within a given analyte concentration range the linear regression coefficient (r^2) value should be between 0.99 and 1 ($0.99 \leq r^2 \leq 1$).

The linear range of the analytical method was determined by preparing analytical standards of withaferin A and withanolide A at 6 different concentrations and injecting them on the HPLC in duplicate. Linear regression analysis was performed on the plot of the peak area versus the average concentration (μg/ml).

The data obtained when evaluating the linearity of an analytical method is best described by the general equation of a line,

$y = mx + c$ where: **Equation A.1**

- y = peak area ratio of the analyte
- m = slope of the line (gradient)
- x = concentration of the analyte in μg/ml
- c = y-intercept

The acceptance criteria for linearity is that the r^2 value should be ≥0.99 (Du Preez, 2010:5). Such a value indicates that the method is highly linear and thus should give accurate and reliable results. Table A.22 – A.3 below show the peak areas that were obtained at each concentration of standard for the determination of linearity.

Table A.2 Results for linearity of withaferin A

Standard (μg/ml)	Peak area ratio
5.00	220.5
10.00	404.2
20.00	795.2
30.00	1192.5
40.00	1591.7
50.00	1988.4
slope (m) **y intercept (c)** **r^2**	**19.705** **13.956** **0.999**

An r^2 value of 0.999 was obtained for withaferin A using this method. This led to the conclusion that this method is accurate and can be used within a withaferin A concentration range of 5.00-50.00 μg/ml.

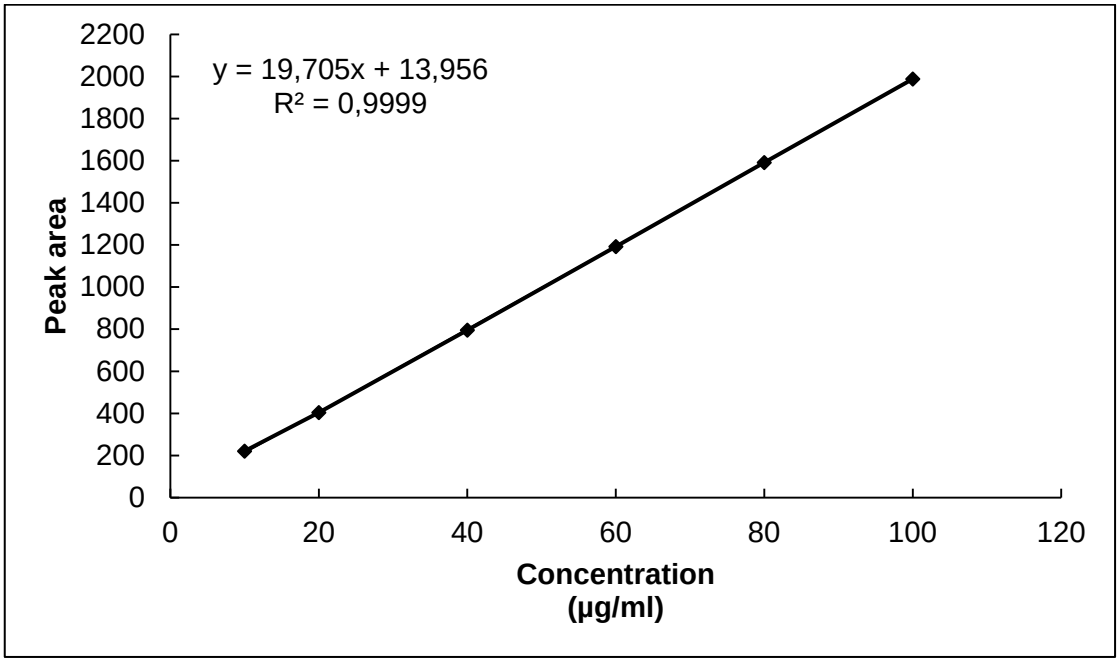

Figure A.6 Linear regression curve of withaferin A

Table A.3 Results for linearity of withanolide A

Standard (μg/ml)	Peak area ratio
5.00	179.5
10.00	355.0
20.00	712.9
30.00	1069.1
40.00	1424.1
50.00	1781.0

slope (m)	**16.184**
y intercept (c)	**0.4327**
r^2	**1**

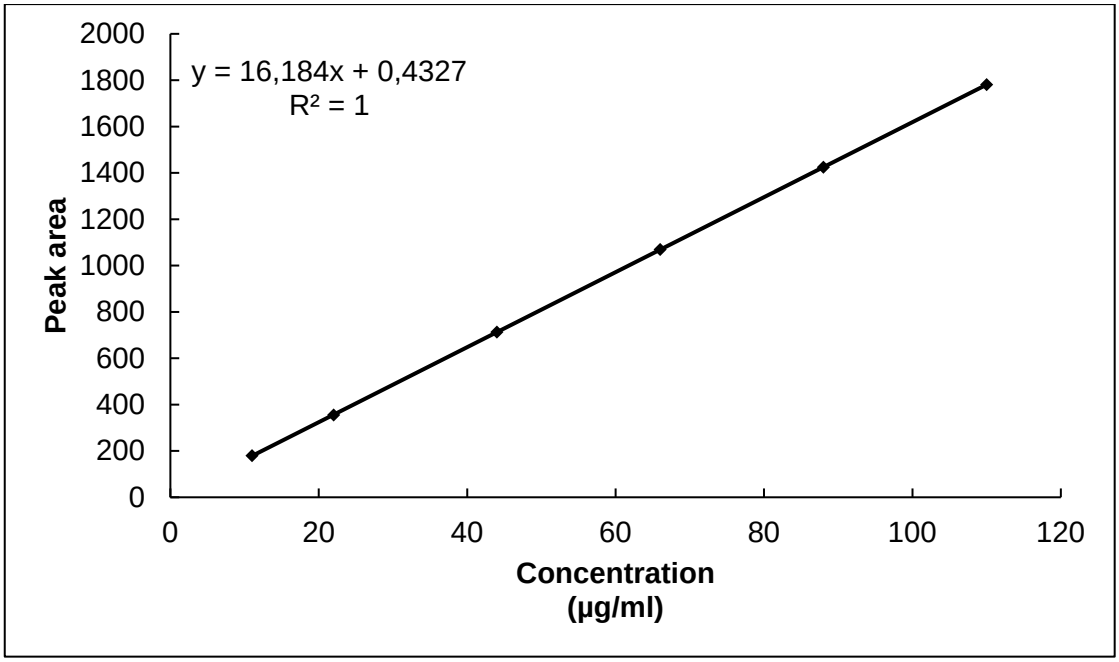

Figure A.7 Linear regression curve of withanolide A

A.5.4 Lower limit of quantification (LLOQ)

The lower limit of quantification of an analytical method is the lowest analyte concentration that can be accurately and precisely determined quantitatively using the set method (FDA, 2001:20). This limit is therefore the lowest concentration that can be quantified by the analytical method. In this study the withaferin A and withanolide A LLOQs of the developed HPLC analytical method were determined. The LLOQ is determined by injecting very low concentrations of standards six consecutive times. The percentage relative standard deviation (%RSD) of the replicate injections should not exceed 15%. The withaferin A and withanolide A LLOQ values for this HPLC analytical method were set at 0.05 and 0.25 µg/ml respectively (see Tables A.4 – A.5. Any results obtained that were below the LLOQ were deemed unreliable and therefore rejected.

Table A.4 Lower limit of quantification of withaferin A

STD[1] (µg/ml)	Peak area (1)	Peak area (2)	Peak area (3)	Peak area (4)	Peak area (5)	Peak area (6)	Mean	SD[2]	%RSD
0,050	2,212	2,006	2,766	2,311	2,110	2,026	2,238	0,258	11,529
0,125	5,064	5,279	5,700	5,419	5,601	5,509	5,429	0,210	3,871
0,250	11,157	12,005	11,926	11,000	11,400	12,176	11,611	0,447	3,848
0,500	22,654	21,985	23,025	22,589	22,897	22,007	22,526	0,402	1,785

1. Standard (STD)
2. Standard deviation (SD)

Table A.5 Lower limit of quantification of withanolide A

STD[1] (µg/ml)	Peak area (1)	Peak area (2)	Peak area (3)	Peak area (4)	Peak area (5)	Peak area (6)	Mean	SD[2]	%RSD
0,100	3,0987	3,0257	4,3047	3,1994	4,2192	4,0730	3,6535	0,552	15,109
0,250	8,4633	9,3125	9,2017	9,4573	8,5996	8,6182	8,9421	0,392	4,383
0,500	17,0721	17,7992	17,8891	18,2027	17,6571	18,5007	17,8535	0,446	2,499
1,000	36,2458	36,0859	35,1807	35,8996	35,9991	36,3505	35,9603	0,379	1,054

1. Standard (STD)
2. Standard deviation (SD)

A.5.5 HPLC analysis of *W. somnifera* crude extracts

In order to determine the amounts of withaferin A and withanolide A present in a plant extract a known amount of crude extract was dissolved in methanol and Milli-Q water as described in section A.5.2. It was then analysed using the described HPLC analytical method. The percentage content of withaferin A and withanolide A that was present in each extract has been tabulated in Table A.6.

Table A.6 Withaferin A and withanolide A content of crude extracts

W. somnifera crude extract	Withaferin A content	Withanolide A content
Ethanol	1.76%	1.21%
50% Ethanol	4.55%	3.04%
Water	0.98%	5.04%

Figure A.8 shows the withaferin A and withanolide A peaks in the standard solution and in the crude extracts. All the crude extract chromatograms had the withaferin A and withanolide A peaks

at the expected time-points. Withaferin A eluted at approximately 7.5 min while withanolide A eluted at approximately 8.5 min. The respective peak areas were representative of the amount of marker compound present in each extract.

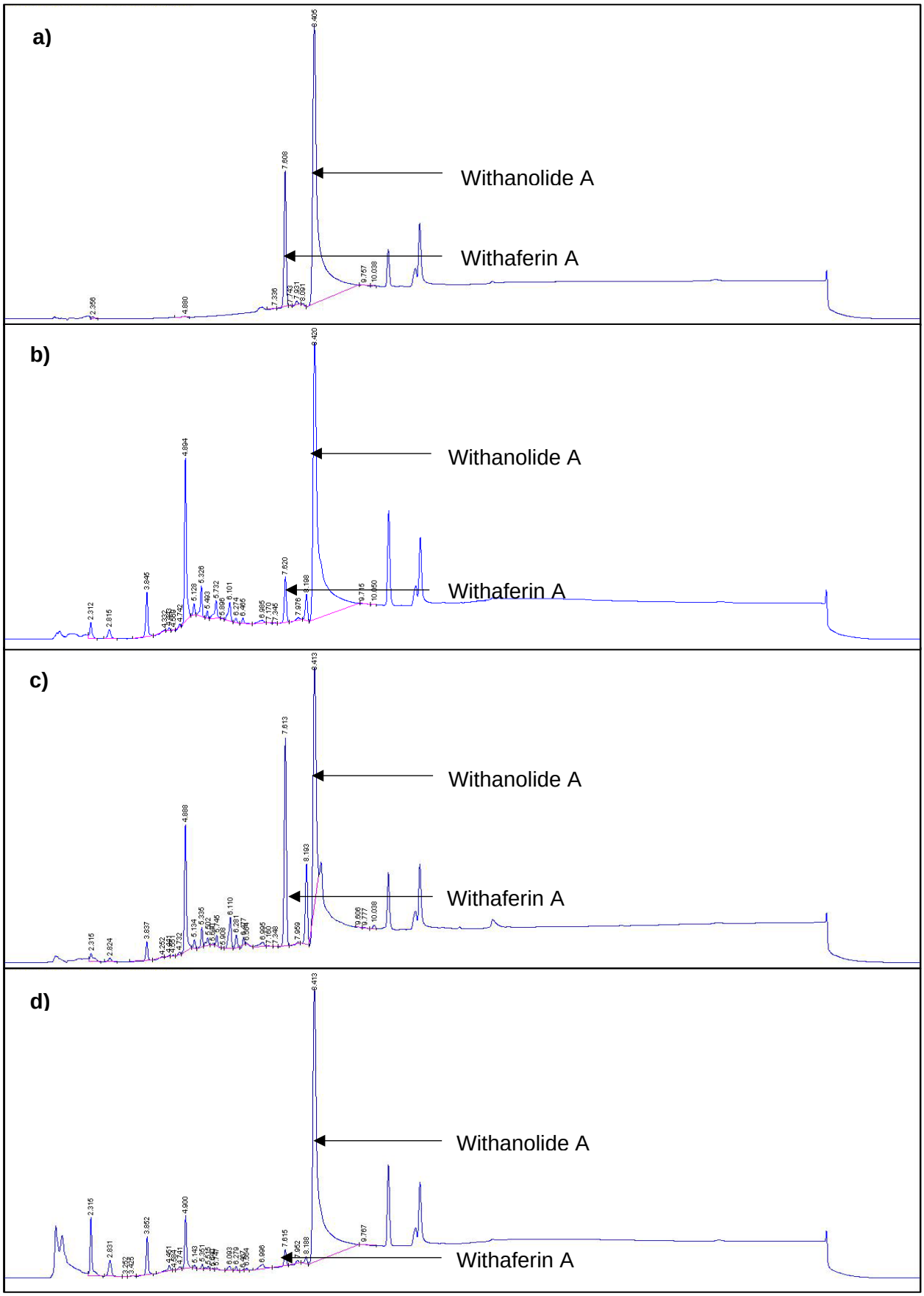

Figure A.8 HPLC chromatograms of withaferin A and withanolide A standards (a), ethanol extract (b), 50% ethanol extract (c) and water extract (d) for HPLC finger-printing

A.6 Nuclear magnetic resonance (NMR) fingerprinting of *W. somnifera* crude extracts

Approximately 10 mg or 50 mg of marker compound or crude extract respectively were weighed and dissolved in 1.5 ml deuterated chloroform ($CDCl_3$; Merck, South Africa). The obtained solutions were filtered using cotton wool into NMR tubes. ^{1}H-NMR and C^{13}-NMR analyses were done using an Avance III 600 Hz NMR Spectrometer (Bruker, Billerica, MA, USA). The NMR spectra were used to indicate the marker compounds and to finger-print the crude extracts.

A.6.1 NMR spectra of pure compounds and crude extracts

NMR spectra were obtained for withaferin A, withanolide A, water extract, ethanol extract and 50% ethanol extract. It was challenging to clearly identify and differentiate the marker compounds in the extracts as the withanolides have similar chemical structures thus making it difficult to separate compounds in the crude extracts. Figures A.9 – A.12 show the NMR spectra that were obtained. The peaks present in the pure compound spectra were seen in the spectra of the crude extracts giving an indication that the marker compounds were present in the extracts. Due to the presence of other compounds in the crude extracts various other peaks were also present.

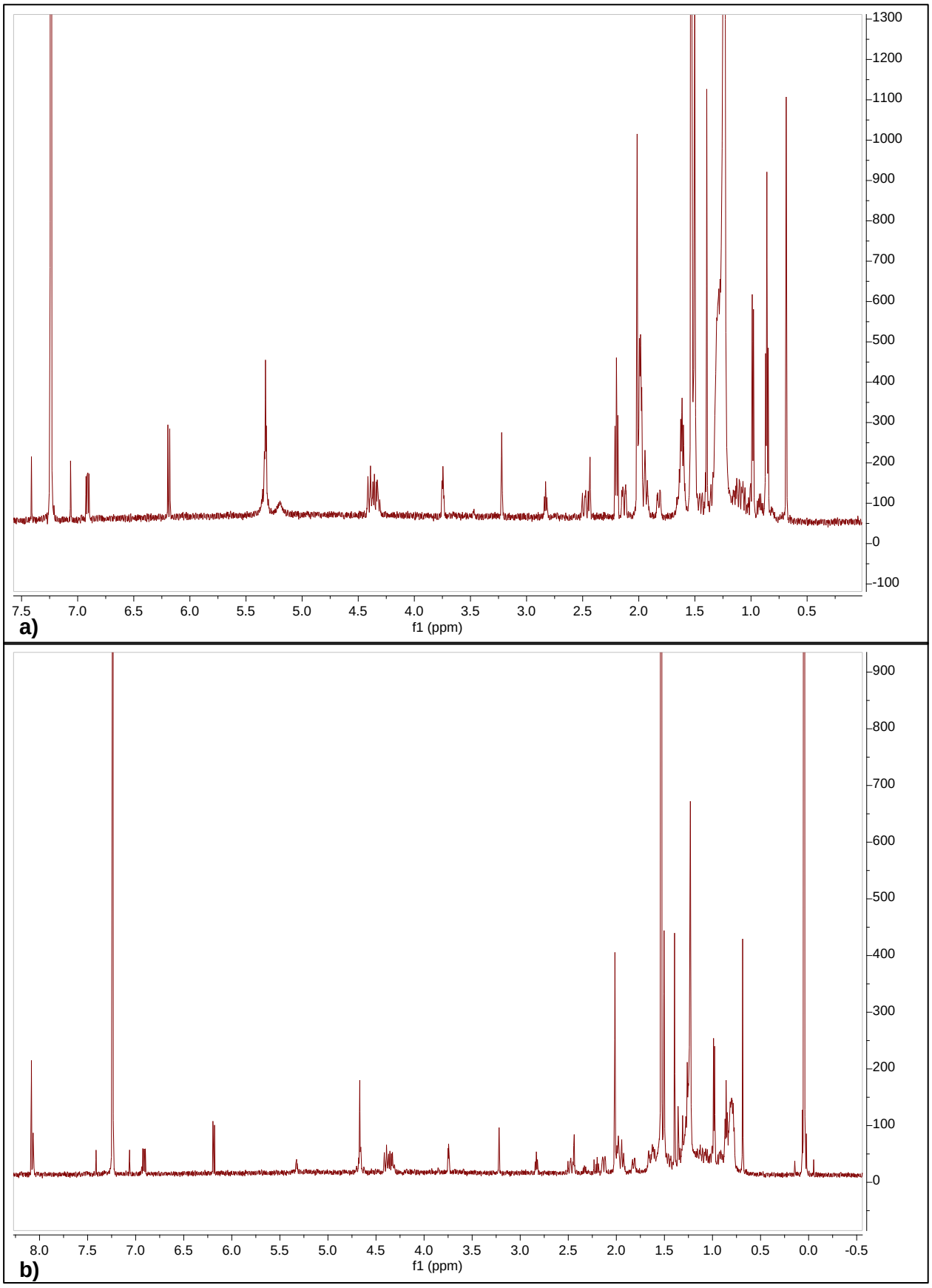

Figure A.9 ^{1}H-NMR spectra of withaferin A (a) and withanolide A (b)

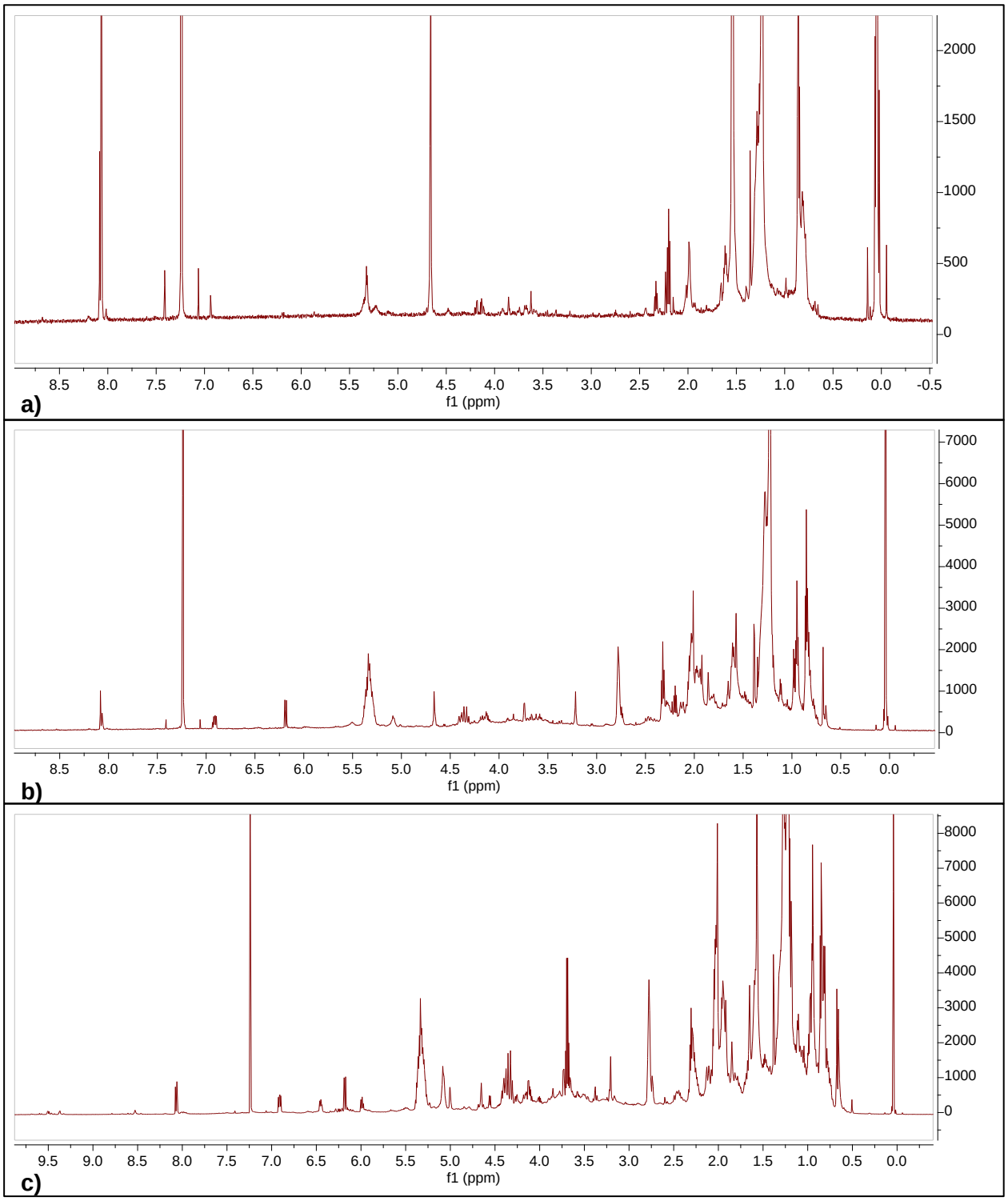

Figure A.10 ^{1}H-NMR spectra of water (a), 50% ethanol (b) and ethanol (c) crude extracts for NMR finger-printing

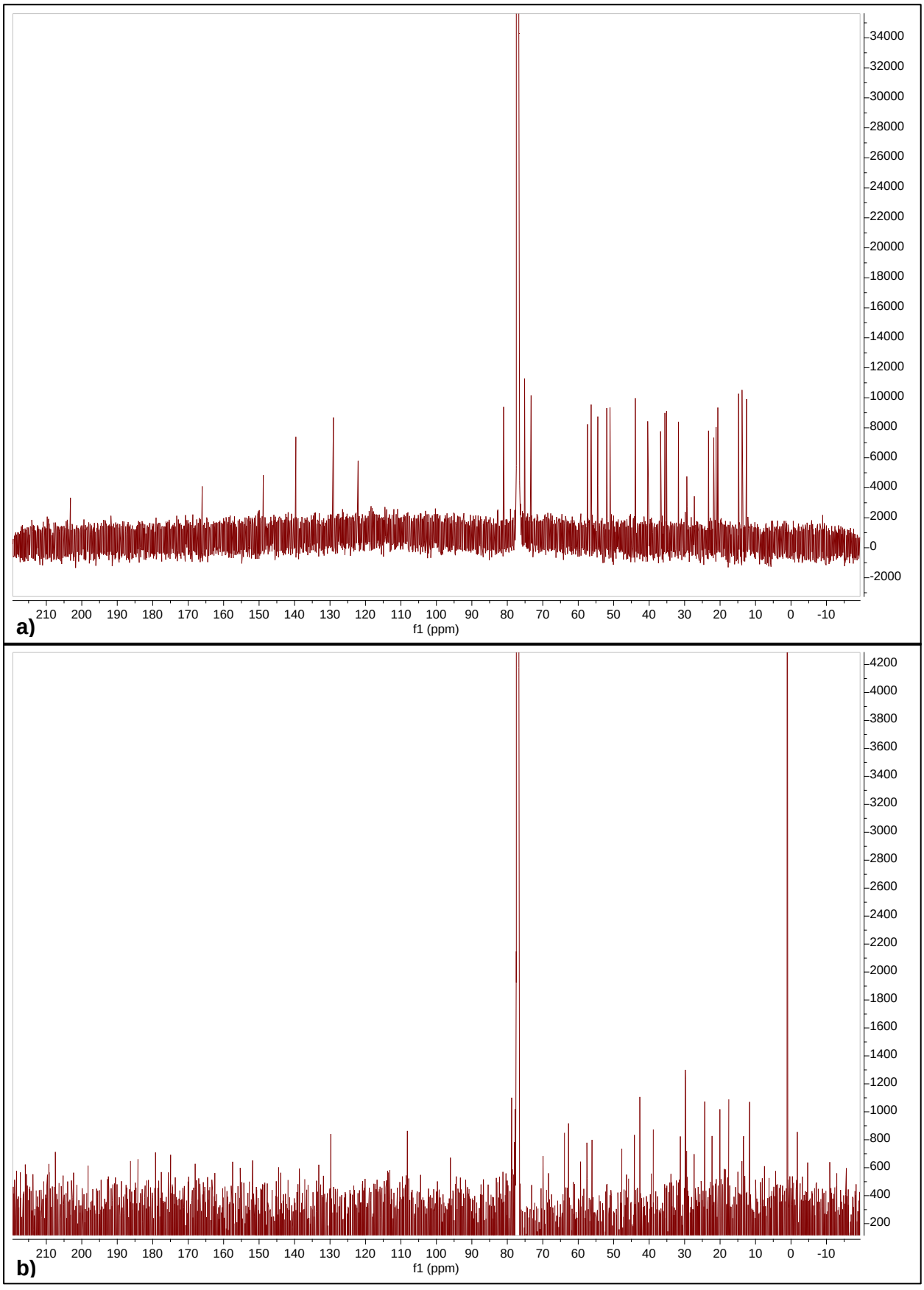

Figure A.11 C^{13}-NMR spectra of withaferin A (a) and withanolide A (b)

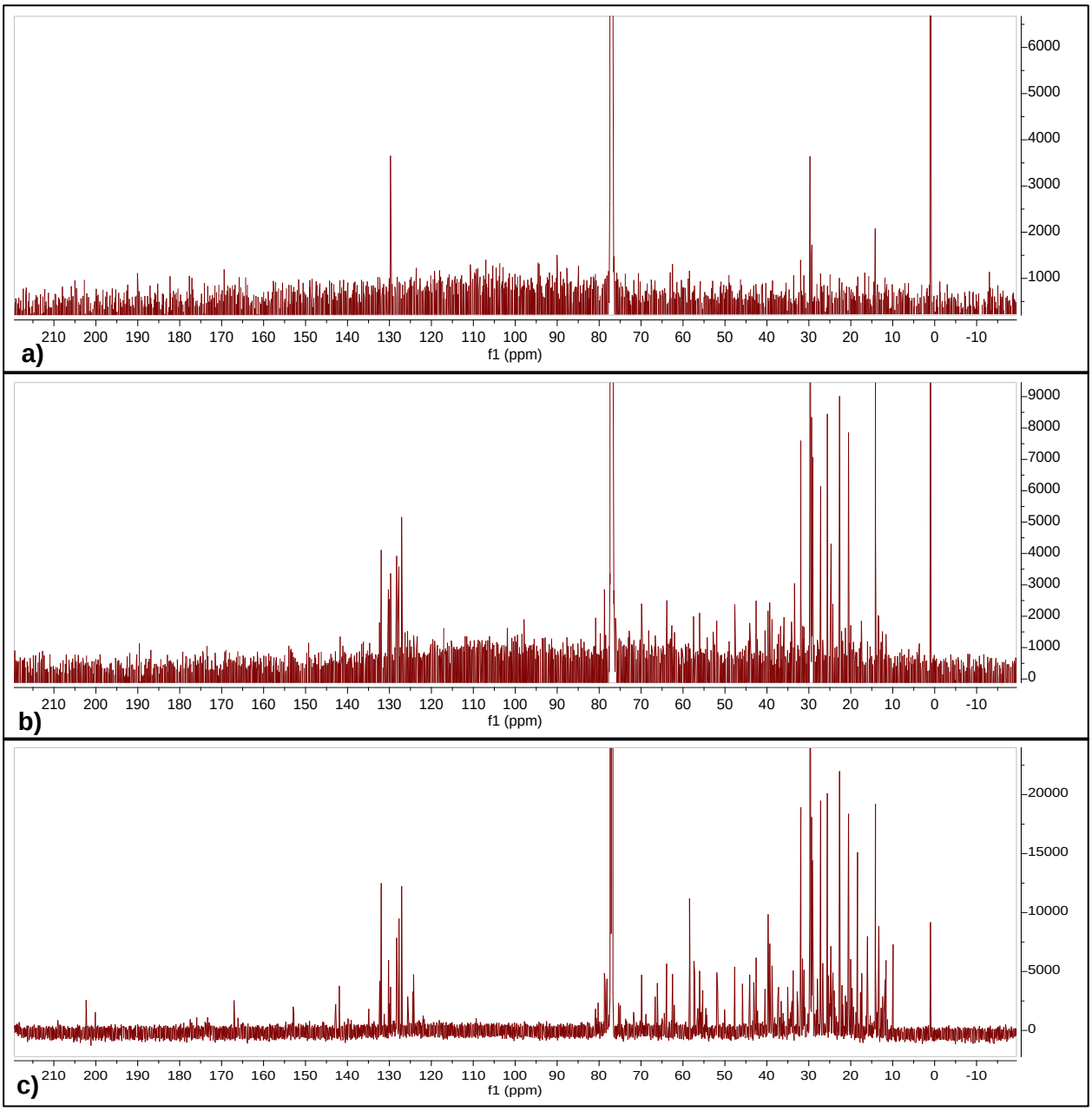

Figure A.12 C^{13}-NMR spectra of water (a), 50% ethanol (b) and ethanol (c) crude extracts for NMR finger-printing

The water extract NMR spectra showed that the water extract did not have as dense a concentration of compounds as was present in the ethanol and 50% ethanol extracts. This may have been due to the compounds present in the water extract failing to fully dissolve in the $CDCl_3$. Thus some compounds may have not been detected by the NMR analysis as they were not in solution in the analysed samples.

A.7 Conclusion

The HPLC analytical method that was used in this study was found to be reliable and gave consistent results throughout the study. If a concentration below the lower limit of quantification was detected the results were deemed unreliable and discarded. According to the spectra and chromatograms, withaferin A and withanolide A were present in all three crude extracts with different percentage compositions.

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ICH **see** International Conference on Harmonisation.

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Appendix B: Formulation of niosomes and solid lipid nanoparticles for topical delivery of Withania somnifera crude extracts

B.1 Background

Due to the selectively impervious nature of the skin, vesicles are being used progressively as drug delivery vehicles for topical and transdermal drug delivery. Vesicles in nanoparticulate form have become the ideal choice for delivery of compounds to the skin in cosmetics and in pharmaceuticals. There are various forms of vesicles that have been formulated to date and these include solid lipid nanoparticles (SLNs), niosomes, nanostructured lipid carriers (NLCs), ufosomes, liposomes, ethosomes and phytosomes to name a few (Yeh *et al.*, 2013:239). These nanovesicles are continuously investigated and used for the delivery of natural and synthetic active pharmaceutical ingredients (APIs) through the skin (Manosroi *et al.*, 2013:474). In this study niosomes and SLNs were selected as the vesicles of choice for the topical delivery of *Withania somnifera* crude extracts.

B.2 Vesicles for topical drug delivery

B.2.1 Niosomes

Niosomes are also known as non-ionic surfactant vesicles. They are similar to liposomes as they also have the bilayer membrane structure that is similar to cell membranes (Wang *et al.*, 2012:1134). They are however made from non-ionic surfactants such as Brij, Spans or Tweens and are stabilised with cholesterol (Mujoriya *et al.*, 2011:21; Rahimpour & Hamishehkar, 2012:142). The API is encapsulated in the hydrophilic core or in the vesicular membrane (illustrated in Figure B.1) which in turn results in prolonged API circulation. Niosomal systems have been gaining more interest as vehicles for targeting API delivery to the skin (Rahimpour & Hamishehkar, 2012:142).

The first commercialisation of niosomes was by L'Oréal in the anti-ageing treatment cream Niosôme by Lancôme in 1986 (LÓréal, 2015). Since then, further research has been done on niosomes in order to improve on their effectiveness, versatility and stability as drug delivery vesicles. Liposomes and niosomes are conventionally known to topically deliver APIs to the upper stratum corneum layer of the skin versus permeating through the skin for a transdermal effect. Therefore, elastic and other innovative niosomes have been developed that are able to enhance

skin permeation and squeeze through the skin pores and reach the deeper layers of the skin (Manosroi *et al.*, 2013:474).

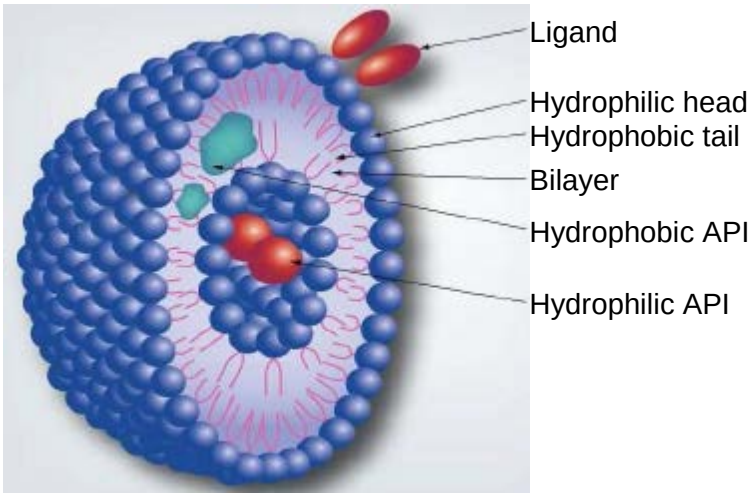

Figure B.1: Illustration of niosomes adapted from Chandu *et al.* (2012:25)

Drug delivery vesicles, including niosomes, have been reported to enhance transdermal absorption, increase physicochemical stability and reduce adverse effects such as irritation (Manosroi *et al.*, 2012:318). The greater stability of niosomes, in comparison to phospholipid based vesicles, makes them more attractive pharmaceutically and cosmetically due to less care required in handling and storage (Marianecci *et al.*, 2012:18). Other advantages of niosomes are good biocompatibility, increased bioavailability, targeted drug delivery and regulated drug distribution (Wang *et al.*, 2012:1134). The targeted drug delivery property of niosomes has been manipulated using pH sensitivity and it was shown that 5-fluorouracil pH-sensitive niosomes had a tumour targeting effect and a low organ damaging potential (Wang *et al.*, 2012:1140). In as much as there are advantages to using niosomes for drug delivery there are many limitations which still have to be overcome such as, reproducibly formulating vesicles in specific shapes and sizes, as well as fully controlling drug delivery and release (Prow *et al.*, 2011:486).

Niosomes have a wide variety of documented uses. These include dermal and transdermal drug delivery, ocular delivery, oral delivery, pulmonary delivery, parenteral delivery, gene delivery, diagnostics and natural product delivery (Marianecci *et al,.* 2014:200). It therefore made sense in this study to investigate the influence of niosomes on the dermal and transdermal delivery of a natural product (*Withania somnifera* extract)

B.2.2 Solid lipid nanoparticles (SLN)

SLNs are vesicles whereby lipids and surfactants that are generally regarded as safe (GRAS) are utilised to make vesicles that have a solid lipid matrix (Jain *et al.*, 2010:443). Depending on the nature of the API it can be entrapped in the core of the lipid matrix or at the surfactant/co-

surfactant interface of the particles as shown in Figure B.2. Initially SLNs were used in parenteral formulations, but the use has spread to other routes of drug delivery (Zur Mühlen *et al.*, 1998:149). In this study it was decided to use SLNs and niosomes as drug delivery vesicles in order to enable the drawing of a comparison between the topical deliveries of the different crude extracts.

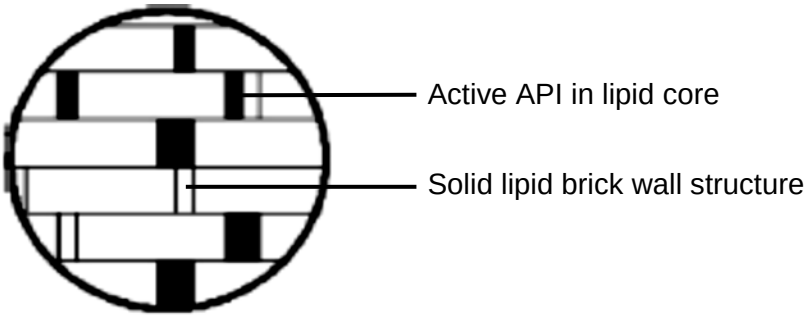

Figure B.2: Illustration of solid lipid nanoparticles adapted from (Pardeike *et al.*, 2009:171)

Relatively lipophilic APIs are usually considered for entrapment in and delivery using SLNs while other vesicles, e.g. liposomes, niosomes, etc. are mainly considered for hydrophilic APIs (Kuchler *et al.*, 2009:169). The marker compounds in question, withaferin A and withanolide A, are both sparingly soluble in water; with withanolide A having a slightly higher reported water solubility (Cayman Chemical, 2015). It was therefore presumed that withaferin A would display better topical delivery using SLNs in comparison to withanolide A. It has however been proven that SLNs are also able to enhance the topical delivery of hydrophilic APIs (You *et al.*, 2007:270; Kuchler *et al.*, 2009:172; Singh *et al.*, 2010:655).

SLNs are advantageous in that they help achieve controlled and targeted release, in turn reducing systemic absorption of API (Jain *et al.*, 2010:443; Musicanti & Gasco, 2012:2471). This is due to the presence of solid lipid in comparison to oils that are present in other drug delivery vesicles such as liposomes; APIs are relatively less mobile in solid lipid as compared to their mobility in liquid oil. Other advantages are: i) they are small in size; thus increasing the surface area for skin permeation; ii) they allow for encapsulation of macromolecules (e.g. peptides and proteins) and both hydrophilic and lipophilic drugs; iii) they are relatively non-toxic to some cells (e.g. Caco-2 cells) at low concentrations (Severino *et al.*, 2014:33) and iv) they are able to protect APIs from photo-degradation when dispersed in a gel matrix (Carlotti *et al.*, 2009:1524). SLNs are not without disadvantages and these include poor API loading capacity, expulsion of API after polymeric transition of lipid during storage and stability problems (e.g. aggregation and component degradation) with long-term storage (Garud *et al.*, 2012:385; Musicanti & Gasco, 2012:2471).

SLNs have been formulated for application in parenteral, nasal, respiratory, ocular, rectal, topical, anti-cancer and anti-tubercular pharmaceutical formulations to name a few (Garud *et al.*, 2012:390). The influence of SLNs on the anticancer efficacy of camptothecin was investigated by Huang *et al.* (2008:1101) and they reported that the most sustained release was from the SLNs versus the free drug, NLCs or lipid emulsions. In this study SLNs were therefore formulated for potential application in topical anti-cancer formulations.

B.3 Formulation of niosomes and solid lipid nanoparticles

There are various methods that have been used for formulation of different types of vesicles. The methods for the different vesicles are usually similar but only differ regarding the different excipients used to come up with a different vesicular end product. The most common methods due to their simplicity, cost and ability to give reproducible results are the film hydration method and the solvent injection method. Other methods are the hand shaking method, high pressure homogenisation, ultrasonication, spray drying and microemulsion based SLNs preparation (Rajera *et al.*, 2011:946; Garud *et al.*, 2012:390).

B.3.1 Excipients used in the formulation of niosomes and solid lipid nanoparticles

Table B.1: Excipients that were used in the formulation of niosomes and solid lipid nanoparticles

Excipient	Reason for inclusion	Supplier	Batch number
Tween® 80	Surfactant	Merck Chemicals, Darmstadt, Germany	1043695
Span® 60	Surfactant	Merck Chemicals, Darmstadt, Germany	S5361721 034
Cholesterol	Stabiliser for niosomes	Sigma Aldrich, St Louis, MO	SLBD3586V
Compritol® 888 ATO (glyceryl behenate)	Solid lipid for SLNs	Gattefosse, Lyon, France	141749
Precirol® ATO 5 (glyceryl distearate)	Solid lipid for SLNs	Gattefosse, Lyon, France	146829
L-α-Phosphatidylcholine	Solid lipid for SLNs	Sigma Aldrich, St Louis, MO	SLBJ3005V
Sodium cholate hydrate	Edge activator	Sigma Aldrich, St Louis, MO	BCBJ9825V
Chloroform	Solvent	Sigma Aldrich, St Louis, MO	STBD3317V

B.3.1.1 Tween® 80

Tween® 80 is also known as polysorbate 80 and the official chemical name is polyoxyethylene 20 sorbitan monooleate. It is a yellow oily viscous liquid at normal room conditions. In

pharmaceutical formulations it acts as a wetting agent, solubiliser and emulsifier. It is on the GRAS list and is therefore used in topical, oral and parenteral formulations (Lawrence, 2005a:581).

It has been documented that surfactants with high hydrophilic-lipophilic balance (HLB) values are unable to form vesicles and rather form lamellar structures due to aggregation and coalescing. HLB values give a guideline as to whether a particular surfactant is able to form vesicles. The HLB value of Tween® 80 is 15.0, which means that it is water soluble and leads to its use in oil in water (o/w) emulsions (Marianecci *et al.*, 2014:189). It was selected as one of the surfactants in this study in order to balance out the low HLB value of Span® 60 and aid solubilisation of the hydrophilic components of the crude extracts. Tween® 80 was used as an emulsifier and solubiliser in both the niosome and the SLN formulations.

B.3.1.2 Span® 60

Span® 60 is a sorbitan fatty acid ester and is often referred to as sorbitan monostearate. It is a cream solid often used in pharmaceutical and cosmetic formulations as an emulsifying agent, solubiliser and as a wetting agent at a concentration range of 0.1-15.0%. Span® 60 is generally regarded as non-toxic and non-irritating; hence, its use in food products, cosmetics, oral formulations and topical formulations. In this study Span® 60 was incorporated for its emulsifying and solubilising properties in the niosome and in the SLN formulations (Lawrence, 2005b:713).

B.3.1.3 Cholesterol

Cholesterol is a lipophilic compound obtained from animal fat and wool fat. It is used in pharmaceutical formulations as an emollient and as an emulsifying agent. In topical and cosmetic formulations cholesterol is used as an emulsifying agent at concentrations of 0.3-5.0% (Peltonen, 2009:179). In this study a cholesterol concentration of 0.4% was used in the niosomes.

Cholesterol is used in the formulation of niosomes as it increases cohesion in the lipid bilayer of the vesicles, thus aiding vesicle formation and vesicle stability. The steroid molecule combines with a single chain of surfactant and thus results in an increased stability (Marianecci *et al.*, 2012:21). Span® 60 and cholesterol are said to interact by way of hydrogen bonding. In any niosome formulation cholesterol influences the fluidity, aggregation, elasticity, size and shape of the niosomes (Kumar & Rajeshwarrao, 2011:212).

B.3.1.4 Compritol® 888 ATO

Compritol® 888 ATO is glycerol dibehenate or glyceryl behenate and it is composed of a mixture of diacylglycerols, monoacylglycerols and triacylglycerols. It is often used in pharmaceutical and

cosmetic formulations as a coating agent, binding agent, lubricant and thickening agent. Compritol® 888 ATO has been investigated as an encapsulating agent and successfully used in encapsulating lipophilic APIs in the formulation of solid lipid microparticles (SLMs), SLNs and NLCs (Pople & Singh, 2009:286). In this study, Compritol® 888 ATO was explored for use in the formulation of the SLNs.

B.3.1.5 Precirol® ATO 5

Precirol® ATO 5 exists as a fine white powder which is a mixture of mono-, di-, and triglycerides of 16C- and 18C-fatty acid chains. It is generally used as a taste masker in oral dosage forms, tablet lubricant and as a matrix for sustained drug release. Due to its relatively high melting point it has been investigated for use in the formulation of SLMs and SLNs (Armstrong, 2009:293).

B.3.1.6 Sodium cholate

Sodium cholate is used as an edge activator in the formulation of vesicles as it gives the vesicles elasticity which allows vesicles to squeeze through the skin pores. So-called “elastic niosomes” were formulated by Manosroi *et al.* (2013:475) and they reported that they showed a lower cytotoxicity and higher physicochemical stability in comparison with the free API. The sodium cholate is said to make the vesicles elastic by accumulating at sites of high stress and reducing the energy required for a change in shape. Another possible mechanism by which sodium cholate brings about vesicle elasticity is by intercalating between the surfactant molecules (e.g. Tween®) and therefore decreasing the packing density which results in elasticity (Manosroi *et al.*, 2013:479).

B.3.1.7 Phosphatidylcholine

Phosphatidylcholine is a phospholipid and is therefore an amphiphilic molecule. It is thus able to form micelles and other unilamellar and multilamellar vesicular structures due to its ability to associate at the hydrophilic/hydrophobic interfaces. Phosphatidylcholine is a well-tolerated excipient in pharmaceutical formulations and in this study it was used in the formulation of the SLNs as part of the lipid phase. Due to its amphiphilic nature it acts as an emulsion stabiliser, surfactant and as a solubilising agent (Lambert *et al.*, 2009:499).

B.3.2 Method for formulation of solid lipid nanoparticles and niosomes

In this study it was decided to use the solvent injection method for formulating the SLNs and the niosomes. Below is a step by step description of how the vesicles were formulated:

1) All the excipients were weighed out.
2) The lipophilic constituents were dissolved in the organic solvent (diethyl ether).

3) The aqueous phase was heated to 60 °C on a hot plate while stirring with a magnetic stirrer.

4) The prepared organic phase was slowly injected into the aqueous phase using an 18 gauge needle.

5) A temperature of 60 ± 2 °C was maintained and stirring continued until all the organic solvent was driven off.

6) Using a Hielscher UP 200ST sonicator (Hielscher Ultrasound Technology, Teltow, Germany), the resulting formulation was sonicated on ice for 1-5 min to come up with a homogenous end-product.

B.4 Physicochemical characterisation of formulations

There are particular properties that are usually determined in order to characterise vesicles so as to control the quality of the end-product. The commonly investigated parameters are size, polydispersity index (PDI), morphology, zeta-potential, number of lamellae, pH, encapsulation efficiency and composition (Marianecci *et al.*, 2014:194). These physicochemical properties of vesicles are influenced by i) hydration temperature, ii) choice of main surfactant, iii) nature of membrane additives, iv) size reduction techniques and v) nature of drug (Uchegbu & Vyas, 1998:34). Therefore these factors were kept uniform in order to obtain a uniform and optimal product.

Diameters of nanovesicles can be determined using dynamic light scattering (DLS), photon correlation spectroscopy (PCS) or some electron microscopy techniques (scanning electron microscopy, negative-staining transmission electron microscope, atomic force microscopy, etc.). In this study we used DLS on the Zetasizer Nano ZS, (Malvern Instruments, Worcestershire, UK) and the transmission electron microscope (TEM) to determine the sizes of the formulated vesicles. The TEM images provided information with respect to the general shape of the vesicles, number of lamellae and the size of the vesicles. A multi-lamellar vesicle is clearly seen on TEM images by the presence of more than one surfactant bilayer in an “onion-like” skin arrangement (Marianecci *et al.*, 2014:194). The Zetasizer measurements gave an indication of the size and the homogeneity of the vesicle suspensions by calculating the PDI of each formulation.

Zeta-potential was measured using the Zetasizer Nano ZS, (Malvern Instruments, Worcestershire, UK). A zeta-potential (mV) measurement gives an indication of API/vesicle interactions and stability of the emulsion in question. It also indicates the charge on the vesicles as it may be desirable at times to form charged niosomes with oppositely charged polyatomic ions.

The pH of freshly prepared formulations was measured using a calibrated Mettler Toledo pH meter (Mettler Toledo, Columbus, OH, USA). All pH measurements were done in duplicate at 25 °C. The pH probe was rinsed after each measurement in order to avoid cross-contamination of samples.

In order to determine the encapsulation efficiency the method as described by Junyaprasert *et al.* (2008:853) was followed with a few modifications. The formulated SLNs or niosomes were centrifuged in an ultracentrifuge at a speed of 30 000 rpm and a temperature of 4 °C for a period of 30 min. Subsequent to centrifuging the supernatant was collected and diluted with Ultra-pure water then analysed using the HPLC method as described in Appendix A, Section A.5. Equation B.1 was then used to calculate the percentages of the marker compounds (withaferin A or withanolide A) encapsulated in the vesicles.

$$\%EE = \left(\frac{\text{Total amount of compound added - Free amount of compound}}{\text{Total amount of compound}}\right) \times 100\% \qquad \textbf{Equation B.1}$$

B.5 Optimisation of formulations

Niosomal dispersions are formed at a surfactant/lipid level within the range 10-30 mM as documented by Uchegbu and Vyas (1998:40). However, a wider range of surfactant/lipid concentrations for niosome formulation is available in literature. In this study 26 mM of surfactant/lipid was used in the formulation of niosomes.

SLNs come about when the liquid lipid (oil) of o/w emulsion is replaced by a solid lipid or a combination of solid lipids in a concentration range of 0.1% to 30.0% (w/w). The solid lipid is dispersed in an aqueous medium such as water or buffer and stabilised using 0.5 to 5.0% (w/w) surfactant (Pardeike *et al.*, 2009:171). Thus after a thorough literature study it was decided to work with lipid and surfactant within the stated concentrations ranges.

In order to conserve the plant extract available; various placebo formulations were made according to Table B.2 and Table B.3. In the trial phase of formulating the SLNs, the influence of the surfactant/lipid ratio, the choice of lipid and duration of sonication on the different formulations was investigated. With the niosomes the three variables that were investigated are surfactant/cholesterol ratio, choice of surfactant and sonication time. Choice of surfactant was done using the HLB values of the excipients as described bySeverino *et al.* (2014:96). The pH, zeta-potential and particle size of each formulation was measured in duplicate. The formulation with the optimum characteristics was chosen for final formulation with the plant extract.

Table B.2: Formulation of niosomes for the purpose of optimisation

	Surfactant: Cholesterol	Surfactant	Sonication time (min)	Formulation code
A	1:1	Tween®	1	AT1
			5	AT5
		Tween® + Span®	1	ATS1
			5	ATS5
B	2:1	Tween®	1	BT1
			5	BT5
		Tween® + Span®	1	BTS1
			5	BTS5
C	4:1	Tween®	1	CT1
			5	CT5
		Tween® + Span®	1	CTS1
			5	CTS5

Table B.3: Formulation of solid lipid nanoparticles for the purpose of optimisation

	Surfactant: lipid	Lipid	Sonication time (min)	Formulation code
A	1:1	Precirol®	1	AP1
			5	AP5
		Compritol®	1	AC1
			5	AC5
B	2:1	Precirol®	1	BP1
			5	BP5
		Compritol®	1	BC1
			5	BC5
C	4:1	Precirol®	1	CP1
			5	CP5
		Compritol®	1	CC1
			5	CC5

B.5.1 Results for optimisation of formulations

The formulations obtained were milky white and the consistency ranged from watery to viscous emulsions.

Table B.4: Average values for optimisation of placebo niosomes (n = 3). Values are presented as mean ± SD.

Formulation	pH	Zeta-potential (mV)	Size (nm)	PDI

AT1	7.11 ± 0.24	-18.6 ± 0.15	112.5 ± 3.00	0.326 ± 0.003
AT5	7.11 ± 0.24	-19.6 ± 0.55	133.9 ± 1.40	0.276 ± 0.004
ATS1	7.21 ± 0.11	-34.5 ± 0.05	287.2 ± 15.95	0.628 ± 0.076
ATS5	7.21 ± 0.11	-33.5 ± 0.05	137.2 ± 3.15	0.332 ± 0.009
BT1	7.84 ± 0.27	-19.0 ± 0.50	77.5 ± 0.17	0.215 ± 0.005
BT5	7.84 ± 0.27	-18.3 ± 0.45	141.4 ± 1.55	0.091 ± 0.015
BTS1	7.73 ± 0.09	-35.1 ± 0.55	189.7 ± 0.15	0.449 ± 0.002
BTS5	**7.73 ± 0.09**	**-34.2 ± 0.10**	**131.7 ± 0.30**	**0.265 ± 0.000**
CT1	7.20 ± 0.13	-18.7 ± 0.65	74.7 ± 0.63	0.270 ± 0.002
CT5	7.20 ± 0.13	-18.5 ± 0.10	142.2 ± 1.00	0.224 ± 0.015
CTS1	7.20 ± 0.16	-33.2 ± 0.35	140.8 ± 2.25	0.370 ± 0.003
CTS5	7.20 ± 0.16	-30.1 ± 0.50	137.5 ± 0.65	0.378 ± 0.007

All the placebo niosome formulations had a pH in the range 7.11-7.84, which is relatively high in comparison to the pH of skin, but still acceptable for skin application. Sonicating for 5 min versus sonicating for 1 min resulted in relatively more homogenous dispersions as indicated by the decrease in PDI when sonicated for longer. The formulations with Tween® 80 as the only surfactant had relatively small sizes, but the lower zeta-potential |values| suggested that they were less stable than the vesicles composed of a mixture of Tween® 80 and Span® 60. In light of all this, Formulation BTS5, with average pH, zeta-potential, size and PDI values of 7.73, -34.2, 131.7 nm and 0.265 respectively, was selected as ideal for formulation of the niosomes.

Table B.5: Average values for optimisation of placebo solid lipid nanoparticles (n = 3). Values are presented as mean ± SD.

Formulation	pH	Zeta-potential (mV)	Size (nm)	PDI
AP1	7.22 ± 0.13	-32.2 ± 0.10	348.1 ± 8.65	0.504 ± 0.023
AP5	7.22 ± 0.13	-29.9 ± 0.35	233.7 ± 2.20	0.476 ± 0.001
AC1	**7.01 ± 0.07**	**-38.5 ± 0.05**	**180.2 ± 3.60**	**0.266 ± 0.003**
AC5	7.01 ± 0.07	-27.3 ± 6.50	398.9 ± 43.45	0.784 ± 0.096
BP1	7.59 ± 0.32	-18.1 ± 0.60	758.4 ± 15.10	0.956 ± 0.011
BP5	7.59 ± 0.32	-28.8 ± 0.35	449.1 ± 7.45	0.728 ± 0.008
BC1	7.16 ± 0.18	-0.27 ± 0.01	133.6 ± 1.60	0.379 ± 0.008
BC5	7.16 ± 0.18	-3.03 ± 0.20	159.6 ± 2.90	0.332 ± 0.039
CP1	7.94 ± 0.11	-0.01 ± 0.00	851.3 ± 44.45	0.459 ± 0.016
CP5	7.94 ± 0.11	-0.09 ±0.01	177.1 ± 7.70	0.785 ± 0.096
CC1	7.07 ± 0.26	-3.32 ± 0.06	785.9 ± 74.05	1.000 ± 0.000
CC5	7.07 ± 0.26	-12.9 ± 0.50	628.3 ± 0.80	0.781 ± 0.016

All the placebo SLN formulations had a pH between 7 and 8, with the Compritol® 888 ATO formulations presenting with lower average pH values than the corresponding Precirol® formulations. No general trend was observed with respect to the rest of the properties of the placebo SLN formulations. Regardless of sonication being done on ice, the heat produced during the sonication process may have had an impact on the properties of the SLNs, due to the high amounts of lipid present. It was therefore decided to use Formulation AC1 as the final formulation for the SLNs, as it had the most optimal and consistent values.

B.5.2 Results for the physicochemical characterisation of the final formulations

Tables B.6 and B.7 show the final niosome and SLN formulas that were used throughout the study. The final ratio (%w/w) of surfactant to cholesterol that was used for the niosomes is 2:1. The formulations only differed with respect to the crude extract that was used as the active constituent. The final formulations had creamy to dirty-green appearances due to presence of the leaf extracts; see Figure B.3 and Figure B.4.

Table B.6: Formula for the optimised formulation of niosomes

Ingredient	Amount (mg)
Tween® 80	16 (0.16%)
Span® 60	64 (0.64%)
Cholesterol	40 (0.40%)
Sodium cholate	6 (0.06%)
W. somnifera extract (ethanol; 50% ethanol or water)	200 (2.00%)
Ultra-pure water	To 10 ml

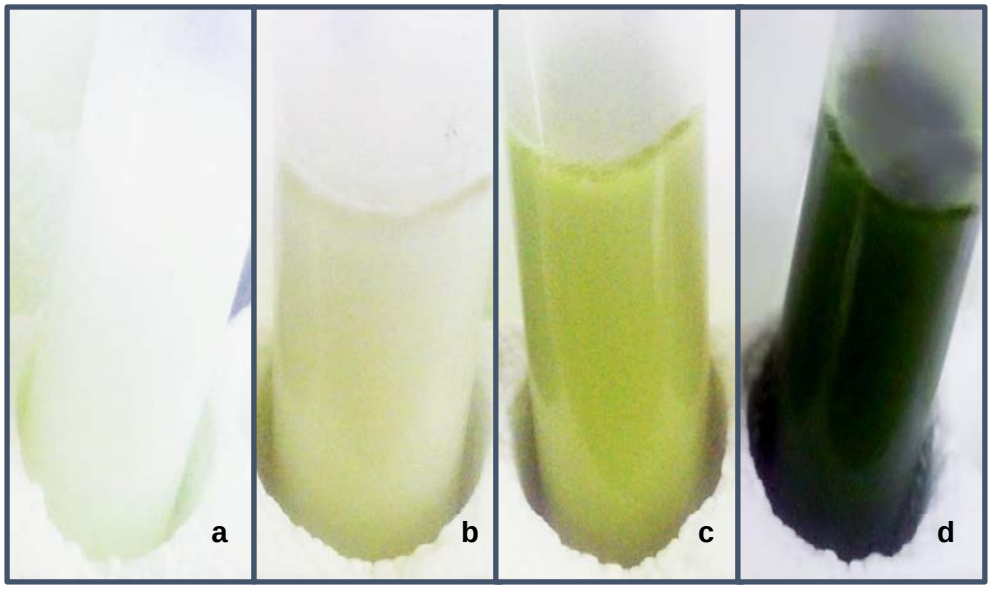

Figure B.3: Images of the final niosome formulations: (a) placebo niosomes, (b) water extract niosomes, (c) 50% ethanol extract niosomes and (d) ethanol extract niosomes

Table B.7: Formula for the optimised formulation of solid lipid nanoparticles

Ingredient	Amount (mg)
Tween® 80	40 (0.40% w/w)
Span® 60	160 (1.60% w/w)
Compritol® 888 ATO	100 (1.00% w/w)
Phosphatidylcholine	100 (1.00% w/w)
Sodium cholate	0.75 (7.50 x 10^{-3}% w/w)
W. somnifera extract (ethanol; 50% ethanol or water)	200 (2.00% w/w)
Ultra-pure water	To 10 ml

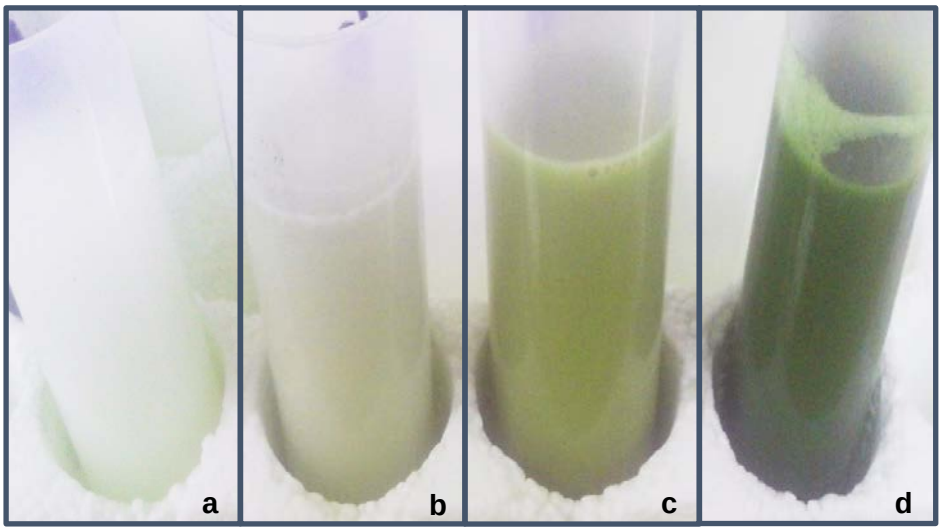

Figure B.4: Images of the final SLN formulations: (a) placebo formulation, (b) water extract SLNs, (c) 50% ethanol extract SLNs and (d) ethanol extract SLNs

B.5.2.1 Size and polydispersity of niosomes and solid lipid nanoparticles

The sizes of the niosomes and SLNs differed with respect to the extract that was incorporated as the active component. The water niosomes had the smallest average size (165.95 ± 9.44 nm) followed by ethanol SLNs (172.27 ± 44.50 nm), ethanol niosomes (173.72 ± 52.60 nm), water SLNs (186.75 ± 4.64 nm), 50% ethanol SLNs (260.83 ± 51.37 nm) and lastly, the 50% ethanol niosomes (304.57 ± 52.36 nm) with the biggest average size. Generally, the SLN and niosome formulations of the same extract had relatively similar average sizes. Since vesicle size plays a role during drug delivery; it is within reason to suppose that the different vesicular sizes of the formulations may have an influence on topical delivery of each of the extracts. Figure B.5 shows the average vesicular sizes of all the formulations ± standard deviation (SD). The Zetasizer also gave information with respect to the homogeneity of the sample.

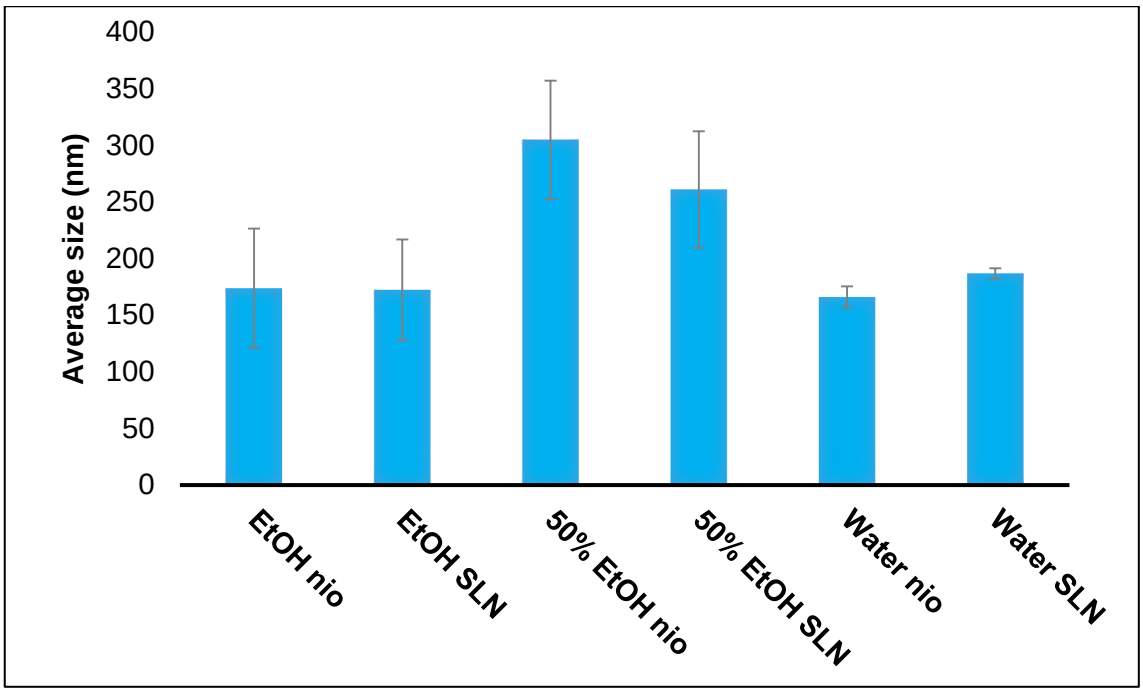

Figure B.5: Average sizes (nm) of niosomes and solid lipid nanoparticles (n = 3). Values are presented as mean ± SD.

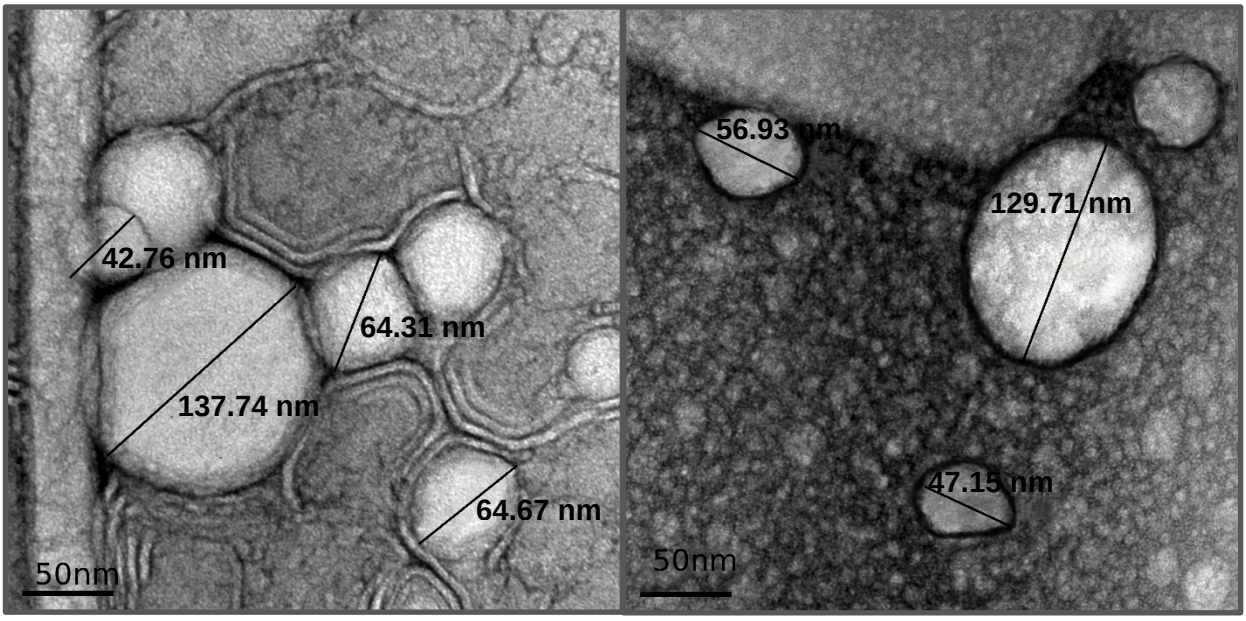

Figure B.6: Transmission electron micrographs of niosomes

It is evident from the TEM images that the niosome formulations contained a mixture of unilamellar and multi-lamellar vesicles. The multi-lamellar niosomes however seem to have been relatively unstable as shown by the broken down lamellar.

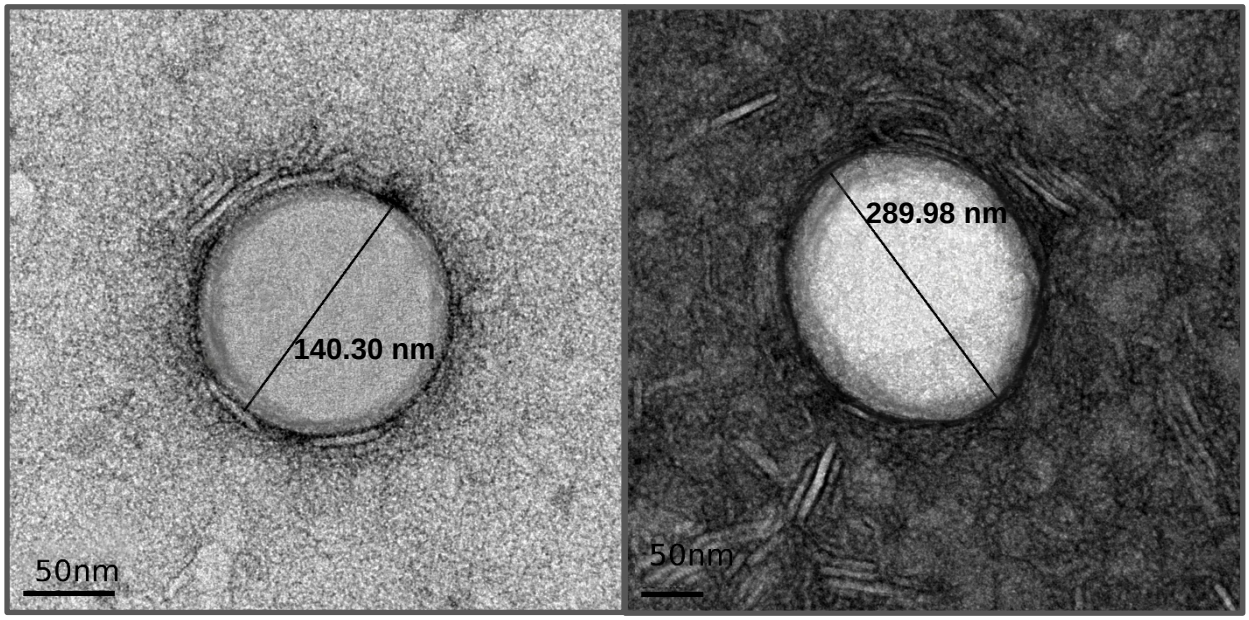

Figure B.7: Transmission electron micrographs of solid lipid nanoparticles

The SLNs had clearly defined circular cross-sections. These TEM images depicted a wide range of sizes which were in line with the particle sizes that were obtained using the Zetasizer.

Table B.8: Average polydispersity indices (PDI) of all optimised formulations (n = 3). Values are presented as mean ± SD.

Formulation name	Average PDI ± SD
Ethanol niosomes	0.231 ± 0.122
Ethanol SLNs	0.419 ± 0.019
50% Ethanol niosomes	0.263 ± 0.033
50% Ethanol SLNs	0.735 ± 0.218
Water niosomes	0.171 ± 0.029
Water SLNs	0.479 ± 0.023

A small PDI indicates that the sample is fairly homogenous; while a larger PDI indicates a polydisperse sample. In this study all the formulations had a PDI value below 0.5 except the 50% ethanol SLN formulation, which had an average PDI value of 0.735 as shown in Figure B.8.

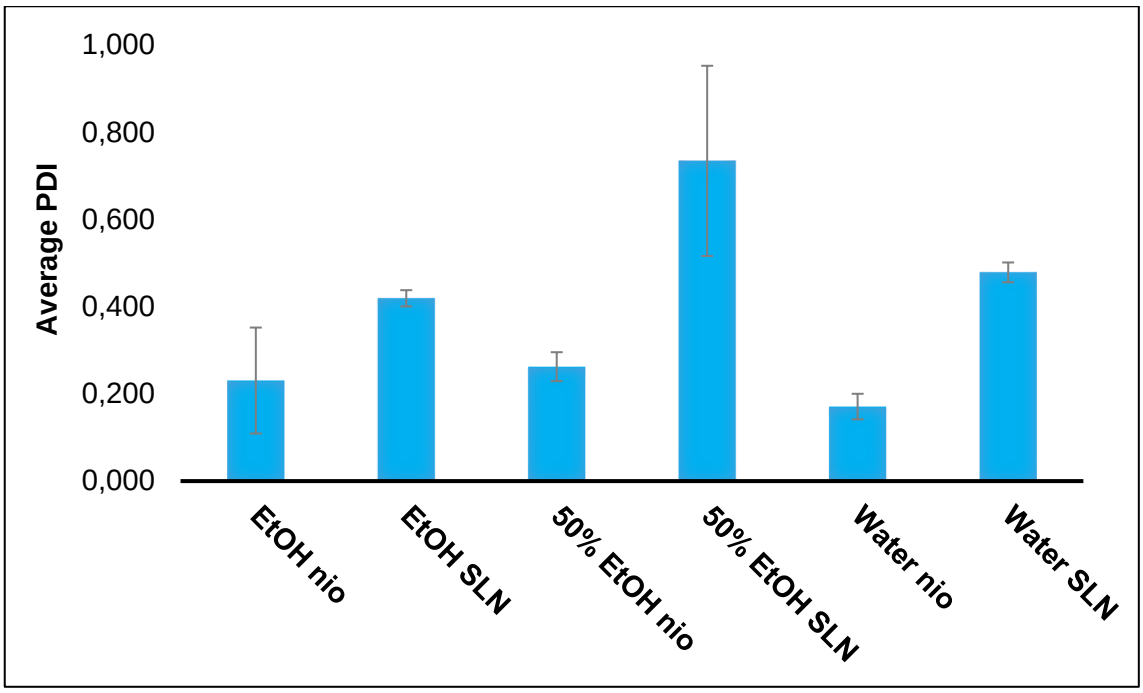

Figure B.8: Average polydispersity indices of niosomes and solid lipid nanoparticles (n = 3). Values are presented as mean ± SD.

From these results we see that the niosomes had more uniform particle sizes as compared to the SLNs. The ethanol extract niosomes were the most homogenous pertaining to size followed by the water niosomes, 50% ethanol niosomes, ethanol SLNs, water SLNs and lastly, the 50% ethanol SLNs. This phenomenon may have been due to the high concentration of lipids in the SLNs, which possibly did not disperse easily within the aqueous dispersant.

B.5.2.2 Zeta-potential of niosomes and solid lipid nanoparticles

The zeta-potential of the formulations ranged from -10.45 to -34.98 mV with the water formulations having the lowest absolute values and the ethanol formulations having the higher absolute values. This indicated that the ethanol formulations had a higher stability potential in comparison to the other formulations. Ethanol niosomes had the highest average absolute value with -34.98 ± 0.80 mV, followed by the ethanol SLNs (-27.32 ± 2.23 mV), 50% ethanol niosomes (-25.83 ± 2.14 mV), 50% ethanol SLNs (-24.95 ± 3.04 mV), water SLNs (-16.68 ± 1.54 mV) and lastly, the water niosomes (-10.45 ± 4.10 mV).

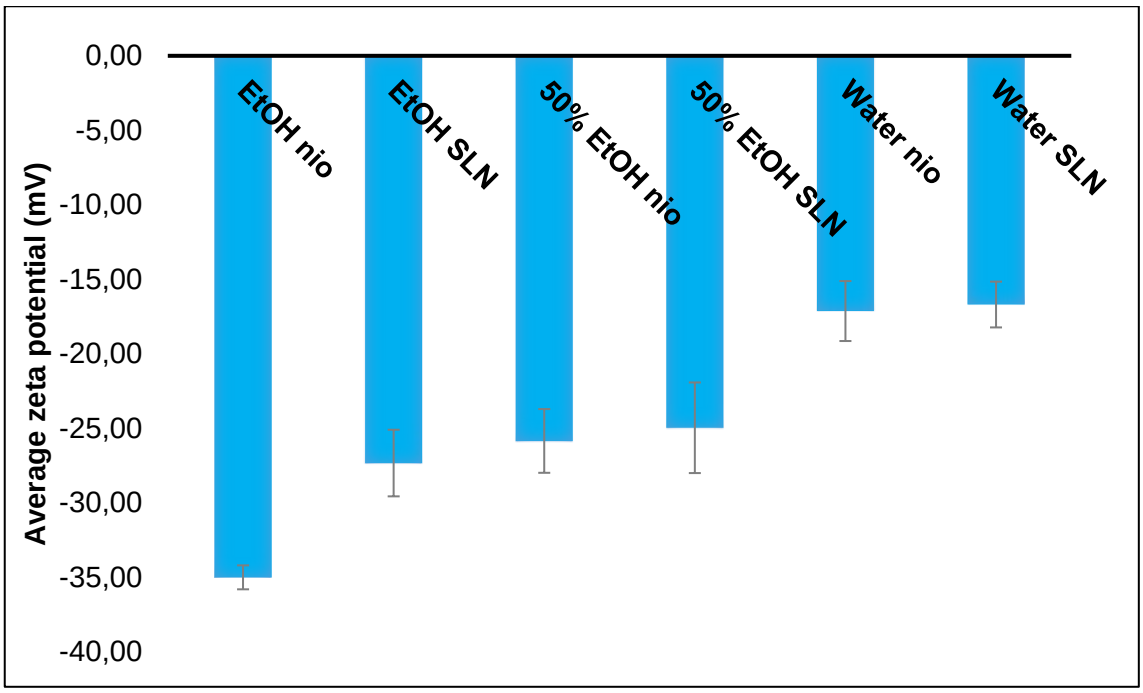

Figure B.9: Average zeta-potential (mV) of niosomes and solid lipid nanoparticles (n = 3). Values are presented as mean ± SD.

Generally the niosomes had a higher absolute value for zeta-potential as compared to the SLNs, thus reflecting a relatively higher stability of niosomes versus the SLNs. The different components of the extracts seem to have had an influence on the aggregation and cohesion properties of the formulations. The water formulations had water soluble compounds which may have reduced the cohesive properties of the formulations and resulted in the unfavourable zeta-potential values.

B.5.2.3 pH of the niosomes and solid lipid nanoparticles

The placebo formulations all had pH values in the range of 7-8. The final formulations however had pH values between 5.183 and 5.709, which is acceptable for topical application as the pH of skin has been determined to be around 5.5. A pH between 4 and 6 has been documented as suitable for topical application, which therefore led to the deduction that the formulations would not lead to any irritation due to pH incompatibilities between the skin and the formulation (Ali & Yosipovitch, 2013:261). It was also observed that in all the comparisons between a niosome formulation and a respective SLN formulation the SLN formulation had a slightly lower average pH than the niosome formulation.

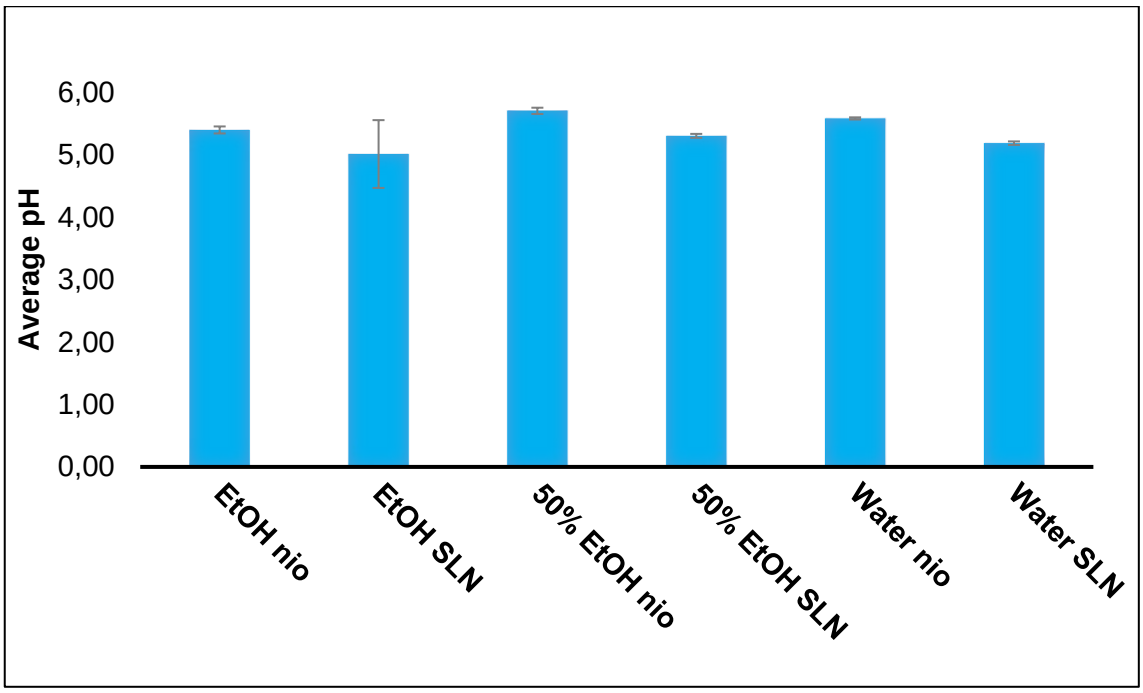

Figure B.10: Average pH of niosomes and solid lipid nanoparticles (n = 3). Values are presented as mean ± SD.

B.5.2.4 Encapsulation efficiency of niosomes and solid lipid nanoparticles

The encapsulation efficiency was only determined for the final formulations, this was due to a limited availability of plant extract. The formulations were made in triplicate and the HPLC analysis to determine the amount of compound encapsulated was done in duplicate for each formulation.

B.5.2.4.1 Withaferin A

The percentage encapsulation of withaferin A ranged from 31.99-70.03% as shown in Figure B.11. The SLN formulations all had a higher withaferin A encapsulation as compared to their respective niosome formulations. The formulation which had the highest withaferin A encapsulation was the ethanol SLNs (70.03 ± 5.74), this was followed by the ethanol niosomes (69.12 ± 1.04), 50% ethanol SLNs (67.72 ± 3.68), 50% ethanol niosomes (65.82 ± 1.02), water SLNs (35.37 ± 1.70) and lastly the water niosomes (31.99 ± 5.94) in that order. The ethanol formulations and the 50% ethanol formulations all managed to encapsulate more than 65.00% of the withaferin A, however, the water formulations had very low withaferin A encapsulation. This may have been due to a higher content of hydrophilic components in the water extracts.

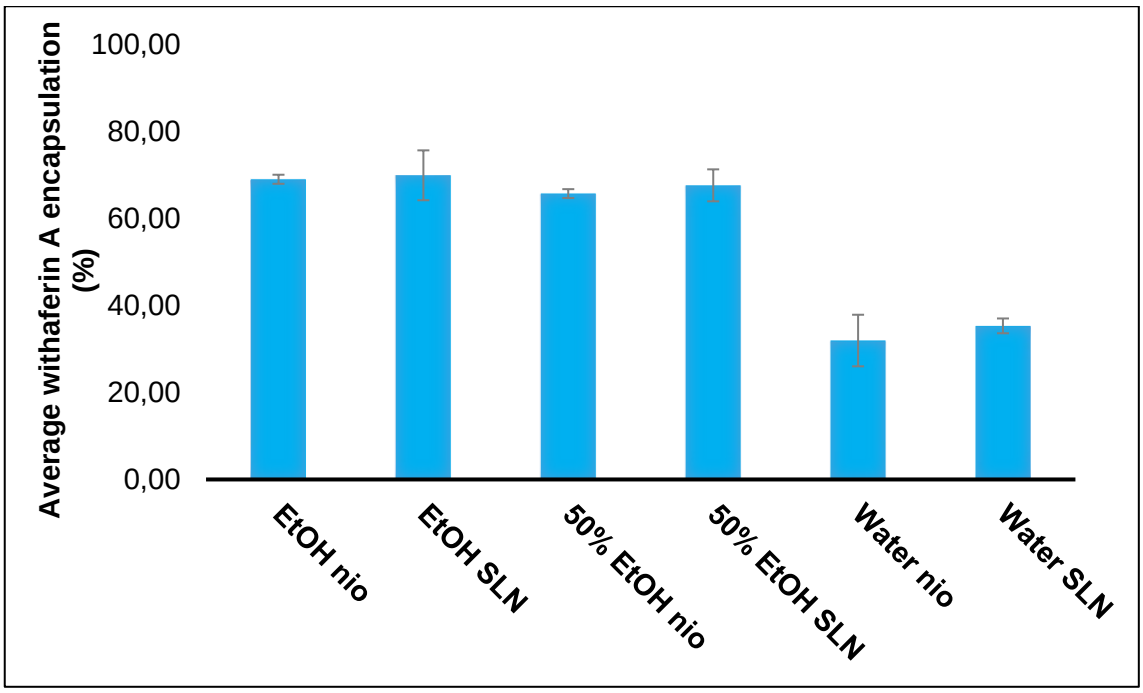

Figure B.11: Average encapsulation efficiency (%) of withaferin A (n = 3). Values are presented as mean ± SD.

B.5.2.4.2 Withanolide A

Withanolide A had a high percentage encapsulation efficiency (above 70.0%) in all the formulations, except the ethanol niosomes; which had a very low encapsulation of withanolide A (41.81%). Figure B.12: shows the values that were obtained for the percentage withanolide A encapsulated.

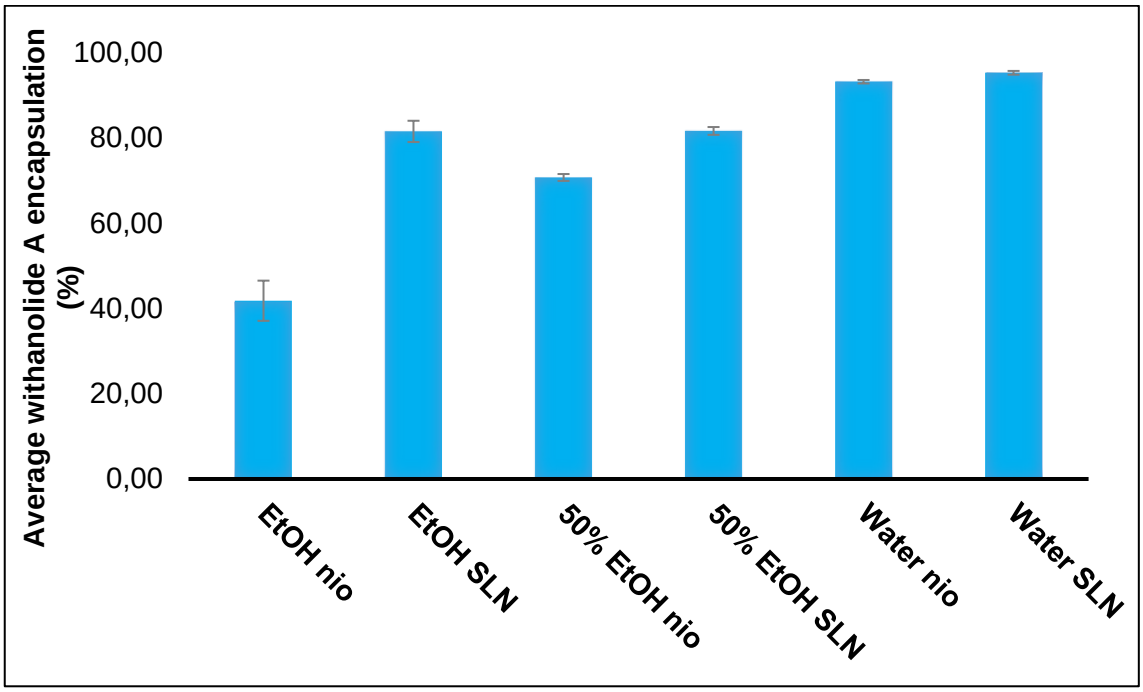

Figure B.12: Average encapsulation efficiency (%) of withanolide A (n = 3). Values are presented as mean ± SD.

The water extract SLNs had the highest percentage encapsulation of withanolide A (95.28 ± 0.44%), followed by water extract niosomes (93.18 ± 0.41%), 50% ethanol extract SLNs (81.64 ± 0.92%), ethanol SLNs (81.53 ± 2.49%), 50% ethanol niosomes (70.72 ± 0.81%) and lastly, the ethanol niosomes (41.81 ± 4.71%). As a general trend the SLN formulations all had a higher percentage encapsulation of withanolide A as compared to their respective niosome formulations.

B.6 Conclusion

The final formulations all had varying characteristics which were possibly influenced by the different excipients and contents of the plant extracts. Stability of the formulations over a three month period was assessed (Appendix C). The formulations prepared as described in this Appendix were then used for the Franz cell diffusion studies (Appendix D) and the *in vitro* efficacy studies (Appendix E). It is possible that the physicochemical properties of the formulations influenced the results that were obtained in this study as a whole.

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Appendix C: Physico-chemical stability testing of Withania somnifera niosomes and solid lipid nanoparticles

C.1 Introduction

Stability is described as the state of being free from change (Lerche & Sobisch, 2011:1799) or the ability of a formulation to remain within its initial physical, chemical, microbiological, therapeutic, toxicological and protective specifications (Kommanaboyina & Rhodes, 1999:858). With respect to pharmaceutical formulations any formulation must be sufficiently stable under the proposed storage conditions and over the proposed shelf-life before it can be offered to the public (Lerche & Sobisch, 2011:1810). It is therefore imperative to take formulation stability into account when working with any new formulations. There are many factors that influence the stability of a formulation and these may include excipients used, pH, moisture, heat, light, exposure to microbes, the use of preservatives and formulation methods (Mitsui, 1997:197; Kommanaboyina & Rhodes, 1999:858; Narayana & Dobriyal, 2009:371).

During stability testing of formulations the first stage is to check on the physical and chemical stability. Chemical parameters that are assessed include colour change, fragrance change, crystallisation, etc. and the physical parameters include solidification, evaporation, separation, gelling, sedimentation, etc. (Mitsui, 1997:191; Matthews, 1999:832). Particle size and polydispersity index (PDI) are also assessed because particle size, size distribution and polydispersity changes are known to be sensitive indicators of the kinetic stability of colloidal dispersions (Csempesz & Puskas, 2007:82). Microbiological, therapeutic and toxicological stability must also be assessed (Allen *et al.*, 2005:113). The parameters of interest are determined by the dosage form of the end-product. Stability tests are usually conducted as stress tests and the formulations are usually exposed to stresses such as thermal, pH, light, humidity and oxidative stress (Bakshi & Singh, 2002:1021).

In this study it was decided to do a three month temperature stability study on lyophilised niosomes and solid lipid nanoparticles (SLNs) at room temperature (≈25 °C) in a temperature controlled laboratory. Freeze-drying technology is commonly used in order to preserve pharmaceutical products especially those that are known to be heat-sensitive. Therefore, freeze-drying was used to preserve the nano-vesicles over the stability testing period. Some of the advantages of freeze-drying pharmaceutical formulations have been reported as follows (Hua *et al.*, 2010:15):

- Compounds dried at these low temperatures will not be lost due to denaturation or biological vitality loss.

- There is a very low probability of microbial growth and enzyme function in freeze dried drugs.
- Drugs can maintain their original volume and shape.
- Protection of pharmaceuticals from oxidation as the drying is carried out under vacuum conditions.
- Allows for storage of most pharmaceuticals at room temperature.

According to the International Conference on Harmonisation (ICH) guidelines, drug substances intended for storage in a refrigerator can be stored at 25 ± 2 °C/60 ± 5% RH for a 6 month stability test. In this study the formulations were stored at room temperature (≈25 °C) as this was a preliminary stability assessment. Instead of six months, a shorter testing period of three months can be used if a significant change has already occurred within the first three months (ICH, 2003:2). The stability testing was therefore conducted for three months as there were some significant changes that had already surfaced by that time-point. The ICH states that a significant change is described as a 5% change from the initial value; whereas Kommanaboyina and Rhodes (1999:858) state that the minimum acceptable potency level is 90% of the initial potency.

The stability testing of herbal medicinal products is usually more complicated than conventional stability testing due to the presence of multiple uncharacterised components that are present in herbal formulations. This was true in this study as crude plant extracts were used as the active pharmaceutical ingredients (APIs). The main parameters that can be readily assessed in order to determine the stability of herbal medicinal products are the aesthetic properties (Narayana & Dobriyal, 2009:375). Stability indicating parameters assessed for herbal medicinal products are the organoleptic properties (taste, colour, smell), consistency (i.e. viscosity), pH and quantitative assay of usually only one or two marker compounds (Narayana & Dobriyal, 2009:372).

Due to the lack of information on all components of an herbal medicinal product it is complex to predict stability issues and attempt to combat them in the initial formulation process. The stability tests that were conducted in this study were a pilot study in an attempt to identify the key areas that need to be addressed when formulating niosomes and SLN using *Withania somnifera* crude extracts as the APIs.

C.2 Materials and methods

Three different batches of each formulation were prepared as described in Appendix B, lyophilised and stored at room temperature in clear glass containers that were covered with foil. The containers were covered with foil in order to protect the formulations from light.

Each test parameter was assessed in duplicate on three samples at each time point. The parameters that were assessed are pH, zeta-potential, particle size, PDI and encapsulation efficiency (EE). A 28 day period was regarded as one month and sampling was done on the day of preparation (day 0), then after 1, 2, 4, 8 and 12 weeks. Table C.1 highlights a summary of the stability testing that was carried out.

Table C.1: Summary of stability testing

Storage conditions	Room temperature (25 °C)
Storage container	Glass container covered with foil
Parameters tested	pH, zeta-potential, particle size, PDI, EE
Duration of stability test	≈3 months
Sampling points	Day 0, 7, 14, 28, 56, 84

C.2.1 Preparation of freeze-dried formulations

During freeze-drying, the prepared formulations were frozen at -80 °C in ice-cube trays. Once frozen the formulations were crushed to increase the surface area then refrozen in the freeze-drying glass jars. The frozen and crushed formulations were then lyophilised in a vacuum using a freeze-dryer (VirTis, United Kingdom) until a dry powder remained.

C.2.2 pH

The lyophilised formulations were suspended in 10 ml of deionised Milli-Q® water (Millipore, Milford, USA) to prepare a 2.0% plant extract formulation (niosome or SLN). The pH of each formulation was measured at 25 °C in duplicate using a Mettler Toledo pH metre (Mettler Toledo, Columbus, OH, USA).

C.2.3 Particle size, polydispersity index and zeta-potential

Formulations were suspended in 10 ml Milli-Q® water and approximately 1 ml of the reconstituted formulation was injected into a disposable folded capillary cell. The zeta-potential was then measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Thereafter, the particle size and PDI were measured on the same samples using the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). All the measurements were done in duplicate at a temperature of 25 °C.

C.2.4 Encapsulation efficiency

The EE was determined using the method as described by Hua *et al.* (2010:212). In order to determine EE the lyophilised formulations were suspended in 10 ml Milli-Q® water and placed in

ultracentrifuge tubes. The suspended formulations were centrifuged at a speed of 30 000 rpm and a temperature of 4 °C for 30 min using an ultracentrifuge. A low temperature was used in order to negate the influence of the high rotor temperature associated with high speed centrifuging. After centrifuging the supernatant which represented the free compound was analysed using HPLC and the EE was calculated according to Equation C.1 (Junyaprasert *et al.*, 2008:853).

$$\%EE = \left(\frac{\text{Total amount of compound added - Free amount of compound}}{\text{Total amount of compound}}\right) \times 100\% \qquad \textbf{Equation C.1}$$

C.3 Results and discussion

Preliminary three-month stability tests were performed for a product intended for refrigerator storage as the tests were conducted at room temperature. A 5.0% change in any parameter was perceived as a significant change reflecting potential instability of the formulation in question. In the figures abbreviations are used for the different formulations as follows: water extract niosomes (water nio), ethanol extract niosomes (EtOH nio), 50% ethanol extract niosomes (50% EtOH nio), water extract SLNs (water SLN), ethanol extract SLNs (EtOH SLN) and 50% ethanol extract SLNs (50% EtOH SLN).

C.3.1 pH

The pH of freshly prepared formulations was measured in duplicate and noted as the initial pH. Subsequent measurements were made after 7, 14, 28, 56 and 84 days. Table C.2 and Figure C.1 show a summary of the pH results that were obtained over the 84 day test period.

Table C.2: Average pH of the formulations at the different time intervals (n = 3).

	Initial	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Day 84**
Water extract niosomes						
Ave	5.588	5.332	5.186	5.270	5.336	5.257
SD	0.017	0.016	0.024	0.030	0.045	0.040
%RSD	0.307	0.299	0.465	0.563	0.838	0.756
Ethanol extract niosomes						
Ave	5.402	4.548	4.514	4.578	4.571	4.512
SD	0.057	0.185	0.036	0.080	0.059	0.071
%RSD	1.059	4.076	0.806	1.743	1.285	1.568
50% ethanol extract niosomes						
Ave	5.709	5.280	5.092	5.189	5.236	5.157
SD	0.050	0.158	0.011	0.012	0.047	0.046
%RSD	0.880	2.990	0.225	0.223	0.894	0.891

Water extract SLNs						
Ave	5.191	5.480	5.317	5.410	5.499	5.299
SD	0.028	0.036	0.030	0.007	0.010	0.123
%RSD	0.545	0.651	0.567	0.137	0.181	2.329
Ethanol extract SLNs						
Ave	5.706	5.086	4.968	5.062	5.035	4.901
SD	0.054	0.053	0.115	0.025	0.050	0.068
%RSD	0.940	1.035	2.322	0.498	0.990	1.390
50% ethanol extract SLNs						
Ave	5.308	5.482	5.342	5.420	5.533	5.397
SD	0.032	0.052	0.027	0.054	0.015	0.060
%RSD	0.594	0.956	0.510	0.988	0.266	1.105

The pH values of the formulations fluctuated between an average of 4.512 and 5.709 over time. This reflected that the formulations were rather unstable with respect to pH. The lowest pH that was recorded was relatively acidic, but it is within the limits of what is acceptable for topical drug delivery.

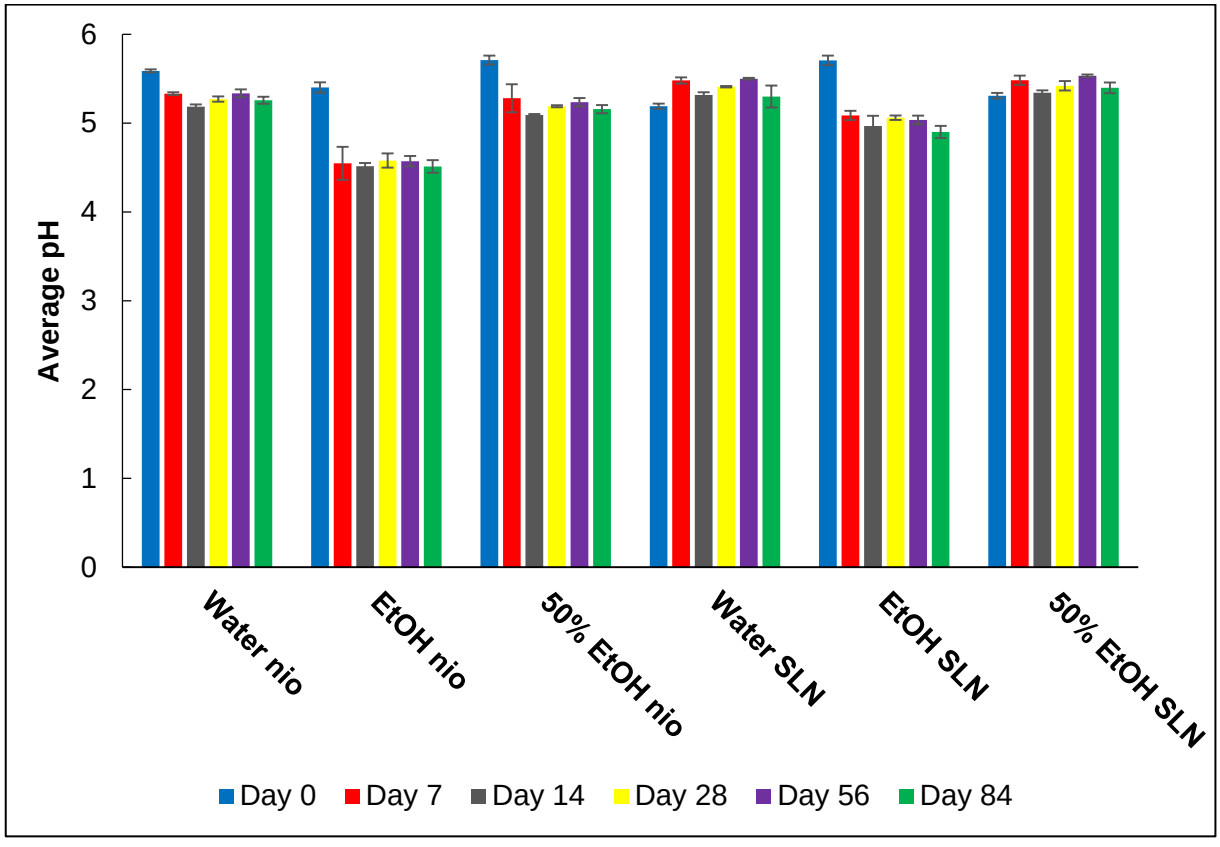

Figure C.1: Average pH of the formulations at the different time intervals (n = 3). Values are presented as mean ± SD.

All the niosome formulations and the ethanol SLN formulations presented with a relatively sharp

drop in pH after 7 days. The ethanol extract formulations (niosomes and SLNs) both displayed the sharpest fall in pH after 7 days. Generally, the 50% ethanol extract SLNs had the lowest recorded percentage pH fluctuation (1.68%) from day 0 to day 84; while the ethanol extract niosomes had the highest recorded percentage pH fluctuation (16.47%). It must however be noted that from day 7 to day 84 all the formulations had a percentage pH change that was less than 5.0%. The initial pH change may have been due to an initial adjustment of the formulations after the freeze-drying process.

C.3.2 Zeta-potential

The zeta-potential of the formulations was measured in duplicate for each of the three samples. The measurements were done on days 0, 7, 14, 28, 56 and 84. Table C.3 and Figure C.2 show the zeta-potential values that were obtained for each formulation over the 84 day test period.

Table C.3: Average zeta-potential of the formulations at the different time intervals (n = 3).

	Initial	Day 7	Day 14	Day 28	Day 56	Day 84
Water extract niosomes						
Ave (mV)	-17.12	-15.17	-14.52	-11.47	-11.98	-4.00
SD	2.01	1.60	1.05	0.84	1.35	0.68
%RSD	-11.75	-10.53	-7.26	-7.33	-11.23	-17.06
Ethanol extract niosomes						
Ave (mV)	-34.98	-34.67	-31.17	-31.33	-35.07	-34.20
SD	0.80	1.21	0.97	0.63	1.81	2.13
%RSD	-2.30	-3.49	-3.11	-2.02	-5.17	-6.23
50% ethanol extract niosomes						
Ave (mV)	-25.83	-24.45	-26.48	-23.55	-23.80	-24.70
SD	2.14	4.67	2.28	1.43	0.92	1.94
%RSD	-8.28	-19.10	-8.60	-6.08	-3.86	-7.87
Water extract SLNs						
Ave (mV)	-16.68	-20.37	-15.45	-18.53	-16.28	-14.02
SD	1.54	1.59	1.07	1.12	2.63	1.70
%RSD	-9.20	-7.81	-6.93	-6.05	-16.17	-12.14
Ethanol extract SLNs						
Ave (mV)	-27.32	-25.72	-27.77	-27.15	-21.28	-20.62
SD	2.23	2.01	1.76	1.37	1.27	0.60
%RSD	-8.18	-7.82	-6.34	-5.05	-5.98	-2.90
50% ethanol extract SLNs						
Ave (mV)	-24.95	-23.10	-20.83	-21.93	-18.98	-12.96
SD	3.04	1.54	1.36	0.75	1.12	2.27
%RSD	-12.18	-6.65	-6.53	-3.42	-5.88	-17.50

All the formulations had electronegative zeta-potential values with the average values ranging between - 4.00 mV and - 35.07 mV. The zeta-potential is a measure of the electro-static repulsion of particles. If the zeta-potential is high, it means that the repulsion is high and the system is regarded as stable. Therefore zeta-potential is measured in colloidal systems (such as niosomes and SLNs) in order to assess the stability of the system. If the zeta-potential value is too low, it implies that there are low repulsive forces to maintain the system which can thus lead to aggregation or coagulation (Lu & Gao, 2010:66). However, zeta-potential that is too high implies that the repulsive forces are too strong to maintain the system and phase separation can occur. It is reported that the optimum zeta-potential values for colloidal systems should be more positive than + 30 mV or more negative than - 30 mV (Dispersion Technology Inc, 2013).

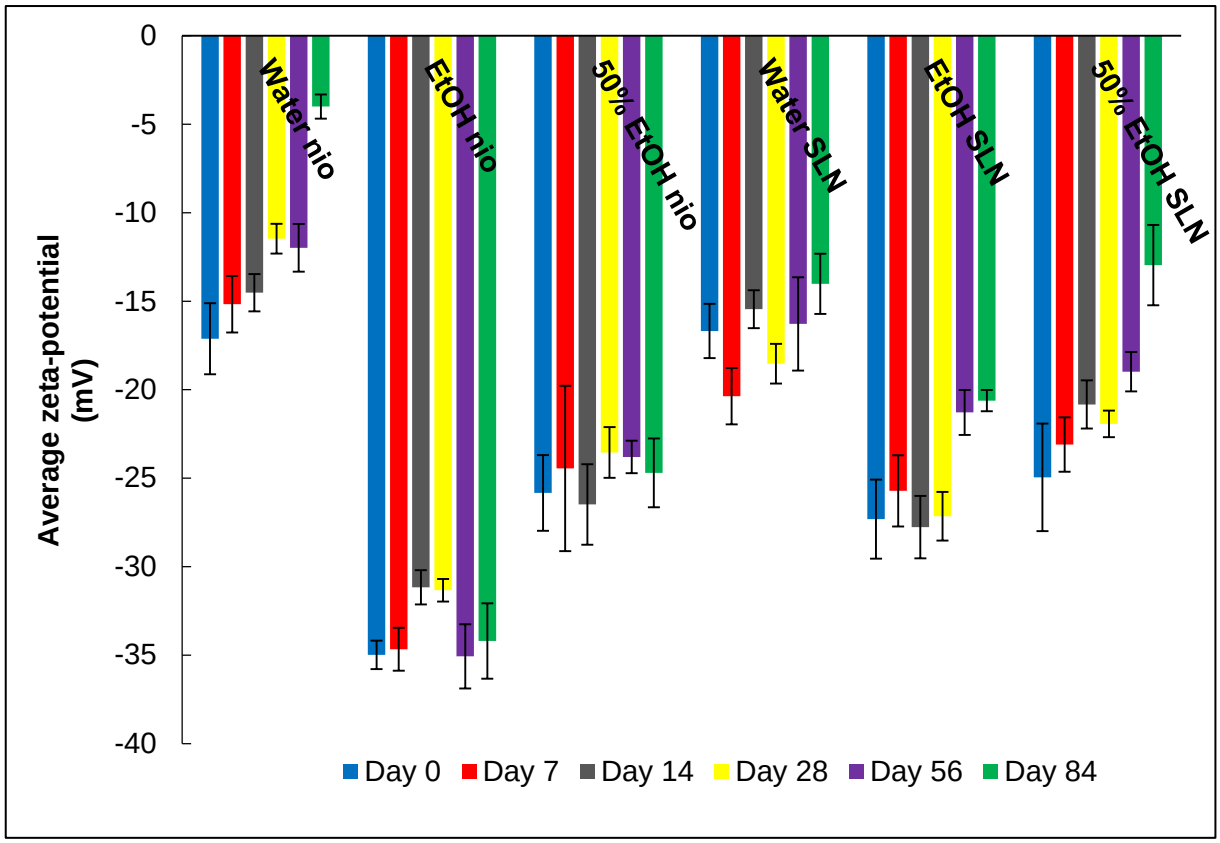

Figure C.2: Average zeta-potential of the formulations at the different time intervals (n = 3). Values are presented as mean ± SD.

The water extract niosomes and water extract SLNs had relatively lower zeta-potential values as compared to the rest of the formulations. On comparing the niosome formulations, we observe that the ethanol extract niosomes had the most electronegative zeta-potential values followed by the 50% ethanol extract niosomes and lastly, the water extract niosomes. The same trend is true for the SLN formulations, leading to an assumption that the compounds in the ethanol extract led to the higher zeta-potential values that were recorded. As a general trend, the three formulations

(ethanol extract niosomes, 50% ethanol extract niosomes and ethanol extract SLNs) which had relatively higher initial zeta-potential values displayed a smaller change in average zeta-potential over the 84 day period. This may have been due to these formulations being initially relatively stable and thus giving them the ability to maintain their zeta-potential within a narrower range as compared to the remaining formulations.

With respect to zeta-potential measurements; the ethanol extract niosome formulation was the most stable with a 2.23% change in measurement from the initial value to the 84th day. All the measurements for the ethanol extract niosome formulation were more electronegative than - 30 mV; showing that this formulation was within the stable range throughout the testing period. The water extract niosomes on the other hand were the most unstable with respect to zeta-potential measurements as the average zeta-potential dropped by 76.64% from the initial measurement to the 84th day measurement.

C.3.3 Particle size

The average particle size of the formulations was measured in duplicate for each sample. All the measurements were taken on days 0, 7, 14, 28, 56 and 84. Table C.4 and Figure C.3 show a summary of the average particle sizes that were recorded over the 84 day period.

Table C.4: Average particle size of the formulations at the different time intervals (n = 3).

	Initial	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Day 84**
Water extract niosomes						
Ave (nm)	165.95	186.82	166.12	243.37	288.50	405.35
SD	9.44	23.87	13.80	9.65	60.48	11.77
%RSD	5.69	12.77	8.31	3.97	20.97	2.90
Ethanol extract niosomes						
Ave (nm)	173.72	154.47	273.80	327.83	216.75	311.45
SD	52.60	15.28	17.40	20.55	16.83	88.23
%RSD	30.28	9.89	6.35	6.27	7.76	28.33
50% ethanol extract niosomes						
Ave (nm)	145.38	135.20	169.93	245.78	304.57	418.43
SD	15.05	3.57	22.32	97.13	52.36	25.38
%RSD	10.35	2.64	13.14	39.52	17.19	6.07
Water extract SLN						
Ave (nm)	186.75	194.72	247.17	450.60	532.42	495.48
SD	4.64	15.04	83.65	47.34	28.01	102.64
%RSD	2.48	7.72	33.84	10.51	5.26	20.71
Ethanol extract SLN						
Ave (nm)	172.27	214.45	179.47	208.73	420.88	476.75

SD	44.50	29.96	2.17	33.54	163.44	22.13
%RSD	25.83	13.97	1.21	16.07	38.83	4.64
50% ethanol extract SLN						
Ave (nm)	260.83	242.38	418.70	908.57	1544.33	1715.17
SD	51.37	5.65	11.59	53.28	239.58	322.90
%RSD	19.70	2.33	2.77	5.86	15.51	18.83

The average particle size of the formulations ranged from 145.30 to 1715.17 nm. All the formulations had a relatively sharp increase in particle size during the two week period between days 14 and 28.

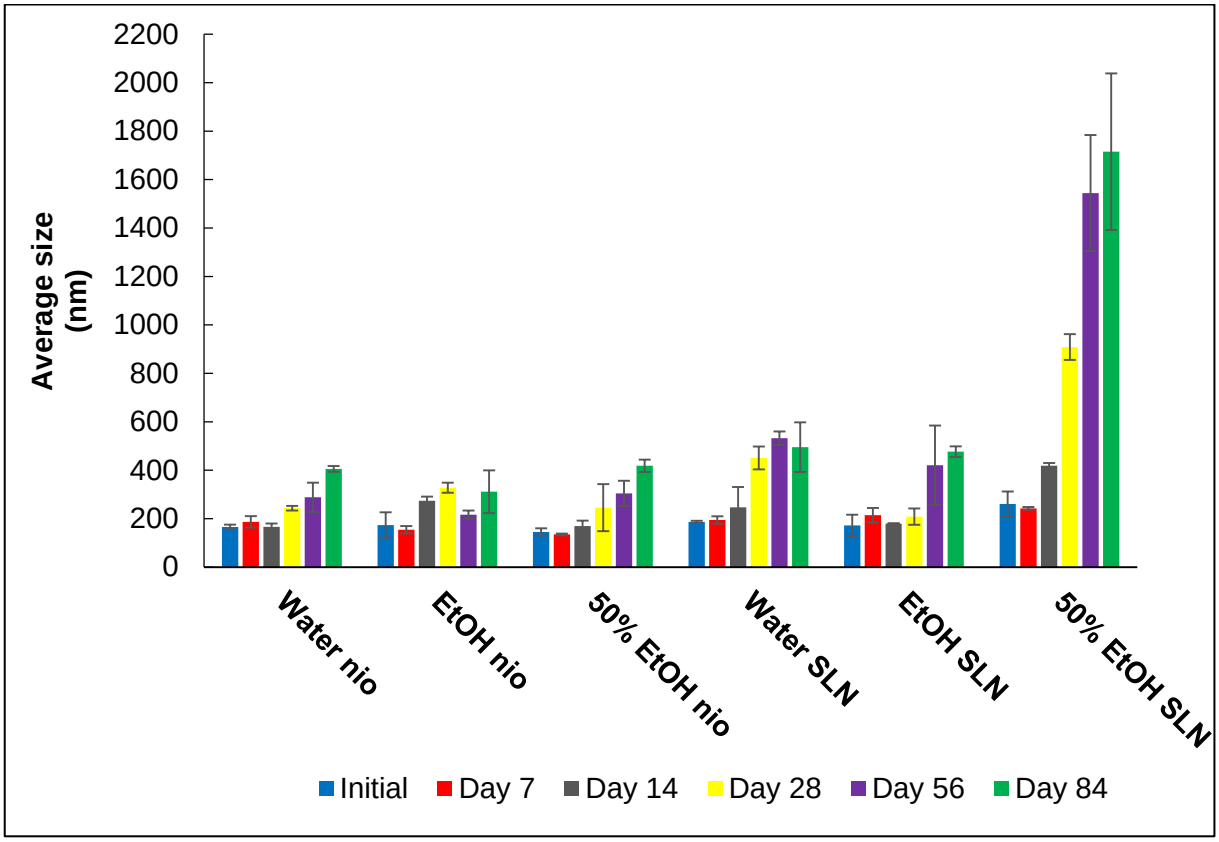

Figure C.3: Average particle size of the formulations at the different time intervals (n = 3). Values are presented as mean ± SD.

The 50% ethanol extract SLNs displayed the biggest rise in average particle size from 260.83 to 1715.17 nm in the 84 day test period. This reflects great instability as the particle size increased by more than 500% of the initial measurement. This somewhat correlates with the sharp drop in average zeta-potential that was observed for the formulations in the last two months. The ethanol extract niosomes had the smallest change in particle size from the initial measurement (173.72 nm) to the measurement on the 84th day (311.45 nm). This correlates with the minimal change in average zeta-potential measurements; thus further supporting that this formulation was

relatively stable in comparison to the other formulations. The ethanol extract SLNs were relatively stable in the first 28 days after formulation, but thereafter the average particle size doubled in the four weeks that followed. The formulation may have reached its stability threshold at the given conditions.

These results generally show us that the formulations failed to maintain their average particle size over the 84 day test period. All the formulations except the 50% ethanol extract SLNs, however, managed to maintain an average particle size within the nano-scale and below 500 nm.

C.3.4 Polydispersity index

The average PDI of the formulations was measured in duplicate on the same samples that were used for the average particle size as these measurements were taken simultaneously. All the measurements were taken on days 0, 7, 14, 28, 56 and 84. Table C.5 and A PDI value below 0.5 is required for a formulation to be regarded as a relatively homogenous formulation. All the formulations except the 50% ethanol extract SLNs had initial PDI values below 0.5 which shows that these formulations were relatively homogenous regarding particle size. The niosome formulations all had PDI values below the homogeneity mark of 0.5 up until the 28th day. On the 56th day the PDI values had shot up to above 0.5 and dropped down to below 0.5; again by the 84th day. The SLN formulations were rather unstable pertaining to PDI values as the values fluctuated throughout the 84 day test period.

show a summary of the average PDI values that were obtained upon measurement.

Table C.5: Average polydispersity index values of the formulations at the different time intervals (n = 3).

	Initial	Day 7	Day 14	Day 28	Day 56	Day 84
Water extract niosomes						
Ave	0.171	0.206	0.365	0.378	0.561	0.288
SD	0.029	0.035	0.050	0.059	0.021	0.049
%RSD	17.126	16.987	13.745	15.589	3.690	17.214
Ethanol extract niosomes						
Ave	0.231	0.270	0.190	0.249	0.526	0.284
SD	0.122	0.027	0.024	0.081	0.083	0.045
%RSD	52.687	9.987	12.486	32.673	15.823	15.717
50% ethanol extract niosomes						
Ave	0.255	0.313	0.260	0.294	0.635	0.345
SD	0.027	0.030	0.011	0.047	0.083	0.035
%RSD	10.492	9.688	4.040	15.971	13.094	10.184

Water extract SLN						
Ave	0.479	0.540	0.417	0.462	0.633	0.478
SD	0.023	0.028	0.033	0.029	0.229	0.061
%RSD	4.737	5.226	7.841	6.344	36.120	12.808
Ethanol extract SLN						
Ave	0.419	0.522	0.436	0.468	0.523	0.451
SD	0.019	0.043	0.060	0.014	0.011	0.039
%RSD	4.437	8.312	13.706	2.924	2.033	8.653
50% ethanol extract niosomes						
Ave	0.735	0.536	0.575	0.858	0.650	0.549
SD	0.218	0.076	0.071	0.201	0.026	0.025
%RSD	29.702	14.132	12.266	23.360	4.068	4.518

A PDI value below 0.5 is required for a formulation to be regarded as a relatively homogenous formulation. All the formulations except the 50% ethanol extract SLNs had initial PDI values below 0.5 which shows that these formulations were relatively homogenous regarding particle size. The niosome formulations all had PDI values below the homogeneity mark of 0.5 up until the 28th day. On the 56th day the PDI values had shot up to above 0.5 and dropped down to below 0.5; again by the 84th day. The SLN formulations were rather unstable pertaining to PDI values as the values fluctuated throughout the 84 day test period.

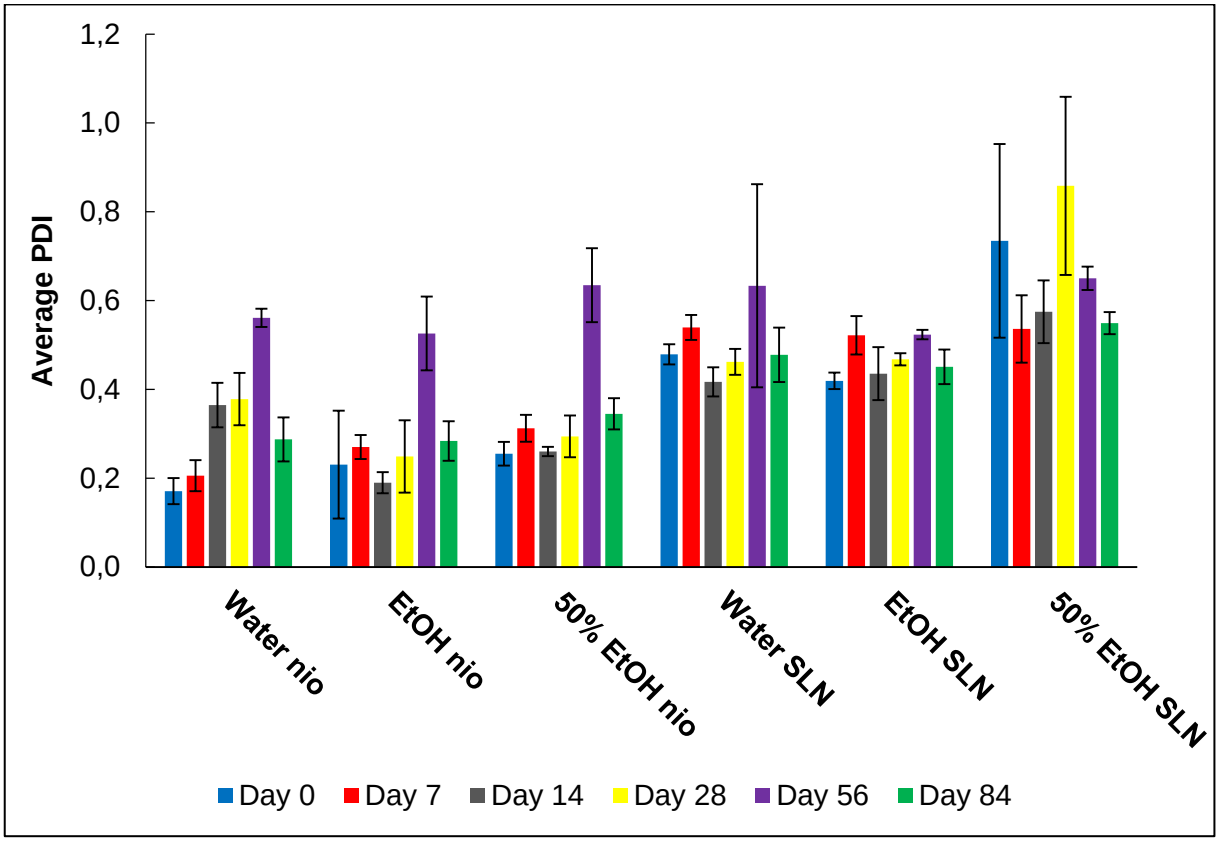

Figure C.4: Average polydispersity index values of the formulations at the different time intervals (n = 3). Values are presented as mean ± SD.

On the 56th day, all the formulations presented with relatively high PDI values; this may have been due to a handling error during sample preparation. The 56th day fell on a day in August which had much higher temperatures than the previous sampling date (July). The formulations may have gotten too warm during the brief sonication of the sample preparation causing some particles to aggregate into bigger particles and others to break down into smaller particles; thus resulting in the highly polydispersed formulations.

C.3.5 Encapsulation efficiency

The encapsulation efficiencies of the two marker compounds were assessed individually using the HPLC method as described in Appendix A. It is crucial for the compound to remain encapsulated within the nanovesicles prior to delivery as these vesicles are the drug delivery vehicles for the compounds. Niosomes and SLNs were tested for changes in withaferin A and withanolide A encapsulation over the 84 day test period.

C.3.5.1 Encapsulation efficiency of Withaferin A

The average EE of withaferin A was calculated in duplicate for three separate samples at each time point. The EE assessments were done on days 0, 7, 14, 28, 56 and 84. Table C.6 and Figure C.5 show the average EE of withaferin A that was determined over time.

Table C.6: Average encapsulation efficiency (%) of withaferin A at the different time intervals (n = 3).

	Initial	Day 7	Day 14	Day 28	Day 56	Day 84
Water extract niosomes						
Ave	31.99	31.21	30.96	29.70	28.20	24.64
SD	5.94	0.61	2.29	1.93	1.66	0.62
%RSD	18.56	1.96	7.39	6.50	5.90	2.53
Ethanol extract niosomes						
Ave	69.12	63.31	60.92	62.41	61.19	58.90
SD	1.04	0.55	1.89	0.32	1.96	1.12
%RSD	1.51	0.88	3.11	0.51	3.20	1.90
50% ethanol extract niosomes						
Ave	65.82	64.96	65.18	64.70	67.53	65.69
SD	1.02	0.69	0.83	0.76	0.77	0.31
%RSD	1.55	1.07	1.27	1.18	1.14	0.47
Water extract SLN						

Ave	35.37	38.74	35.97	35.27	32.68	17.06
SD	1.70	6.16	5.74	3.36	1.82	2.07
%RSD	4.81	15.89	15.95	9.53	5.58	12.11
Ethanol extract SLN						
Ave	70.03	56.63	54.93	54.41	49.11	44.03
SD	5.74	2.37	0.87	1.43	1.23	0.67
%RSD	8.20	4.19	1.59	2.63	2.50	1.52
50% ethanol extract SLN						
Ave	67.72	54.02	50.00	49.38	46.33	47.41
SD	3.68	1.43	1.33	0.61	1.26	0.29
%RSD	5.44	2.64	2.66	1.24	2.71	0.62

The water extract niosomes and SLNs generally had the lowest EE values in comparison with all the formulations with initial values of 31.99% and 35.37%, respectively. Due to the initial low values, at the end of the 84 day test period the water extract niosomes contained only 24.64% of the withaferin A added during formulation; while the water extract SLNs contained 17.06%. The highest initial EE values were recorded for the ethanol extract niosomes and ethanol extract SLNs with average values of 69.12 and 70.03%, respectively. All the formulations except the 50% ethanol extract niosomes exhibited a significant drop in percentage EE, especially in the first 7 days. These results imply that the 50% ethanol extract niosomes were able to retain most of the extract within the vesicles over the 84 day period. Withaferin A encapsulation was most stable in the ethanol extract niosomes (from 69.12 to 58.90%) and 50% ethanol extract niosomes (from 65.82 to 65.69%) during the stability assessment period, as these formulations presented with the least overall change.

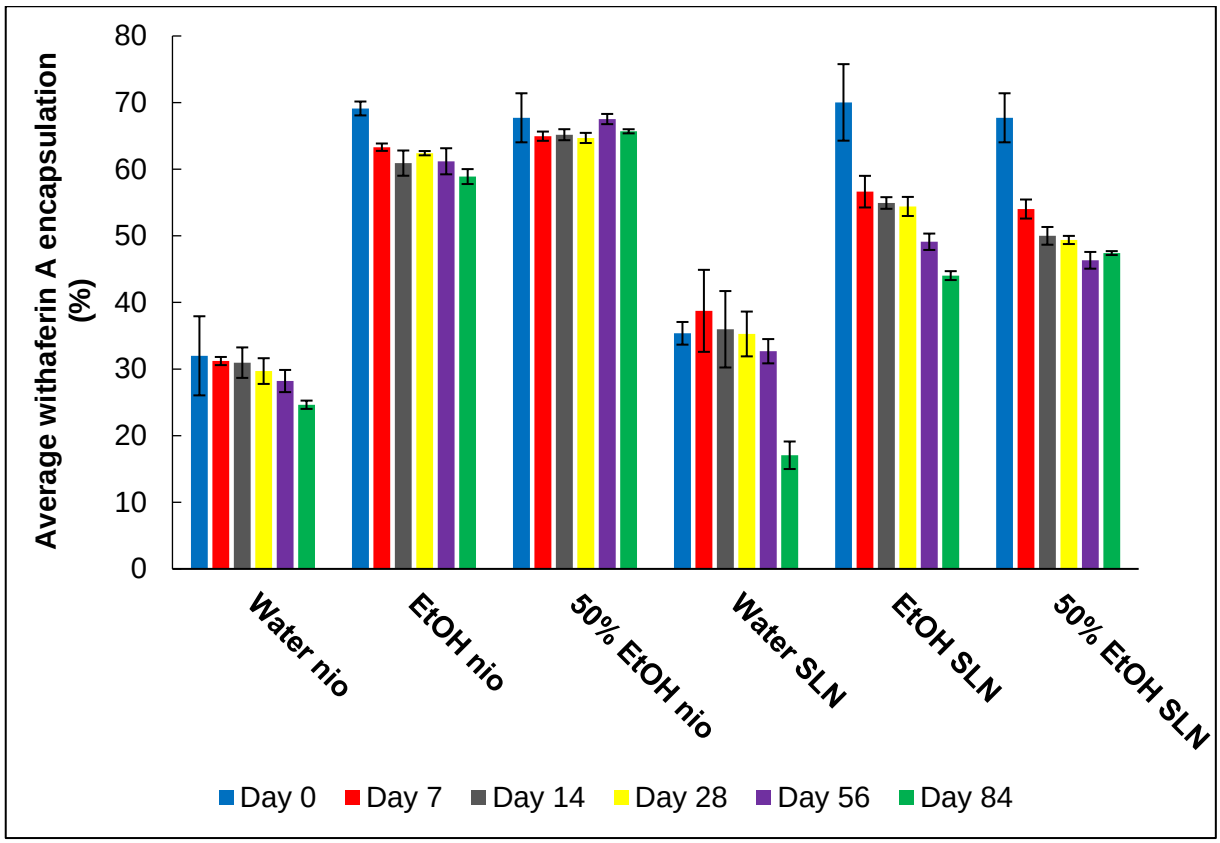

Figure C.5: Average encapsulation efficiency of withaferin A at the different time intervals (n = 3). Values are presented as mean ± SD.

From Figure C.5 it is apparent that the most distinct changes in withaferin A encapsulation either occurred in the first 7 days or between the 56th and 84th days. The initial drop in encapsulation may have occurred due to some compounds in the extract such as withaferin A, leaking out of the vesicles, as the lyophilised vesicles acclimatised to the test conditions (room temperature) after the freeze-drying process. While the drop in EE that is observed towards the end of the test period as with the water extract niosomes, water extract SLNs and ethanol extract SLNs may have been due to the formulations reaching their stability limit and resulting in withaferin A leaking out of the vesicles.

It is evident that the niosome formulations had a better ability to retain the encapsulated withaferin A within the vesicles. The SLN formulations displayed relatively erratic withaferin A encapsulation over the test period as compared to the niosome formulations.

C.3.5.2 Encapsulation efficiency of withanolide A

The average EE of withanolide A was calculated in duplicate for three replicates at each time point. The EE assessments were done on days 0, 7, 14, 28, 56 and 84. Table C.7 and Figure C.6 show the average EE of withanolide A that was determined over time.

Table C.7: Average encapsulation efficiency of withanolide A at the different time intervals (n = 3).

	Initial	Day 7	Day 14	Day 28	Day 56	Day 84
Water extract niosomes						
Ave	93.18	92.04	92.60	92.76	93.68	92.20
SD	0.41	0.52	0.12	0.67	0.12	0.29
%RSD	0.44	0.57	0.12	0.72	0.13	0.31
Ethanol extract niosomes						
Ave	41.81	40.76	39.60	37.81	40.60	32.78
SD	4.71	2.25	1.07	3.39	1.52	1.97
%RSD	11.28	5.53	2.70	8.98	3.75	6.00
50% ethanol extract niosomes						
Ave	70.72	69.59	68.42	68.16	64.35	65.40
SD	0.81	1.28	0.73	1.32	0.26	0.56
%RSD	1.14	1.84	1.07	1.94	0.41	0.86
Water extract SLN						
Ave	95.28	94.60	92.46	97.02	97.14	96.00
SD	0.44	1.28	0.74	0.14	0.68	0.21
%RSD	0.46	1.35	0.80	0.14	0.70	0.22
Ethanol extract SLN						
Ave	81.53	84.29	69.97	67.21	54.06	52.03
SD	2.49	1.21	2.12	0.48	1.74	0.72
%RSD	3.06	1.44	3.04	0.72	3.22	1.38
50% ethanol extract SLN						
Ave	81.64	61.74	54.12	50.10	45.96	44.03
SD	0.92	3.91	3.16	0.76	0.36	0.67
%RSD	1.12	6.33	5.84	1.52	0.78	1.52

Withanolide A displayed very high initial EE values. The water extract formulations had the highest encapsulation efficiencies; followed by the 50% ethanol extract SLNs, ethanol extract SLNs, 50% ethanol extract niosomes and lastly, the ethanol extract niosomes. At the end of the test period the water extract formulations still had withanolide A encapsulation above 90.00%. This occurrence may have been influenced by the presence of water soluble compounds present in the water extract and the relatively high withanolide A content of the water extract (5.04% w/w). The ethanol extract SLNs and 50% ethanol extract SLNs displayed the largest drops in withanolide A encapsulation with differences between days 0 and 84 going beyond 30.00%.

Generally all the formulations had relatively high initial encapsulation efficiencies of withanolide A, with the exception of the ethanol extract niosomes. It is a noteworthy observation that the

formulations which had a high initial encapsulation of withanolide A had a low initial encapsulation of withaferin A. There is no clear explanation for this observation, but the niosomes seemed to have a preference for encapsulating withanolide A in the water extract versus withaferin A.

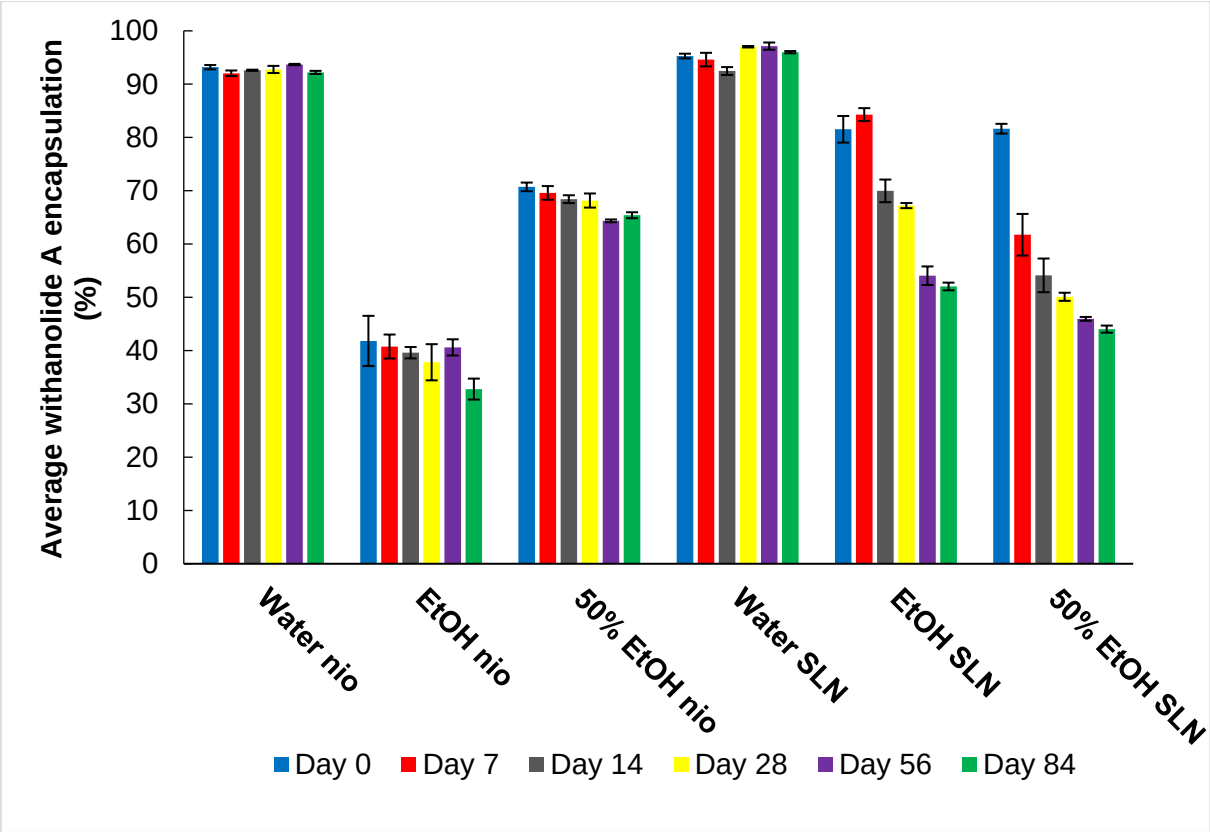

Figure C.6: Average encapsulation efficiency of withanolide A at the different time intervals (n = 3). Values are presented as mean ± SD.

C.4 Conclusion

The results of this stability study showed that instability issues were most apparent with the particle size measurements, PDI and zeta-potential measurements. Nanoparticles can result in decreased stability due to the exposure of a higher surface area as compared to conventional formulations. In order to increase stability of nanoformulations a sufficiently high concentration of nanoparticles may be required (Shah & Khan, 2009:629).

The absence of a lyoprotectant such as sucrose, mannitol or trehalose during the freeze-drying process may have been responsible for some of the instability issues (Hua *et al.*, 2010:8). The initial physicochemical analyses were made on freshly prepared formulations while the subsequent stability-determining measurements were done after the lyophilisation process. Lyophilisation of nanoformulations can result in instability such as changes in particle aggregation, physical properties, osmolarity, pH and drug loading (Majuru & Oyewumi, 2009:614).

According to Hua *et al.* (2010:213) the absence of lyoprotectants during freeze-drying can affect the EE of compounds in liposomes. The same may be true of encapsulation in niosomes and SLNs. This in combination with the presence of many unknown compounds might be the reason for the changes that were observed with the formulations.

There was no formulation that presented with excellent stability throughout the stability study. Overall, it can however be said that the niosome formulations were relatively more stable than the SLN formulations. Further studies and adjustments will need to be conducted in order to address the stability issues that were identified in this study. However, the issue of herbal medicinal products being multi-component systems will continue to be a problem until a standardised method of doing in-depth stability studies in these systems is devised.

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Appendix D: Franz cell diffusion studies

D.1 Introduction

Transdermal and topical drug delivery are essential routes of drug delivery in cases where the target site is the skin. In the treatment of skin cancer the first line of treatment is usually surgical excision, but situations do arise where surgical excision is not possible due to the patient's health or location of the cancer lesion (Telfer *et al.*, 2008:36). Topical drug delivery refers to whereby an active pharmaceutical ingredient (API) is applied to the skin for a local effect (dermal penetration only); whereas transdermal drug delivery refers to whereby an API is delivered to the systemic circulation through the intact skin (percutaneous absorption) (Allen *et al.*, 2005:276; Shivakumar & Murthy, 2010:3). In this study we investigated the transdermal and topical delivery of *Withania somnifera* (*W. somnifera*) crude extracts with a main focus on topical delivery. Topical delivery was required, as the target site for treatment of primary cutaneous melanoma is the melanocytes which are found within the stratum basale of the skin epidermis (Williams, 2003:7).

Advantages of topical drug delivery include the avoidance of first-pass metabolism, it is a simple non-invasive method of drug delivery resulting in high patient compliance and the target site is readily accessible (Cleary, 1993:19; Naik *et al.*, 2000:319). There are also drawbacks with topical drug delivery as the stratum corneum, the body's first line of defence, is a relatively impenetrable barrier to the entry of molecules (Flaten *et al.*, 2015:11).

The absorption of an API through the skin is a passive process that is best described using Fick's law of diffusion (Equation D.1). Where J is the flux, K is the partition coefficient, D is the diffusion coefficient in the membrane, h is the membrane thickness in centimetres and ΔC is the concentration difference across the skin membrane in µg/cm^3 (Shivakumar & Murthy, 2010:5).

$$J = \left(\frac{K \times D}{h}\right) \Delta C \qquad \textbf{Equation D.1}$$

Flux refers to the amount of API that diffuses through the skin and it is dependent on the available surface area plus the contact time of the delivery vehicle and the skin (Smith & Surber, 2000:25). The diffusion coefficient of an API is described as the ease with which the API is able to diffuse through a particular solvent (Smith & Surber, 2000:29). Whereas partition coefficient is a measure of the affinity that an API has for the skin membrane versus the drug delivery vehicle and it thus determines the ease with which an API will diffuse from the drug delivery vehicle into skin membrane. During preformulation and formulation, the water/octanol partition coefficient ($pK_{o/w}$) is used as an indication of the vehicle/skin partition coefficient (Smith & Surber, 2000:27). Withaferin A and withanolide A have log P values of 3.508 and 3.503 respectively, which are

indicative of an ability to diffuse through the skin (Sigma Aldrich, 2014a:4; Sigma Aldrich, 2014b:4).

There are three distinct steps that an API must go through during transdermal drug delivery and these are: i) penetration (entering the stratum corneum), ii) permeation (moving through the different skin layers) and iii) resorption (reaching the dermis and/or vascular system). As an API moves through the skin it can follow any one of the three different pathways that have been described in literature for skin transport. The three pathways are intracellular/transcellular, follicular and intercellular. The chemical and physical properties of the molecule in question will influence the pathway taken (Liuzzi *et al.*, 2016:295). When an API is delivered to the skin by a delivery vesicle the API may permeate independently after leaving the vesicle or it may permeate the skin while within the vesicles using any of the pathways (Figure D.1).

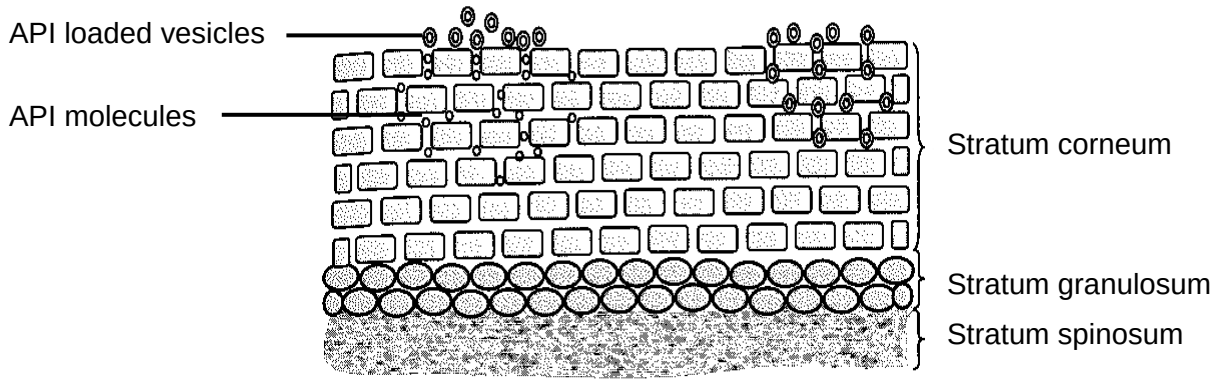

Figure D.1: Proposed mechanism to explain intercellular vesicle permeation across the skin (Shivakumar & Murthy, 2010:11).

Delivery of an API through the skin to deeper skin layers or to the systemic circulation requires the API to be sufficiently lipophilic to penetrate the lipophilic stratum corneum; while at the same time sufficiently hydrophilic to gain entry into the hydrophilic epidermis and dermis. There are very few APIs that naturally possess such characteristics; therefore various methods and technologies have been developed in order to circumvent the barrier function of the skin (Lam & Gambari, 2014:27; Liuzzi *et al.*, 2016:295). Reports have been made that nanoparticulate drug delivery vesicles can aid the penetration of APIs into the skin and protect APIs from untimely degradation once in the body (Honeywell-Nguyen & Bouwstra, 2005:68).

In this study niosomes and solid lipid nanoparticles (SLNs) were selected for use as the drug delivery vehicles for topical application of *W. somnifera* crude extracts. Niosomes have been found to be safe, effective and permeation enhancing topical drug delivery vesicles (Paolino *et al.*, 2008:238; Junyaprasert *et al.*, 2012:309; Marianecci *et al.*, 2012:22; Yeh *et al.*, 2013:243). Topically applied SLNs result in prolonged and targeted release of APIs; while reducing the

amount of API reaching the systemic circulation. SLNs are also able to increase API deposition within the skin (Godin & Touitou, 2012:523; Madan *et al.*, 2014:63).

For the purpose of the Franz cell diffusion studies each of the three extracts (prepared as described in Appendix A) were incorporated into niosomes and SLNs and then used as is for the diffusion studies. This gave a total of six formulations that were used for the skin diffusion and membrane release studies. HPLC analysis was conducted thereafter to determine whether withaferin A and withanolide A had diffused into or through the skin. The aim of the Franz cell diffusion studies was to:

- Investigate the release of withaferin A and withanolide A (in the crude extracts) from niosomes and SLNs.
- Investigate the topical and transdermal delivery of withaferin A and withanolide A in *W. somnifera* crude extracts.
- Investigate the topical and transdermal delivery of *W. somnifera* marker compounds in niosomes vs. SLNs.
- Investigate the retention of withaferin A and withanolide A within the skin layers during a 12 h skin diffusion study.

D.2 Materials and methods

D.2.1 HPLC method for sample analysis

Analysis of all extracted samples was carried out using high performance liquid chromatography (HPLC). The HPLC analytical method for withaferin A and withanolide A was developed in the Analytical Technology Laboratory (ATL) of the North-West University with the assistance and expert knowledge of Professor Jan du Preez. All the analyses were carried out in the ATL lab.

The analytical instrument was an Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump, degasser, an auto-sampler injection mechanism and a diode array detector. ChemStation Rev. A.10.02 software was used for data acquisition and analysis. A gradient elution method was used and mobile phase A consisted of HPLC-grade Milli-Q® water (Millipore, Milford, USA); while mobile phase B consisted of HPLC-grade 100% acetonitrile. A flow rate of 1 ml/min and an injection volume of 50 µl was used. The detection wavelength was set at 210 nm and the stop time at 22 min. Withaferin A eluted at approximately 7.5 min, while withanolide A eluted at 8.5 min.

Prior to injection of any sample set withaferin A and withanolide A standards were injected at different concentrations in order to obtain a linear regression curve. The standards for the linear

curve were prepared using methanol and Milli-Q® water as the solvents. Table D.1 is a summary of the mobile phase run-times that were used with the HPLC analytical method.

Table D.1: HPLC mobile phase conditions for the detection of withaferin A and withanolide A

Time (min)	Mobile phase A (%v/v)	Mobile phase B (%v/v)	Comment
0.0 - 10.0	90.0→0.0	10.0→100.0	Linear gradient
10.0 - 15.0	0.0	100.0	Isocratic
15.0 - 15.1	0.0→90.0	100.0→10.0	Linear gradient
15.1 - 20.0	90.0	10.0	Re-equilibration

D.2.2 Preparation of receptor phase

Phosphate buffer solution (PBS) at a pH of 7.4 was used as the receptor phase for the skin diffusion and membrane release studies. To prepare the phosphate buffer solution, 3.147 g of sodium hydroxide (NaOH) was weighed out and diluted to 786.8 ml with Milli-Q® water and 13.620 g of potassium orthophosphate dihydrogen (KH_2PO_4) was weighed out and diluted to 500 ml with Milli-Q® water. The NaOH and KH_2PO_4 solutions were mixed together and stirred with a magnetic stirrer. The pH of the prepared solution was then measured using a Mettler Toledo pH meter (Columbus, Ohio, USA) and adjusted to pH 7.4 accordingly; using 10% NaOH or 10% orthophosphoric acid.

D.2.3 Preparation of donor phase

W. somnifera extracts in niosomes or SLNs constituted the donor phase for each experiment. The niosome and SLN formulations were prepared using the solvent injection method with a crude extract concentration of 2.0%. Due to the different compositions of each extract the concentrations of withaferin A and withanolide A in the formulations differed with each extract (Table D.2).

Table D.2: Concentrations of withaferin A and withanolide A in donor phase

Formulations	Withaferin A concentration (µg/ml)	Withanolide A concentration (µg/ml)
Water extract niosomes **(NW)** and water extract SLN **(SW)**	352.0	242.0
EtOH extract niosomes **(NE)** and EtOH extract SLN **(SE)**	910.0	608.0
50% EtOH niosomes **(N50)** and 50% EtOH SLN **(S50)**	196.0	1008.0

D.2.4 Skin preparation

The skin that was used for the skin diffusion studies was female Caucasian skin obtained from abdominoplasty surgeries. The patients gave informed consent and ethical approval for obtaining, preparing and using the human skin for research purposes was obtained from the NWU Research Ethics Committee (Ethical approval number - NWU-00114-11-A5).

The skin samples were collected promptly after the surgery and transported in cooler boxes with ice packs. Freshly excised skin was visually inspected and any imperfections (i.e. holes and stretch marks) were clearly marked in order to ensure that these areas would not be used for experimentation as the imperfections would compromise validity of results obtained. Split-thickness skin was then prepared using a Zimmer® dermatome (Warsaw, Indiana, USA) at a thickness of 400 µm and placed on Whatman® filter paper. Holes of 15 mm diameter were punched into the skin which was then wrapped in foil and clearly labelled with the date. The skin was placed in Ziploc® bags and frozen at -20 °C until needed. The 400 µm skin thickness included the stratum corneum, the epidermis and part of the dermis (Flaten *et al.*, 2015:17). Diffusion of an API through the split-thickness skin was taken to imply that transdermal drug delivery had occurred.

D.2.5 Franz cell diffusion studies

The diffusion studies were conducted using static Franz diffusion cells. A Franz cell has two compartments – a donor compartment and a receptor compartment which are both made of inert glass. The chambers of the receptor compartments had volume capacities of at least 2 ml and exposed diffusion surface areas of 1.075 cm^2. A water bath set at 37 °C was used to maintain the temperature of the receptor phase at 37 °C; while maintaining the temperature of the donor phase at 32 °C in order to mimic *in vivo* conditions (Williams, 2003:62; Clares *et al.*, 2014:593). The following general setup described was used for the membrane release studies as well as the skin diffusion studies:

i. The prepared PBS (pH 7.4; receptor phase) was placed in a 37 °C water bath for at least 1 h for it to get to the desired temperature of 37 °C.

ii. The prepared test and placebo formulations (donor phase) were placed in a separate water bath set at 32 °C for at least 1 h for them to reach the desired temperature of 32 °C.

iii. During this period the Franz cells were prepared for the experiment.

iv. The donor and receptor compartments were liberally smeared with Dow Corning® vacuum grease to ensure that a good seal would be obtained.

v. A magnetic stirring rod was placed into the receptor compartment.

vi. Skin (with stratum corneum towards the donor compartment) or a polytetrafluoroethylene (PTFE) membrane filter was then placed between the donor compartment and receptor compartment.

vii. The donor compartment and receptor compartments were sealed together using Dow Corning® vacuum grease and securely fastened together using a metal horseshoe clamp to ensure that no leaks would occur.

viii. The 37 °C PBS (2.0 ml) was then added to the receptor phase ensuring that no air bubbles entered the system. The donor formulation (1.0 ml) was then added to the donor phase above the skin or membrane.

ix. Parafilm® and a cap were used to cover the donor compartment to ensure that no formulation constituents were lost due to evaporation.

x. Fully assembled Franz cells were put on a Franz cell stand and placed in a 37 °C water bath ensuring that only the receptor compartment was immersed. A Variomag® magnetic stirrer at the bottom of the water bath ensured that the receptor phase was constantly stirred and remained homogenous.

xi. At predetermined time intervals the entire receptor phase (PBS, pH 7.4) was removed and filtered into clearly labelled HPLC vials. The removed receptor phase was replaced with fresh buffer in order to maintain the sink conditions.

xii. The HPLC method described in Section D.2.1 was used to analyse the extracted samples.

D.2.5.1 Membrane release studies

Membrane release studies were performed in order to determine the drug release profiles of the niosomes and the SLNs. Results of membrane release studies give an indication as to whether an API is released from the drug delivery vehicle and in turn available for skin diffusion. Membrane release studies are therefore performed prior to skin diffusion studies. In this study, the general method described for Franz cell diffusion studies was followed for the membrane release studies. Instead of skin, 0.45 µm thick PTFE membrane filters were placed between the donor compartment and receptor compartment. Extractions (step ix) were done at hourly intervals up to six hours, giving extraction times of 1; 2; 3; 4; 5 and 6 h.

D.2.5.2 Skin diffusion studies

After the membrane release studies; 12 h skin diffusion studies were conducted. In this study a single extraction was done at the end of the twelve hour period because it was observed in a pilot study that there was no withaferin A or withanolide A detected in the receptor phase after multiple

staggered extractions. This may have been due to the concentrations in the receptor phase being below the limits of detection of the HPLC method used.

D.2.6 Tape-stripping studies

The tape-stripping technique is used in *in vitro* studies to investigate the percutaneous absorption of permeants. Tape-stripping is a relatively simple method in which adhesive tape is applied to the skin surface and rapidly removed; thus removing part of the stratum corneum with it. This is repeated several times; thereafter the amount of permeant on the tape strips is determined using a suitable analytical method such as HPLC (Brain *et al.*, 2002:215; Williams, 2003:213). The following steps ((i) to x)) describe in more detail the tape-stripping method that was performed for each Franz cell:

i. Sixteen pieces (for each Franz cell) of 3M Scotch® Magic™ tape were cut before starting the tape-stripping process. The strips had to be large enough to sufficiently cover the diffusion area.

ii. The pieces of skin were removed from the Franz diffusion cells after the skin diffusion studies and firmly secured onto Whatman® filter paper using pins.

iii. It was ensured that the skin was straightened while securing it on the filter paper to ensure that the whole diffusion area was clearly visible.

iv. The piece of skin was then wiped clean with a paper towel.

v. A piece of 3M Scotch™ Magic™ tape was firmly applied to the diffusion area and removed and thereafter discarded, as this would contain unabsorbed drug on the skin surface and was still part of the cleaning procedure.

vi. The stripping process was repeated with 15 further strips until the skin glistened. These strips were then placed into a polytop containing 5 ml PBS (pH 7.4). The tape-strips represented the stratum corneum that had been stripped off the skin samples and a part of the epidermis (stratum corneum-epidermis (SCE)).

vii. The remaining skin was cut into small pieces and placed in a polytop containing 5 ml PBS (pH 7.4). These skin pieces represented part of the epidermis and dermis (epidermis-dermis (ED)).

viii. The polytops containing the tape-strips and the skin pieces were stored overnight in a refrigerator (4 °C).

ix. The following morning the buffer containing the tape-strips and the skin samples was filtered into respectively labelled HPLC vials and the samples were analysed using HPLC.

x. Steps i - ix were repeated for each individual Franz cell.

D.2.7 Statistical and data analysis

The percentage compound diffused after 6 h and the average cumulative amount of compound per area were calculated for the membrane release data. For the tape-stripping data, the average concentrations of compound in the SCE and in the ED were calculated. The limit of detection for withaferin A was 0.05 µg/ml and that of withanolide A was 0.50 µg/ml. Therefore any results obtained that were below the limit of detection were discarded.

Statistical analysis of all the Franz cell diffusion data was done using STATISTICA data analysis software system (StatSoft, Inc. (2015), version 12). Descriptive statistics of flux were calculated per extract, formulation and compound including the mean, median and standard deviation. T-tests and one-way analysis of variance (ANOVA) analyses were done in order to test for significant differences between the mean flux values. The ANOVA was used as it is a flexible and powerful tool in statistics that can analyse the interaction effects between variables, taking various factors into account (Statsoft Inc, 2013). Comparisons were made with respect to the vesicle type, crude extract and marker compound.

D.3 Results and discussion

D.3.1 Membrane release studies

The results of the membrane release studies were processed to calculate the average percentage of compound diffused after the 6 h period and the average cumulative amount of compound diffused per unit area. Table D.3 shows a summary of the results that were obtained for the release of withaferin A and withanolide A during the membrane release studies.

At the end of the 6 h membrane release studies no analyte was detected by the HPLC method for the **NW**. This led to the assumption that this formulation either had a very slow release which could not be detected within the 6 h period or the formulation generally had a negligible release of withaferin A and withanolide A. Figures D.2 – D.11 show the average cumulative amount of compound that was released per unit area from **SW**, **NE**, **SW**, **N50** and **S50**.

Table D.3: Total amount of marker compound released as a percentage of initial amount in donor formulation and average cumulative amount of marker compound released after the 6 h membrane release studies (n = 10)

Formulation	Average %withaferin A released (%)	Average cumulative withaferin A per area (µg/cm^2)	Average %withanolide A released (%)	Average cumulative withanolide A per area (µg/cm^2)
Water extract niosomes **(NW)**	-	-	-	-
Water extract SLN **(SW)**	12.308	44.856 ± 3.579	0.578	10.835 ± 0.936
EtOH extract niosomes **(NE)**	54.379	355.933 ± 70.491	33.370	150.164 ± 27.355
EtOH extract SLN **(SE)**	10.048	65.771 ± 7.586	4.741	21.332 ± 1.197
50% EtOH extract niosomes **(N50)**	1.250	21.151 ± 0.703	1.941	21.943 ± 0.633
50% EtOH extract SLN **(S50)**	2.842	48.097 ± 2.825	5.398	61.034 ± 2.521

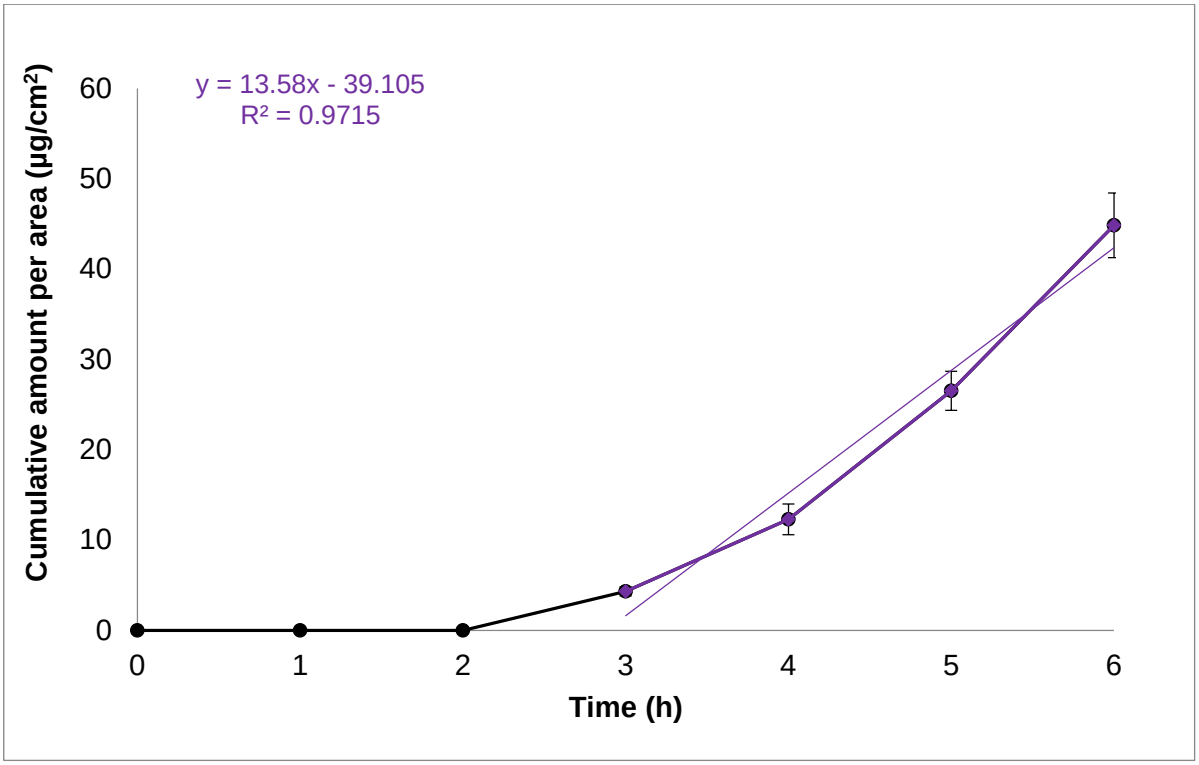

Figure D.2: Average cumulative amount per area (µg/cm^2) of withaferin A released from the **SW** after the 6 h membrane release study (n = 10)

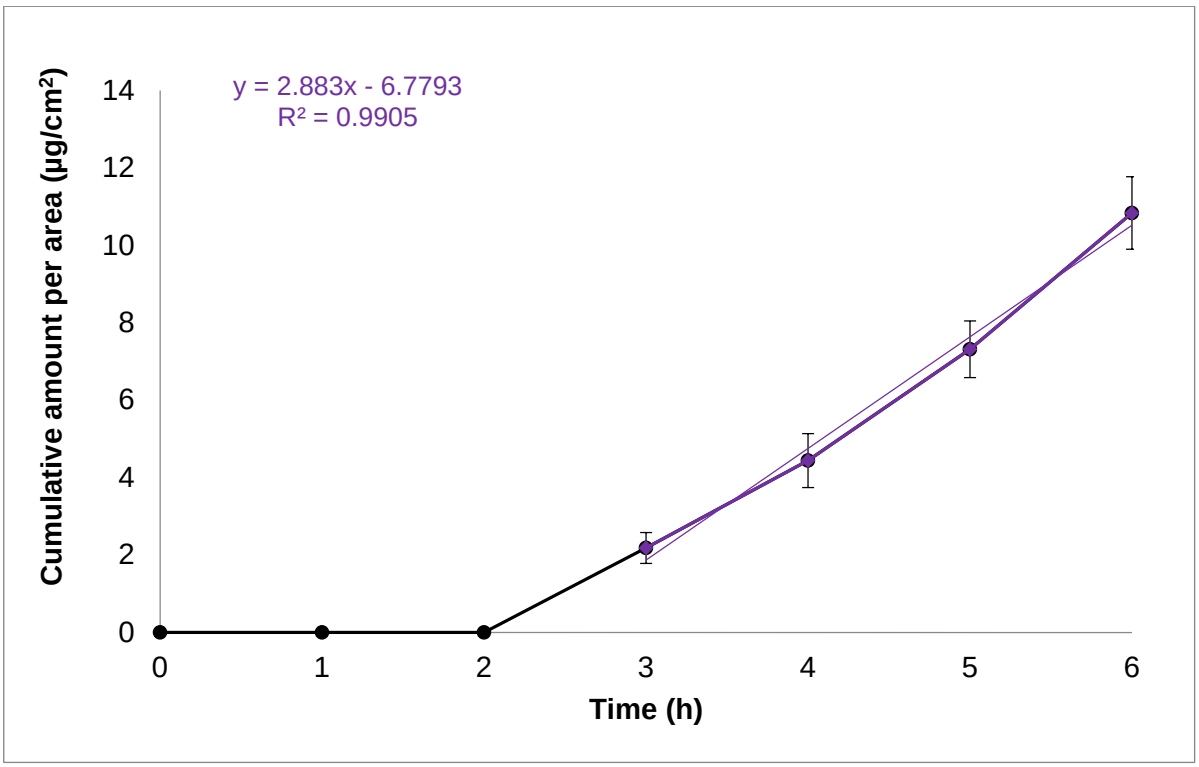

Figure D.3: Average cumulative amount per area (µg/cm^2) of withanolide A released from the **SW** after the 6 h membrane release study (n = 10)

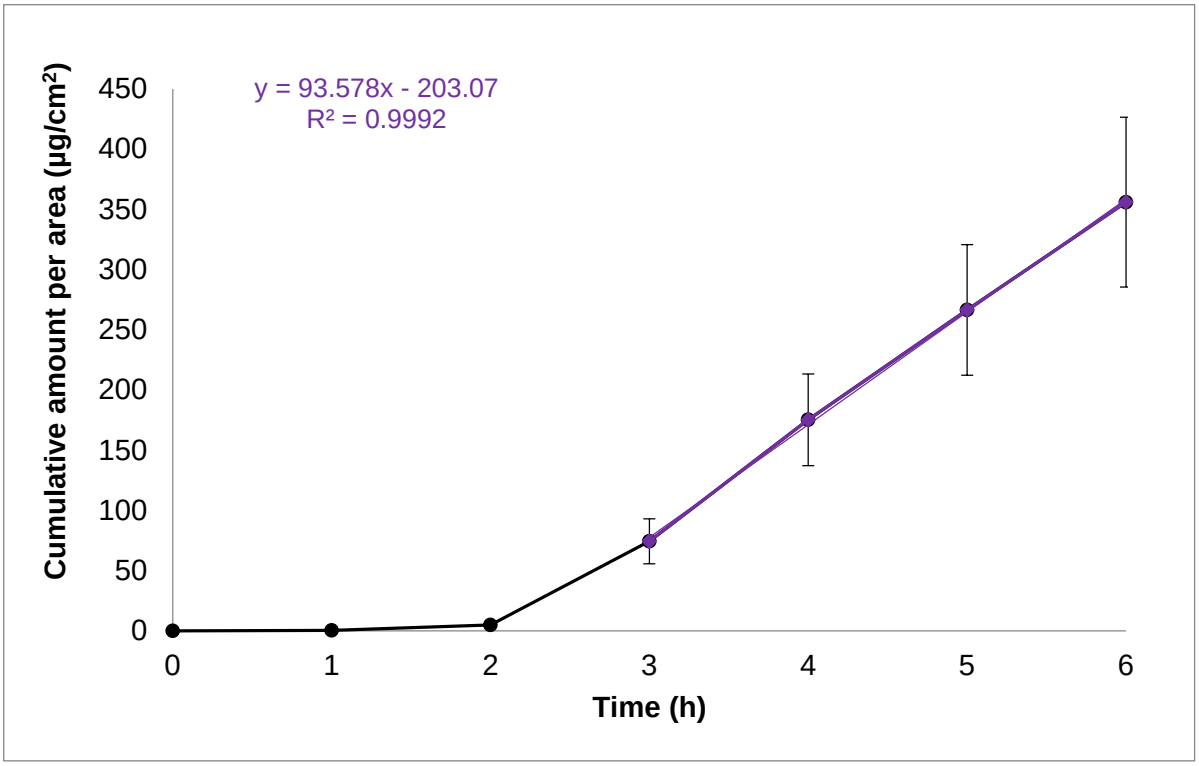

Figure D.4: Average cumulative amount per area (µg/cm^2) of withaferin A released from the **NE** after the 6 h membrane release study (n = 10)

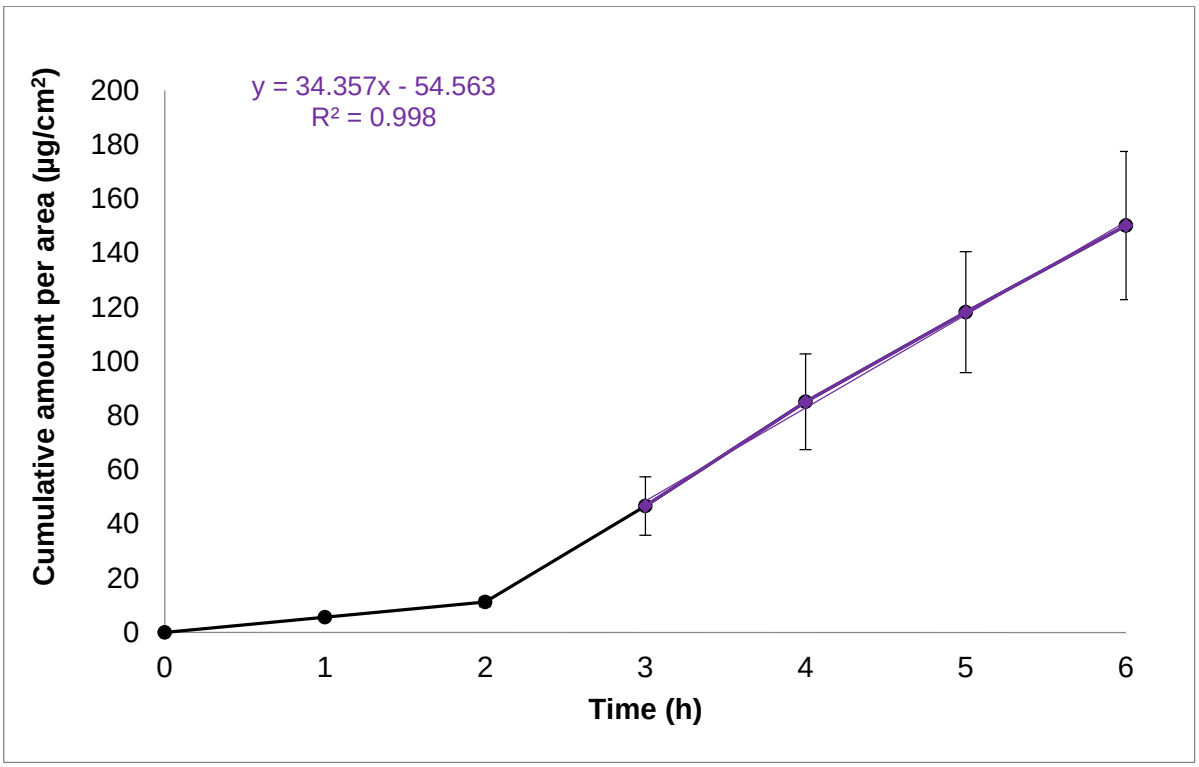

Figure D.5: Average cumulative amount per area (µg/cm^2) of withanolide A released from the **NE** after the 6 h membrane release study (n = 10)

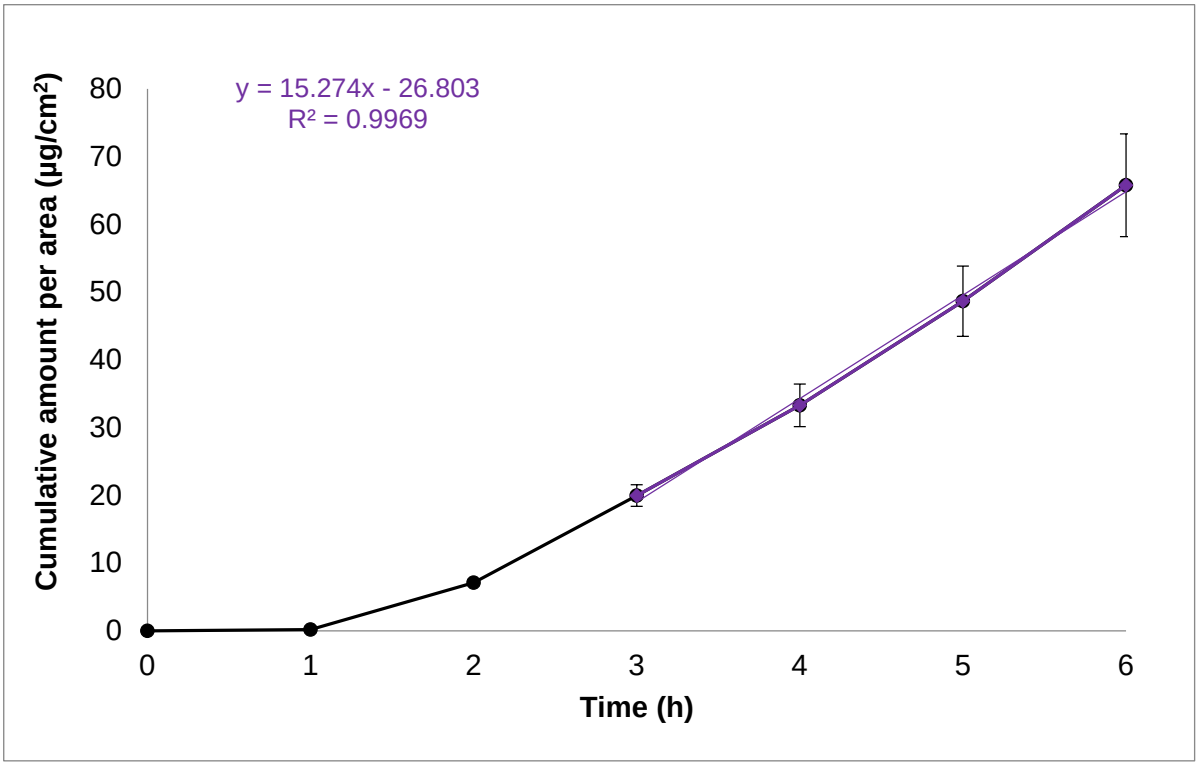

Figure D.6: Average cumulative amount per area (µg/cm^2) of withaferin A released from the **SE** after the 6 h membrane release study (n = 10)

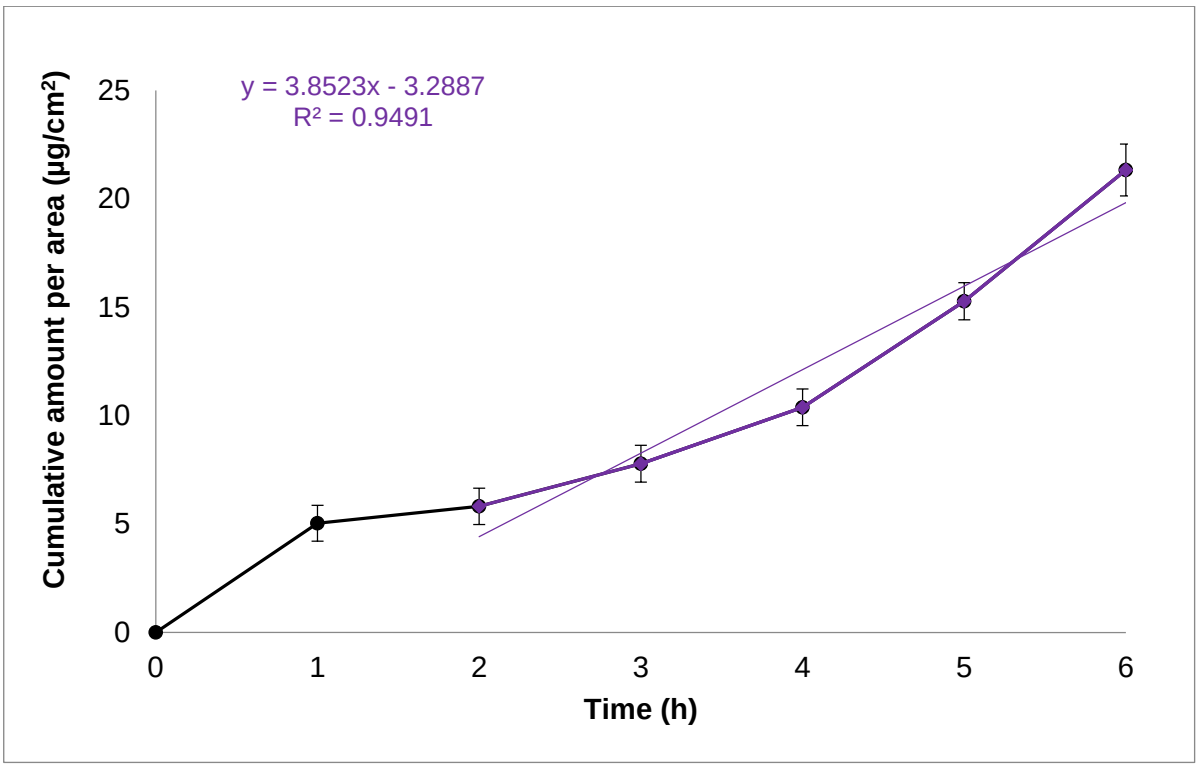

Figure D.7: Average cumulative amount per area (µg/cm^2) of withanolide A released from the **SE** after the 6 h membrane release study (n = 10)

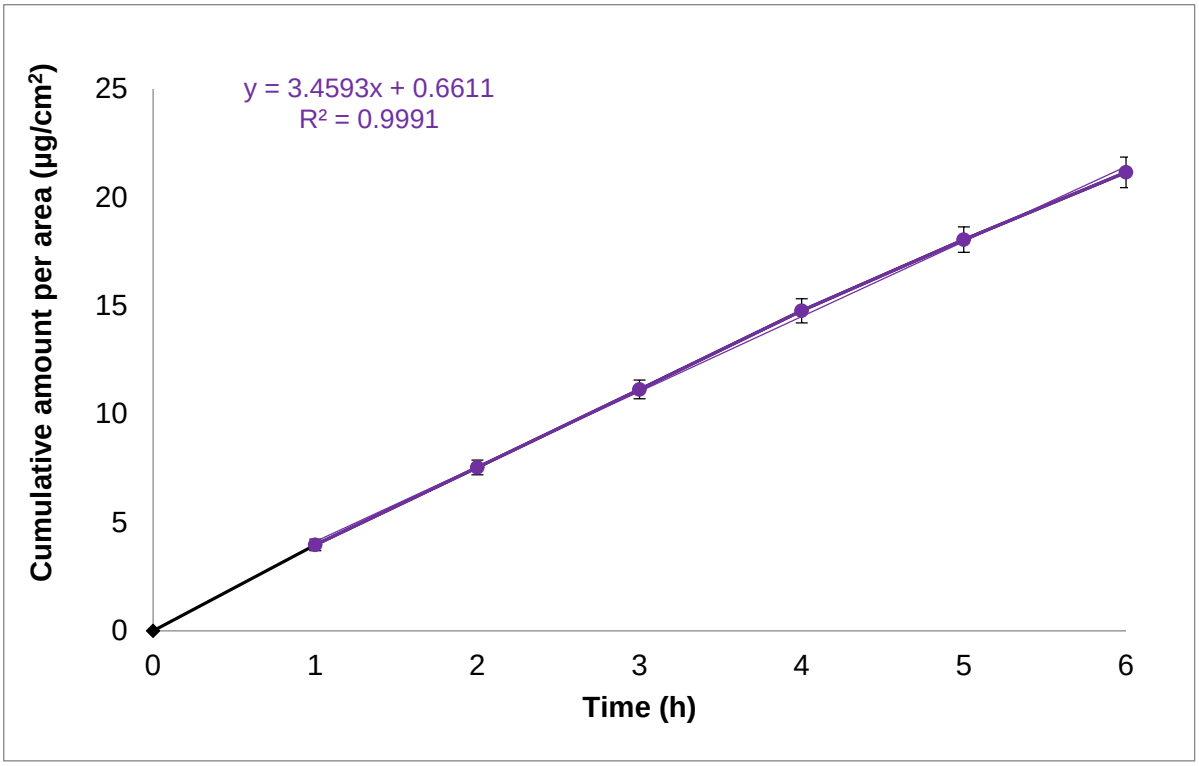

Figure D.8: Average cumulative amount per area (µg/cm^2) of withaferin A released from the **N50** after the 6 h membrane release study (n = 10)

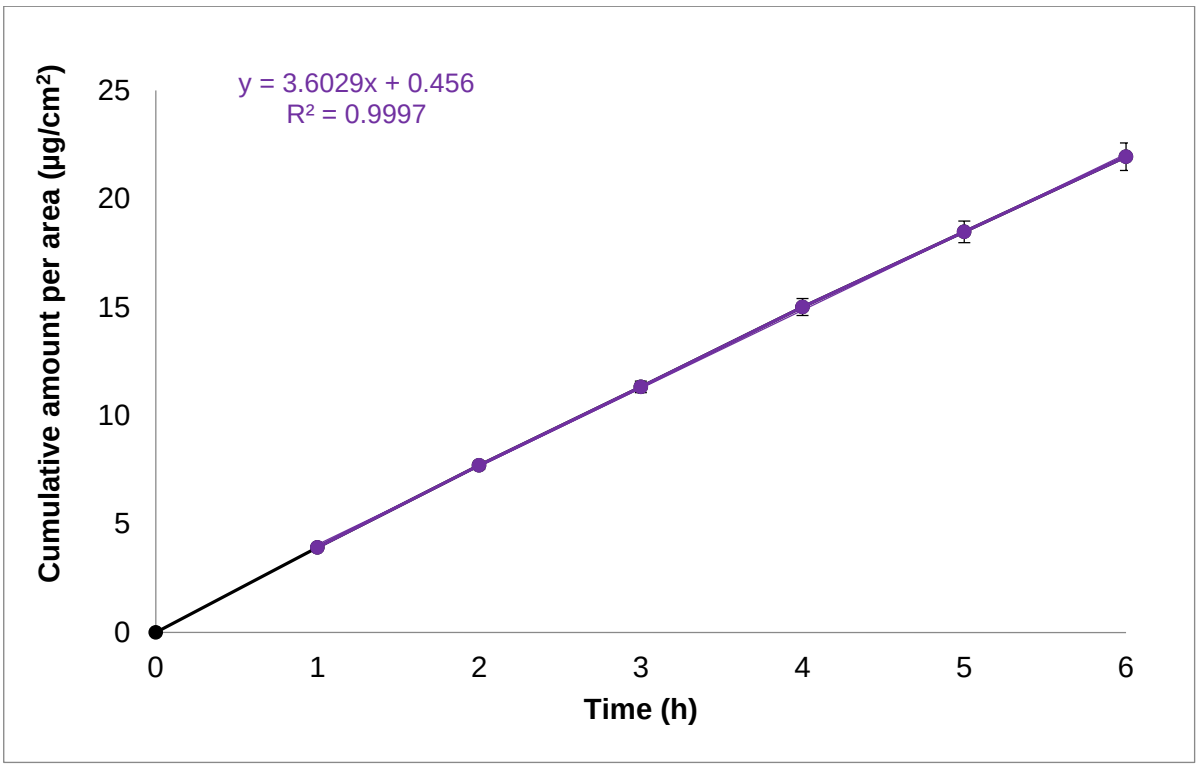

Figure D.9: Average cumulative amount per area (µg/cm^2) of withanolide A released from the **N50** after the 6 h membrane release study (n = 10)

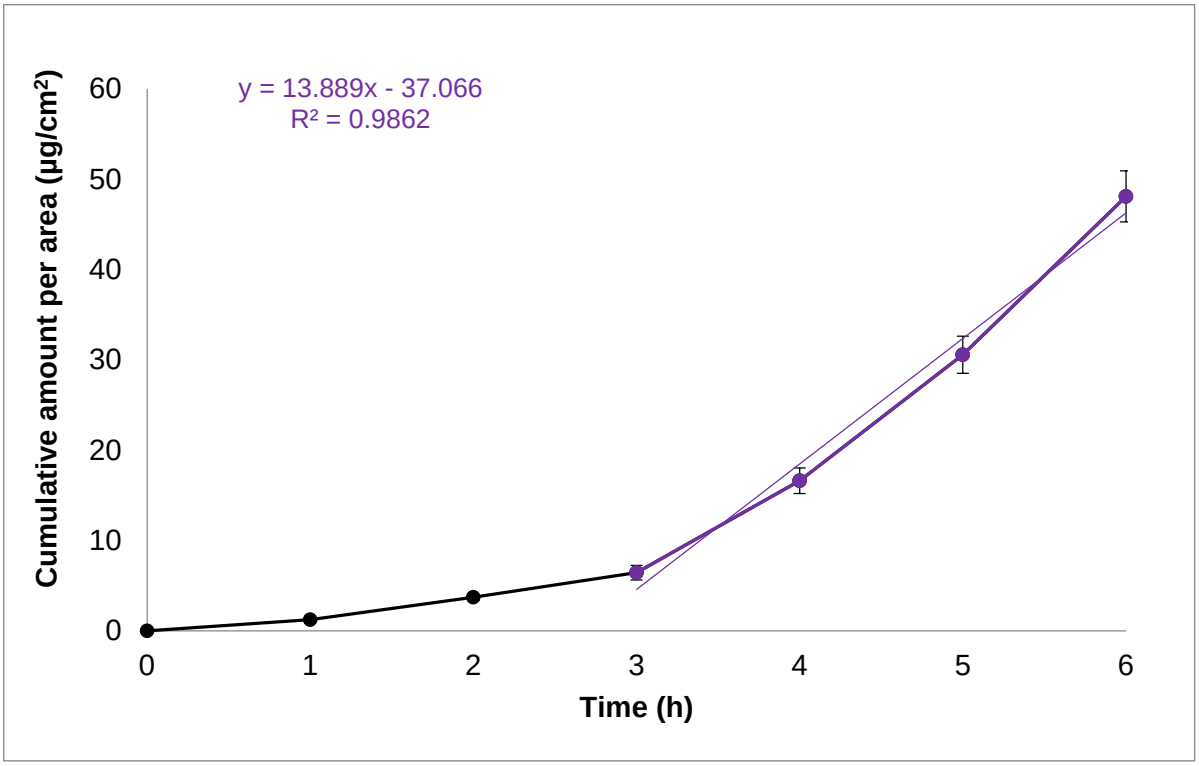

Figure D.10: Average cumulative amount per area (µg/cm^2) of withaferin A released from the **S50** after the 6 h membrane release study (n = 10)

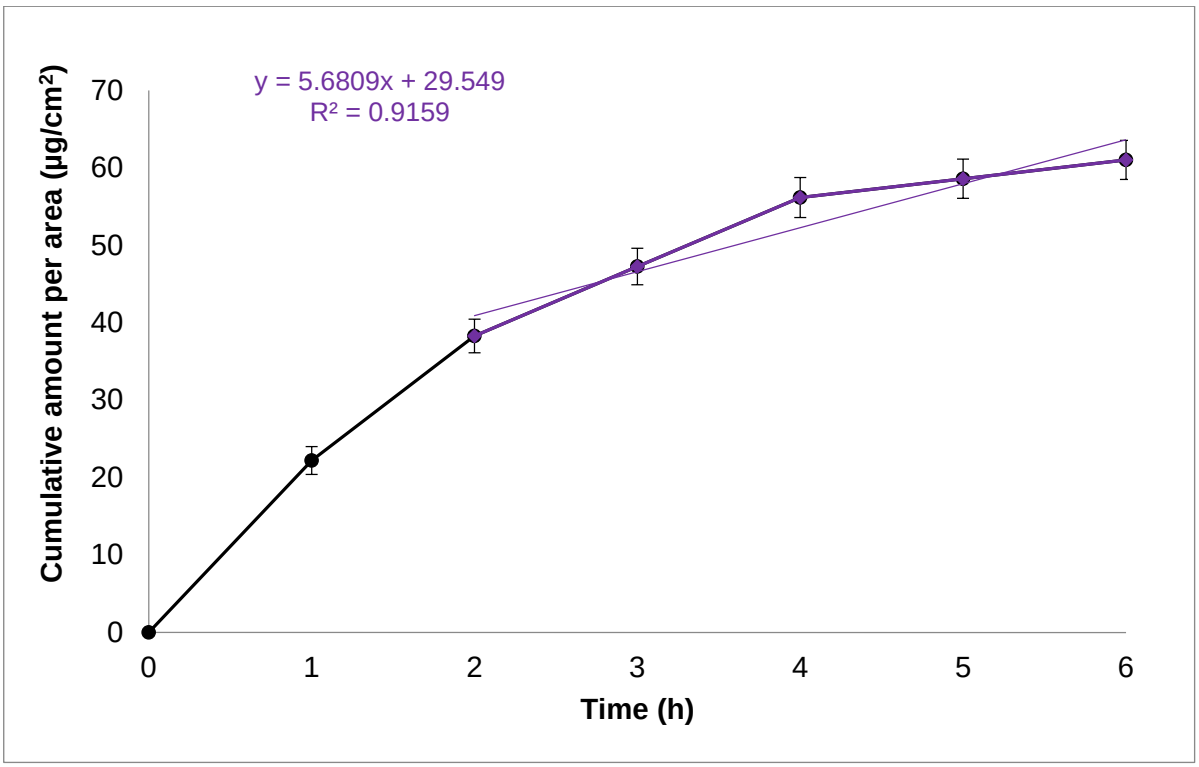

Figure D.11: Average cumulative amount per area (µg/cm^2) of withanolide A released from the **S50** after the 6 h membrane release study (n = 10)

The formulation which had the highest %release of withaferin A was **NE** (54.379%) followed by **SW** (12.308%), **SE** (10.048%), **S50** (2.842%) and lastly, the **N50** (1.250%) formulation. The average cumulative amount diffused did not follow this same trend as it was dependent on the initial amount of withaferin A that was present in each individual extract. The differences in the drug release profiles of the different formulations may have been due to the effects of the different compounds present in the extracts. The **NE** released the highest cumulative amount of withaferin A (355.933 µg/cm^2) followed by **SE** (65.771 µg/cm^2), **S50** (48.097 µg/cm^2), **SW** (44.856 µg/cm^2) and lastly, **N50** (21.151 µg/cm^2). **NE** and **SE** had the highest initial concentrations of withaferin A so it followed that they would have the highest cumulative amounts diffused, due to a higher concentration being the driving force for drug release and flux (Lu & Gao, 2010:74). The **S50** formulation together with **N50** however, had the lowest initial concentrations but **S50** managed to result in the release of a relatively high cumulative amount of withaferin A.

The **NE** had a very high withanolide A %release of 33.370% followed by **S50** (5.398%), **SE** (4.741%), **N50** (1.941%) and lastly, **SW** with 0.578%. With respect to average cumulative amount diffused **NE** also had the highest cumulative amount of withanolide A (150.164 µg/cm^2) released in the 6 h period. The other formulations released an average cumulative amount of withanolide A in this order: **S50** (61.034 µg/cm^2), **N50** (21.943 µg/cm^2), **SE** (21.332 µg/cm^2) and lastly, **SW**

(10.835 µg/cm^2). **N50** and **S50** had the highest initial withanolide A concentrations followed by **NE** and **SE** and lastly, the **SW** formulation. Cumulative withanolide A release followed that hierarchy, except for **NE** which had a higher release than the 50% extract formulations.

All the formulations except **NW** released withaferin A and withanolide A within the 6 h period leading to the conclusion that each of these formulations would have withaferin A and withanolide A available for the skin diffusion studies. Skin diffusion studies were conducted with the **NW** formulation, regardless of the lack of data obtained during the membrane release study; since the skin diffusion studies comprised of a single extraction at the 12 h versus the hourly extractions up to 6 h during the membrane release study.

D.3.2 Skin diffusion studies

After the 12 h skin diffusion studies, none of the marker compounds were detected in the receptor phase for all the formulations. This implied that the concentration of the compounds in the formulations was possibly too low for the compounds to reach significant (detectible by the HPLC method) concentrations in the receptor phase. Another possible explanation is that the formulations promoted the retention of the various compounds within the skin layers and thus deterred the diffusion of the compounds right through the skin to reach the receptor phase. Topical application of SLN encapsulated API has been reported to result in diminished delivery of API to the systemic circulation, while increasing API skin deposition; which may explain the results obtained (Madan *et al.*, 2014:63). Tape-stripping was done after the 12 h skin diffusion study as per norm.

D.3.3 Tape-stripping studies

Results of the tape-stripping studies were processed to calculate the average concentrations of the marker compounds that were present in the SCE and in the ED. The results are presented in Table D.4 and Figure D.12.

Table D.4: Average concentrations (µg/ml) of withaferin A that remained in the SCE and ED after the 12 h skin diffusion studies (n = 10)

Formulation	Average concentration in the SCE (µg/ml)	Average concentration in the ED (µg/ml)
Water extract niosomes **(NW)**	-	-
Water extract SLN **(SW)**	0.299 ± 0.077	-
EtOH extract niosomes **(NE)**	0.298 ± 0.044	-
EtOH extract SLN **(SE)**	0.061 ± 0.012	-

50% EtOH extract niosomes **(N50)**	1.364 ± 0.256	-
50% EtOH extract SLN **(S50)**	0.489 ± 0.079	0.129 ± 0.013

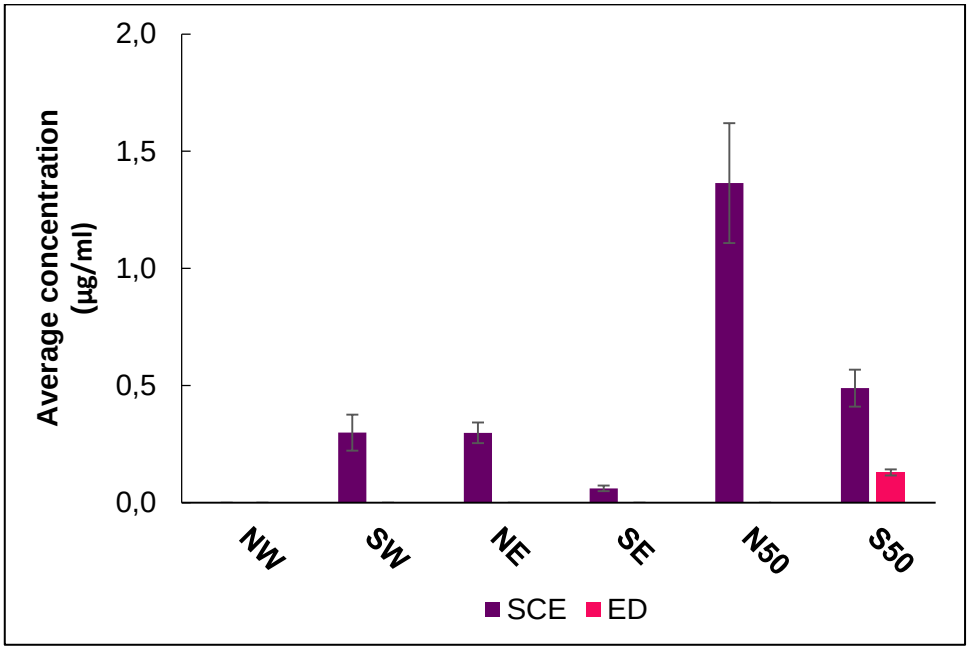

Figure D.12: Average concentrations of withaferin A that remained in the SCE and ED after the 12 h skin diffusion studies (n = 10)

The tape-stripping results indicated that only the **S50** formulation was able to deliver withaferin A to both the SCE and deeper into the ED. This highlighted that the **S50** formulation would be best for future studies as it had the capability to deliver the highly potent withaferin A to deeper skin layers. Delivery of an API into the ED is particularly important with respect to treatment of cutaneous melanoma as melanoma has a tendency to penetrate deep into the skin prior to metastasising. However, the **N50** formulation depicted the highest average concentration of withaferin A in the SCE (1.364 µg/ml), followed by **S50** (0.489 µg/ml), **SW** (0.299 µg/ml), **NE** (0.298 µg/ml) and lastly, **SE** (0.061 µg/ml). The formulations containing the 50% ethanol extract (**N50** and **S50**) presented with the highest concentration of withaferin A in the SCE. This was possibly due to the fact that the 50% ethanol extract (4.55%) had a significantly higher percentage content of withaferin A versus the water (0.98%) and ethanol extracts (1.76%).

Withaferin A mainly permeated into the SCE but not deeper into the hydrophilic ED as it is a relatively lipophilic compound. The combined effect of the occlusive effect of SLN and a high initial concentration of withaferin A in the **S50** formulation is conceivably the reason it is the only formulation that resulted in withaferin A reaching the ED.

Table D.5: Average concentrations of withanolide A that remained in the SCE and ED after the 12 h skin diffusion studies (n = 10)

Formulation	Average concentration in SCE (µg/ml)	Average concentration in ED (µg/ml)
Water extract niosomes **(NW)**	-	-
Water extract SLN **(SW)**	0.837 ± 0.160	0.642 ± 0.161
EtOH extract niosomes **(NE)**	-	-
EtOH extract SLN **(SE)**	0.828 ± 0.098	0.970 ± 0.011
50% EtOH extract niosomes **(N50)**	0.360 ± 0.153	-
50% EtOH extract SLN **(S50)**	0.311 ± 0.046	0.579 ± 0.046

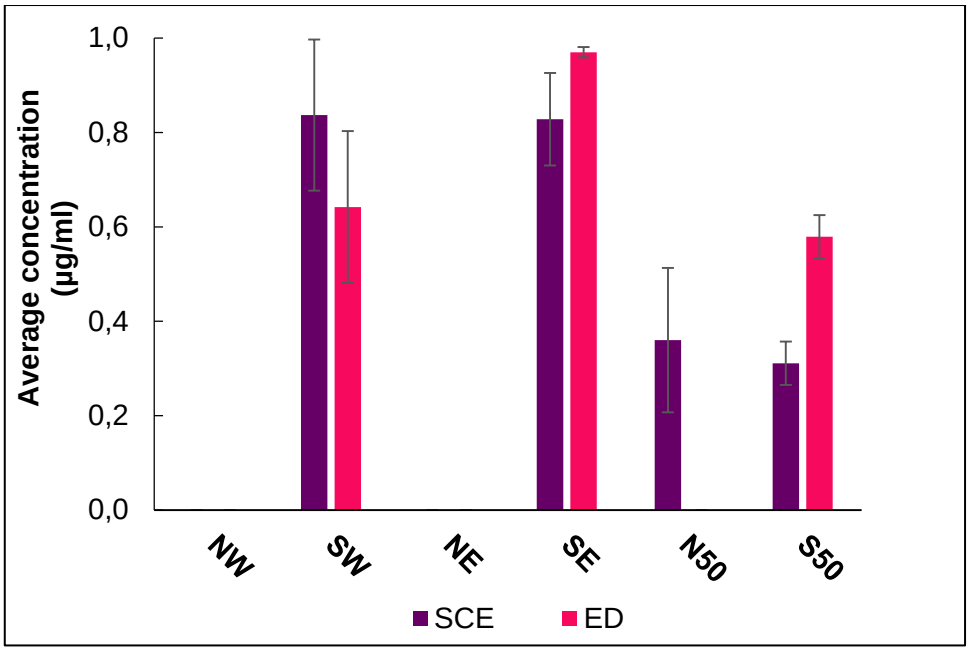

Figure D.13: Average concentrations of withanolide A that remained in the SCE and ED after the 12 h skin diffusion studies (n = 10)

Average concentrations of withanolide A in the SCE ranged from 0.311 to 0.837 µg/ml, while the concentrations in the ED ranged from 0.579 to 0.970 µg/ml. The **SW** formulation resulted in the highest concentration of withanolide A (0.837 µg/ml) in the SCE, followed by **SE** (0.828 µg/ml), **N50** (0.360 µg/ml) and lastly, **S50** (0.311 µg/ml). All the SLN formulations (**SW**; **SE**; **S50**) and the **N50** formulation resulted in withanolide A reaching the SCE. Withanolide A was able to move deeper into the skin due to the increased water content in the deeper layers of the skin. Only the three SLN formulations managed to result in withanolide A reaching the ED, namely **SE** (0.970 µg/ml), **SW** (0.642 µg/ml) and **S50** (0.579 µg/ml) in descending order of average withanolide A concentration in ED. The occlusive effect of SLNs may have played a role in this observed outcome (Majuru & Oyewumi, 2009:610).

The tape-stripping results for the **NW** formulation were compatible with the membrane release and skin diffusion results in that none of the marker compounds were detected in either the SCE or ED. Some of the formulations only managed to result in permeation of a single marker compound into the SCE or ED as summarised in Table D.6.

Table D.6: Summary of tape-stripping results

Formulation	SCE	ED
Water extract niosomes **(NW)**	ND*	ND
Water extract SLN **(SW)**	WFA**	WFA ; WNA***
EtOH extract niosomes **(NE)**	WFA	ND
EtOH extract SLN **(SE)**	WFA ; WNA	WNA
50% EtOH extract niosomes **(N50)**	WFA ; WNA	ND
50% EtOH extract SLN **(S50)**	WFA ; WNA	WFA ; WNA

*ND – Not detected; **WFA – withaferin A; ***WNA – withanolide A

D.3.4 Statistical analysis

The statistical analysis of the Franz cell diffusion data was complex due to the lack of information with some of the samples in which no marker compound was detected. However, the statistical analysis was carried out on the data that was available as it is important to determine whether any differences found are due to random variation or real effects (Machin *et al.*, 2007:3). Mean and median flux was calculated for all the membrane release data (Table D.88). The one-way ANOVA was then done in order to test for significant differences between mean flux and concentration values by comparing variances. A difference in results between groups was taken to be statistically significant if $p < 0.05$. Generally, a lower *p*-value represents an increasing index of the reliability of a result. Comparisons were made on variances due to differences between groups and differences within groups. Each group had 10 replicates.

D.3.4.1 Membrane release studies

All the results of the membrane release studies were assessed for any significant differences between results. It was found that there were significant differences ($p < 0.05$) for the following group comparisons: withaferin A vs. withanolide A; niosomes vs. SLNs and water extract vs. ethanol extract vs. 50% ethanol extract. When taking into consideration all the results it was seen in majority of the cases that a change in any factor (marker compound, extract or vesicle type) resulted in a statistically significant change (shown in Table D.7).

All the calculated flux values for withaferin A were higher than the respective withanolide A values for all the formulations in which diffusion occurred. The only exception was the **N50** in which

withaferin A flux (3.459 ± 0.102 µg/cm^2.h) was slightly lower than the withanolide A flux (3.603 ± 0.128 µg/cm^2.h). Regardless of the small difference, there was a statistically significant difference between the withaferin A flux and withanolide A flux for the **N50** with p = 0.012.

Table D.7: Statistical analysis of membrane release data showing p-values for each comparison

Comparison	p-value
Withaferin A vs. withanolide A	0.000000*
Niosome vs. SLN	0.000000*
Water vs. ethanol vs. 50% ethanol extract	0.000000*
Water vs. ethanol vs. 50% ethanol (SLN)	0.785671
Withaferin A vs. Withanolide A (SLN)	0.000000*
Withaferin A vs. withanolide A (ethanol)	0.000000*
SLN vs. niosome (ethanol)	0.000000*
Withaferin A vs. withanolide A (50% ethanol)	0.000000*
SLN vs. niosome (50% ethanol)	0.000000*
Withaferin A vs. withanolide A (**NE**)	0.000000*
Withaferin A vs. withanolide A (**N50**)	0.012361*
Withaferin A vs. withanolide A (**SE**)	0.000000*
Withaferin A vs. withanolide A (**S50**)	0.000002*
Withaferin A vs. withanolide A (**SW**)	0.000000*
SE vs. **S50** (withaferin A)	0.000132*
SE vs. **SW** (withaferin A)	0.866998
SW vs. **S50** (withaferin A)	0.000132*
SE vs. **S50** (withanolide A)	0.000132*
SE vs. **SW** (withanolide A)	0.084056
SW vs. **S50** (withanolide A)	0.000132*
NE vs. **N50** (withaferin A)	0.000000*
NE vs. **N50** (withanolide A)	0.000000*

* - a statistically significant difference exists

Table D.8: Average flux of withaferin A and withanolide A from niosomes and SLNs (n = 10)

Formulation	Withaferin A flux (µg/cm^2.h)	Withanolide A flux (µg/cm^2.h)
Water extract SLN **(SW)**	13.580 ± 1.104	2.883 ± 0.247
EtOH extract niosomes **(NE)**	76.075 ± 15.396	30.916 ± 5.813
EtOH extract SLN **(SE)**	13.307 ± 1.602	3.213 ± 0.170
50% EtOH extract niosomes **(N50)**	3.459 ± 0.102	3.603 ± 0.128
50% EtOH extract SLN **(S50)**	9.285 ± 0.541	7.541 ± 0.473

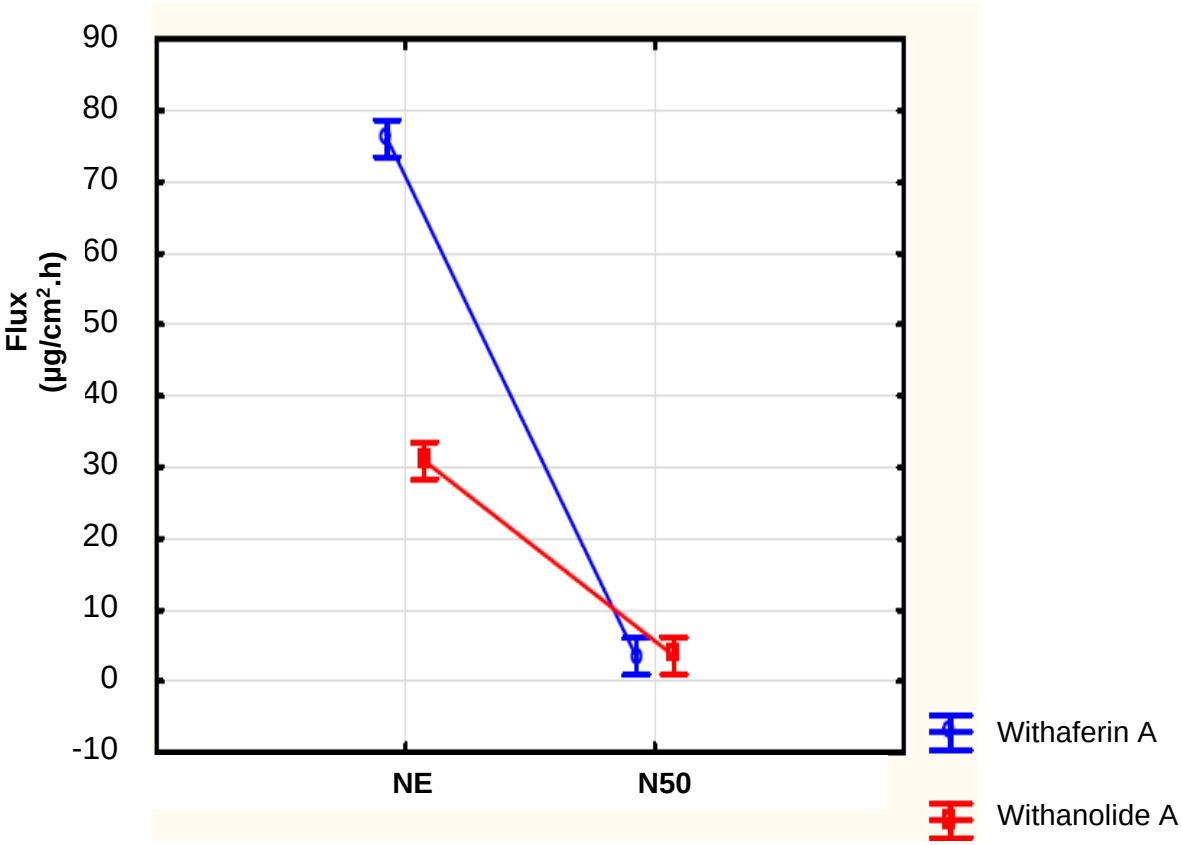

Figure D.14: Comparison of withaferin A and withanolide A flux from the niosome formulations (n = 10)

Flux values for withaferin A and withanolide A from each niosome formulation are presented for comparison in Figure D.14. **NE** had withaferin A (76.075 ± 15.396 µg/cm^2.h) and withanolide A (30.916 ± 5.813 µg/cm^2.h) flux values that were much higher than the **N50** flux values for withaferin A (3.459 ± 0.102 µg/cm^2.h) and withanolide A (3.603 ± 0.128 µg/cm^2.h). The differences were probably due to the fact that the extracts in the formulations had differing initial withaferin A and withanolide A compositions.

When focus was placed on the SLN formulations the ANOVA showed statistically significant differences between withaferin A and withanolide A release. However, there was no significant difference with a change in extract type. A statistically significant difference was identified with either a change from SLNs to niosomes or from withaferin A to withanolide A for the ethanol extract (**SE**) and 50% ethanol extract (**S50**) formulations.

A comparison among all the SLNs was made for the withaferin A flux and for the withanolide A flux. It was found that there was no significant difference ($p = 0.786$) amongst the flux values due to differences between **SW** and **SE**. The *p*-value for withaferin A flux comparison between the **SW** and **SE** was 0.867 and for withanolide A it was 0.084. Figure D.15 gives a representation of

the relationship between withanolide A and withaferin A flux for the different SLN formulations (**SW**, **SE**, **S50**).

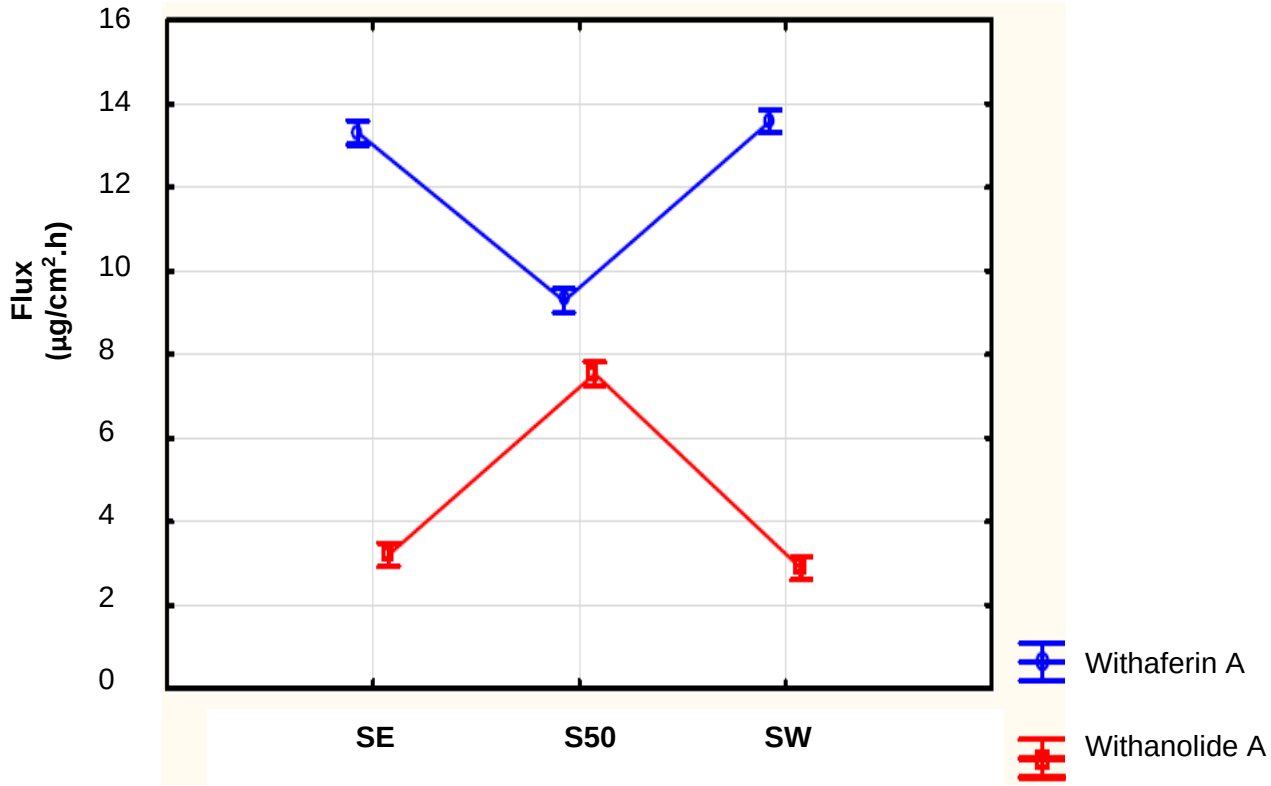

Figure D.15: Comparison of withaferin A and withanolide A flux from the SLN formulations (n = 10)

SE and **SW** both had relatively high withaferin A flux values and relatively low withanolide A flux values. There were statistically significant differences between **S50** and both **SE** and **SW** ($p < 0.05$). **S50** had the optimum release characteristics as it had a relatively average flux for both withaferin A and withanolide A.

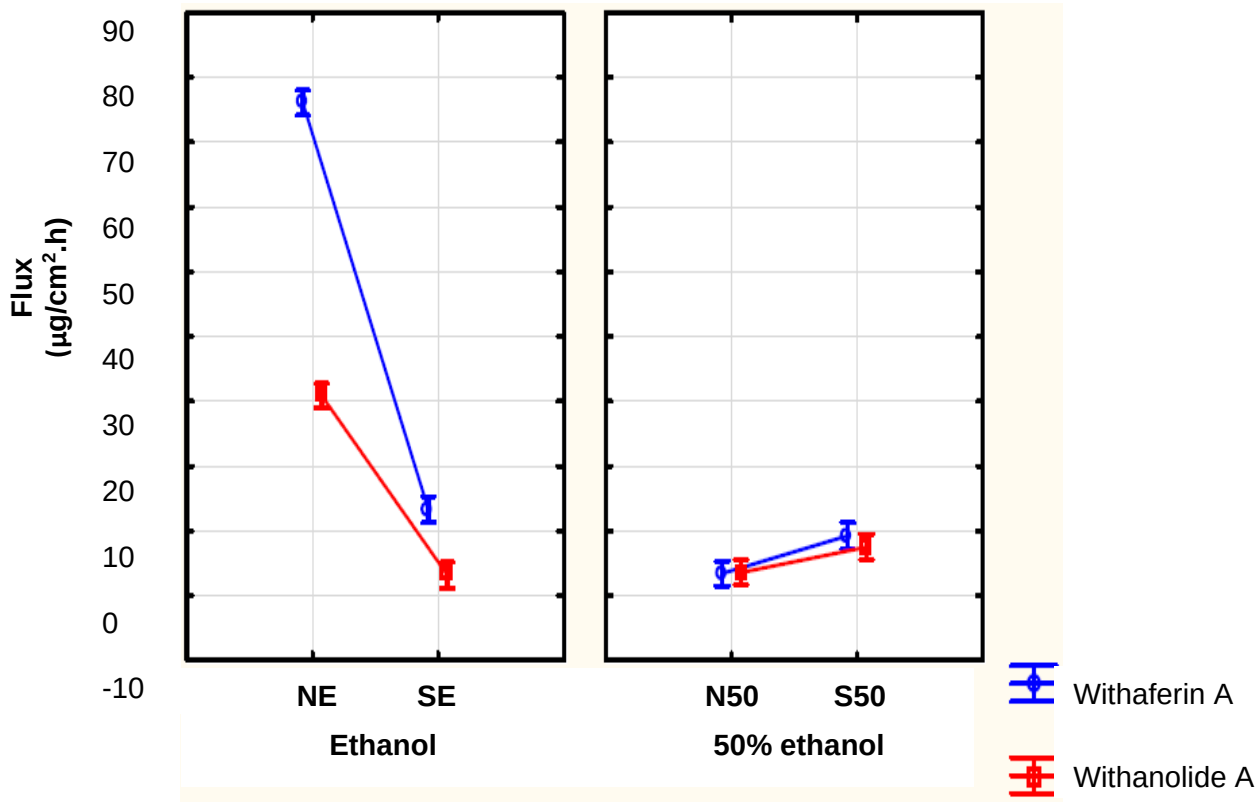

Figure D.16: Influence of drug delivery vesicle on the flux values from ethanol and 50% ethanol formulations (n = 10)

Niosomes resulted in a greater withaferin A and withanolide A flux as compared to the SLNs for the ethanol extract formulations. The opposite was true for the 50% ethanol extract formulations, SLNs resulted in the greater fluxes for the marker compounds. These differences can be attributed to the uncharacterised mixture of compounds in the crude extracts. **NE**, **N50**, **SW**, **SE** and **S50** all released withaferin A and withanolide A during the 6 h membrane release study. Therefore they would theoretically be able to release withaferin A and withanolide A for diffusion into the skin.

D.3.4.2 Tape-stripping

Mean concentrations in SCE and ED were calculated for each marker compound and these values were used for the statistical analysis. P-values for the different comparisons are shown in Table D.9. There was a significant difference between the withanolide A concentrations in the SCE and the ED after the **SW** ($p = 0.019$), **SE** ($p = 0.0004$) and **S50** ($p = 0.0002$) skin diffusion studies. A significant difference was also seen between the withaferin A concentrations in the SCE and ED after the **S50** ($p = 0.000001$) skin diffusion. This implied that the differences in concentration between the two skin layers were not by chance, but due to the influence of the physical, biological and chemical differences between the SCE and ED.

Withaferin A and withanolide A average concentrations in the SCE varied with a change in extract incorporated in the niosomes and SLNs. These variations were found to be statistically significant ($p < 0.05$). The average SCE withanolide A concentration due to the **SW** was not significantly different from that due to the **SE** ($p = 0.984$). This absence of a significant difference goes hand in hand with what was determined for withanolide A membrane release from these formulations.

Table D.9: Statistical analysis of tape-stripping data showing p-values for each comparison

Comparison	p-value
SCE vs. ED (**SW** – withanolide A)	0.018820*
SCE vs. ED (**S50** – withanolide A)	0.000204*
SCE vs. ED (**SE** – withanolide A)	0.000429*
SCE vs. ED (**S50** – withaferin A)	0.000001*
SW vs. **S50** vs. **SE** (SCE – withanolide A)	0.000000*
SW vs. **S50** vs. **SE** (SCE – withaferin A)	0.000000*
SW vs. **S50** (SCE – withanolide A)	0.000127*
SW vs. **SE** (SCE – withanolide A)	0.984374
SE vs. **S50** (SCE – withanolide A)	0.000127*
SW vs. **S50** (SCE – withaferin A)	0.002715*
SW vs. **SE** (SCE – withaferin A)	0.000127*
SE vs. **S50** (SCE – withaferin A)	0.000860*
NE vs. **N50** (SCE – withaferin A)	0.000000*
Withanolide A vs. withaferin A (SCE – **N50**)	0.000000*

* - a statistically significant difference exists

D.4 Conclusion

The results of the membrane release studies showed that withaferin A and withanolide A were released from the niosome and SLN formulations. Therefore these compounds would be available for diffusion into and through the skin. The different release characteristics of the formulations and the differences in skin samples were partly responsible for the differences that were observed for the skin diffusion studies.

During the 12 h skin diffusion study, low concentrations of withaferin A and withanolide A diffused into and through the skin. Possibly a longer time frame would have resulted in higher concentrations of compound being detected; since SLNs are said to allow for sustained release of encapsulated APIs into the skin as they must firstly diffuse through the solid lipid matrix (Godin & Touitou, 2012:523). Niosomes have also been conventionally used for topical delivery of APIs to the stratum corneum versus transdermal delivery of APIs (Manosroi *et al.*, 2013:474). In this study we were interested in topical delivery of APIs for the purpose of reaching and treating melanoma lesions prior to metastasis. The absence of withaferin A and withanolide A in the

receptor phase after the skin diffusion study indicated that the niosomes and SLNs mainly delivered the two marker compounds to the upper layers of the skin for a topical effect.

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Appendix E: In vitro anti-melanoma efficacy of Withania somnifera

E.1 Introduction

Cell culture is a laboratory technique that is used to isolate and maintain cells from biological tissues *in vitro* (Baydoun, 2010:38). This technique makes it possible to study the regulation of cell proliferation, cell differentiation and product formation (Freshney, 2000:1). Cells can thus be used for studying disease states, drug metabolism, drug pharmacology and protein synthesis. In the pharmaceutical field cell culture is mainly used to test the pharmacological and toxicological effects of active pharmaceutical ingredients (APIs) (Baydoun, 2010:38).

The anti-melanoma efficacy of withaferin A (**WFA**), withanolide A (**WNA**) and *Withania Somnifera* extracts was investigated in this study. *W. somnifera* extracts that were used are water extract, ethanol extract and 50% ethanol extract. *W. somnifera*, is a medicinal plant commonly known in Ayurveda as ashwagandha. This plant is known to have various medicinal properties such as anti-cancer, anti-ageing, anti-arthritic and immunostimulatory properties (Malik *et al.*, 2009:1508; Vel Szic *et al.*, 2014).

In vitro cell culture studies have been used to investigate the depigmenting (Nakajima *et al.*, 2011:1405), antiproliferative (Jayaprakasam *et al.*, 2003:130; Zhang *et al.*, 2012:1360), cytotoxic (Al-Fatimi *et al.*, 2005:356), pro-apoptotic and anti-cancer (Malik *et al.*, 2009:1498; Mayola *et al.*, 2011:1017) activities of *W. somnifera*. Conventional cell culture studies have major differences from what occurs in *in vivo* because they focus on what happens to cells but do not account for conditions and events that occur at a tissue level. The main limitations of *in vitro* cell culture are pharmacokinetics, metabolism, tissue responses and systemic responses (Freshney, 2005:360) therefore *in vivo* toxicity and efficacy studies are done in order to gain better insight on the biological effects of compounds.

Due to the limitations of *in vitro* studies the anticancer activities of *W. somnifera* have also been investigated *in vivo* on murine models. *In vivo* studies that have been done show that *W. somnifera* and its metabolites can increase tumour response to treatment (Kalthur *et al.*, 2009:97), inhibit metastasis (Leyon & Kuttan, 2004:119), radiosensitise cancer cells for radiotherapy (Kalthur & Pathirissery, 2010:372), stimulate and protect the immune system, and result in diminished tumour growth (Diwanay *et al.*, 2004:53; Malik *et al.*, 2009:1504). *In vivo* tests are criticised because the animals are often exposed to high and dangerous drug concentrations, which cannot be accurately extrapolated to human use. The inhumane issues associated with

animal testing are the main reason why a lot of energy is being invested in alternative drug toxicity and drug efficacy testing (Holmes *et al.*, 2010:15).

In order to avoid the unnecessary use of animals this study focused on *in vitro* cell culture studies in the conventional two dimensional (2D) and three-dimensional models (3D). Three-dimensional cell culture models are able to close the gap between *in vitro* and *in vivo* results (Li *et al.*, 2011; Mathes *et al.*, 2014:96). Skin cells grown in a 3D matrix behave and interact with other cells differently from cells growing on plastic in normal cell culture (Mathes *et al.*, 2014:96). These cells can mimic critical features of healthy or diseased human skin. Tumour spheroid models (Thoma *et al.*, 2014:33) and 3D skin models (Mathes *et al.*, 2014:81) for psoriasis, melanoma and wound healing have been used in order to investigate disease etiology, drug development and screening (Groeber *et al.*, 2011:353).

In this study, 2D and 3D cell culture were utilised to study the apoptosis inducing effect of *W. somnifera* on melanoma cells. Apoptosis is described as programmed cell death that is initiated by internal signals or due to inappropriate signals coming from the external environment (Kill & Faragher, 2000:282). If apoptosis is not well regulated this may result in diseases such as cancer, reperfusion injury and auto-immune diseases (Barisic *et al.*, 2003:151). Apoptosis is accompanied by characteristic morphologic and biochemical changes that lead to cell death (Pollard *et al.*, 2008:852). These changes can be probed and analysed using flow cytometry and fluorescence microscopy. *In vitro* cell culture studies were utilised to determine whether the crude plant extracts and marker compounds induce apoptosis in melanoma cells. An investigation into possible mechanisms of action was done. The roles of caspases, mitochondrial membrane potential and deoxyribonucleic acid (DNA) damage were investigated during the mechanistic studies.

E.1.1 Flow cytometry

Flow cytometry is an analytical technique which quantitatively analyses and measures the physical characteristics of single cells within a population. This technique uses fluorescence activated cell sorting (FACS) and measures the relative fluorescence on the cell surface and can in turn assemble cells carrying the required marker (Hudson, 2000:270).

Cells can also be separated according to differences detected by light scatter (forward scatter and side scatter). Forward scatter gives an indication of cell size or volume and side scatter correlates with how complex the cell is. Cell complexity refers to nucleus shape, size or amount of cytoplasmic granules and membrane roughness. Flow cytometry allows for analysing cell volume, morphology, DNA content, RNA content, chromosomes, proteins and cell surface antigens (Shapiro, 2003:3). Scattered and fluorescent light is collected by lenses, then beam splitters and filters direct the light to the appropriate detectors. The optical signal received is

processed into electronic signals that are displayed as graphs on a monitor (BD Biosciences, 2000:5; Freshney, 2005:244).

In this study a BD FACSVerse™ (BD Bioscience, Mountain View, CA) flow cytometer was used for all the flow cytometry analyses. A BD FACSVerse™ flow cytometer has a 2-laser, 6 colour configuration with a 488 nm laser and a 633 nm laser. In this study only the 488 nm laser was used, the corresponding filters and mirrors for the parameters analysed in this study are shown in Table E.1.

Table E.1 BD FACSVerse™ filters and mirrors for analysed parameters

Dyes/parameter	Application	Filters	Mirrors
Side scatter (SSC)	Live/dead cell discrimination and cell cycle	488/15	None
FITC/CellEvent®/YO-PRO®-1	Live cells/Casapse-3/7	527/32	507 Longpass (LP)
PI (Propidium Iodide)	Dead cells/DNA binding/ cell cycle	586/42	560 LP
PerCP/7-AAD/ SYTOX®AADvanced®	Dead cells/DNA binding/ cell cycle	700/56	665 LP

All the dyes that were used in this study are similar in that they detect apoptosis and they all bind to DNA but there are differences in the kits and what they measure. Cell preparation procedures also differed in that the TUNEL assay measured the “free” DNA broken strands in fixed cells while the membrane permeability assay used PI to measure DNA in living intact cells. Differences in cell preparation for each assay resulted in differences between the FSC/SSC scatter plots of the same cells.

E.1.2 Confocal microscopy

Confocal microscopy is an imaging system used in cell culture to restrict the illumination to a thin plane in the specimen. Instead of using a wide beam of light for illumination, a point of laser light is sharply focused in the x, y and z planes to excite fluorescent molecules (Pollard *et al.*, 2008:90). Cell events can therefore be imaged by tracking the distribution, relocation and staining intensity of fluorescent probes (Freshney, 2005:479). Confocal microscopy is used for viewing cells in a 3D culture system since it presents the cells in one focal plane and avoids interference that can be caused by cells that are not in the same focal plane (Freshney, 2005:70). This technique was used for viewing the cells that were in 3D culture.

E.2 Materials and Methods

E.2.1 Materials

All the materials used in the cell culture studies were sterile. The plastic consumables were procured from Separations (Johannesburg, South Africa) in sterile individual wrapping. Table E.2 shows the reagents that were used for the cell culture experiments. Experiments were carried out in the laboratory for molecular biology (LAMB, North West University) in which room temperature was maintained at 22 °C.

Table E.2 Reagents and assay kits used in the *in vitro* apoptosis studies

Material	Storage	Supplier	Batch #
Human melanoma cell line (skin origin): A375	Liquid nitrogen vapour phase	ATCC, USA	-
Human keratinocyte cell line: HaCaT	Liquid nitrogen vapour phase	University of Witwatersrand (donation)	-
Apo-BrdU™ TUNEL assay kit	5 °C; -20 °C	Thermo Fisher Scientific, Waltham, MA, USA	1513067; 1572496
Chromatin condensation & membrane permeability dead cell kit	5 °C	Thermo Fisher Scientific, Waltham, MA, USA	1252237
Vybrant MTT cell proliferation assay kit	5 °C	Thermo Fisher Scientific, Waltham, MA, USA	1558729
CellEvent® Caspase-3/7 green flow cytometry kit	5 °C	Thermo Fisher Scientific, Waltham, MA, USA	1583072
Mitochondrion membrane potential kit	-20 °C	Sigma Aldrich, St Louis, Missouri, USA	080002
XTT TOX 2	-20 °C	Sigma Aldrich, St Louis, MO, USA	SLBJ6046V
Hyclone™ Dulbecco's Modified Eagles Medium (DMEM)	5 °C	GE Life Sciences, Little Chalfont, UK	AZL196034
Hyclone™ Phosphate buffered saline 0.0067 M (PBS)	Room temperature	GE Life Sciences, Little Chalfont, UK	AAD201744
Biochrom Foetal Bovine Serum (FBS)	-20 °C	Merck Millipore, Billerica, MA, USA	0888B
Corning Matrigel®	-20 °C	Corning, New York, USA	3318554

E.2.2 Cell line selection and cell maintenance

In this study, one of the aims was to determine the anti-melanoma efficacy of *W. somnifera*. Therefore, A375 human melanoma and HaCaT human keratinocyte cells were selected for use in order to determine if *W. somnifera* had any selectivity for melanoma cells versus keratinocytes.

The A375 and HaCaT cells were cultured in 75 cm^2 T flasks in an incubator set at 37 °C and 5% CO_2. Cells were fed every second day and split every fourth day when they reached approximately 80% confluency. The complete culture media consisted of DMEM, 10% FBS and 1% PenStrep. Prior to any cell manipulation the cells were viewed under an inverted microscope in order to ascertain that they were healthy and to check for any irregularities. Periodically excess cells were frozen in 10% DMSO and stored in liquid nitrogen at -150 °C for long-term storage.

The trypan blue dye exclusion test was used to determine the viability of the cells. Only cells that were at least 90% viable were used in the apoptosis assays or frozen for future use. Dead cells are able to take up trypan blue while viable cells do not take up trypan blue. Cells (10 µl) were exposed to 10 µl of 0.4% trypan blue dye for 3 min and cell viability was detected using a Countess Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA). The results obtained included total number of cells, dead cells, live cells and %viability expressed as cells/ml.

E.2.3 Seeding cells in Corning® Matrigel® Matrix

Corning® Matrigel® Matrix is a solubilised basement membrane gel preparation which contains laminin, collagen IV, heparin sulphate proteoglycans, entactin/nidogen and other growth factors (Corning, 2016). For the cells to grow in a 3D environment cells were seeded in Matrigel® as it better mimics *in vivo* conditions. The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cytotoxicity test was conducted on cells grown in Matrigel® in order to determine the IC_{50} value and compare it with that obtained for cells grown in 2D culture.

The A375 and HaCaT cells were embedded in Matrigel® and grown overnight in 96-well culture plates for the cells to acclimatise to the conditions. A day before seeding cells in Matrigel®, pipette tips, Eppendorf tubes, cell culture plates and Matrigel® were placed at the back of the refrigerator overnight in order to maintain them at a low temperature. To embed cells in Matrigel® the following steps were followed in an aseptic environment.

- Matrigel®, pipette tips, cell culture plate and complete media were kept on ice or in the refrigerator at all times as Matrigel® starts to gelatinise at temperatures above 15 °C. Thus all items that were to get in contact with the Matrigel® were maintained at a cool temperature.
- The bottom of a pre-chilled 96-well pate was evenly coated with 10 µl of Matrigel®.

- The cell culture plate was placed in the incubator (37 °C and 5% CO_2) for 20 min. Care was taken to ensure that the gel did not get too dry.
- During the incubation period cells ($1.0x10^6$ cells/ml) were prepared from normal 2D culture and suspended in cell culture tubes using complete media.
- The cells in complete media were centrifuged for 5 min in cell culture tubes at 200 g and the media was discarded.
- The resulting cell pellet in the tubes was flicked to loosen it then Matrigel® was added to give a final cell concentration of approximately $1x10^6$ cells/ml.
- Cells suspended in Matrigel® (70 µl) were then added to the cell culture wells
- The cell culture plate was placed in the incubator (37 °C and 5% CO_2) for 30 min then 60 µl of media were added to each well.
- The seeded cells were allowed to acclimatise to the Matrigel® overnight before starting an experiment.
- Cells in Matrigel® were used for cytotoxicity (XTT) and apoptosis (FITC annexin V) assays.

E.2.4 MTT and XTT cytotoxicity assays (2D)

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay used to measure cell viability. The MTT dye (yellow) is reduced to a purple insoluble formazan salt when exposed to mitochondrial enzymes in living cells. DMSO can then be used to dissolve the formazan crystals and colour intensity of the dissolved formazan is taken to be proportional to the number of live cells in the population. The same is true for XTT except that the formazan end-product is soluble thus removing the need for a solubilisation step in the assay (Wilson, 2000:190; Freshney, 2005:366).

The MTT assay was performed in order to determine the A375 and HaCaT IC_{50} values of WFA, WNA, WFA+WNA, water extract, ethanol extract and 50% ethanol extract. A375 and HaCaT cells were also treated with placebo SLNs and 50% ethanol extract SLNs in order to determine whether the presence of drug delivery vesicles had an impact on the IC_{50} value. The XTT assay was used to determine the A375 and HaCaT IC_{50} values of 50% ethanol extract SLNs and placebo SLNs.

E.2.4.1 Day one

On the first day 100 µl of A375 or HaCaT cells in suspension were seeded in 96-well cell culture plates. The cell culture plates were placed in an incubator for 24 h for the cells to attach and acclimatise to the culture conditions. A cell concentration of $5x10^5$ cells/ml was used for all the

cytotoxicity assays giving a final number of 50 000 cells per well. Each experiment had three replicates and was repeated thrice to give a total of nine samples per treatment.

E.2.4.2 Day two

On day two WFA, WNA, WFA+WNA, water extract, ethanol extract and 50% ethanol extract suspensions were prepared in PBS (pH 7.4) and the cells were treated with 50 µl of the treatment suspensions. Each treatment was done in triplicate. Methanol treatment was used as a positive control for cytotoxicity and complete media treatment was used as a negative control. Cells were also treated with PBS (pH 7.4) in order to determine whether the solvent for the treatment suspensions had any influence on results obtained. In a trial run of the experiment cells were treated with 0.001, 0.01, 0.1, 1.0 and 10 mg/ml of pure compounds in order to obtain a narrower range to use in the subsequent attempts. The final concentrations that the cells were exposed to are 5, 25, 50, 75, 100, 150, 200 and 250 µg/ml. The WFA+WNA combination consisted of WFA and WNA in equal amounts to give each compound a final treatment concentration of 5, 25, 50, 75, 100, 150, 200 and 250 µg/ml. For the SLN formulation treatments cells were treated with 50% ethanol SLNs and placebo SLNs at final concentrations of 5, 25, 50, 75, 100, 150, 200 and 250 µg/ml.

E.2.4.3 Day three

On day three the protocols as described on manufacturer's guidelines were followed. The cells were centrifuged to ensure that cells were sitting at the bottom of the plate. For the MTT cytotoxicity assay, media was aspirated out and replaced with 100 µl of fresh DMEM then 10 µl of a 12 mM MTT stock solution was added to 100 µl of cell suspension and incubated at 37 °C for 4 h. After the incubation period 85 µl of the medium was removed from the wells and 50 µl of DMSO was added to each well. Absorbance was measured at 540 nm using a microplate reader. Prior to measuring, the contents of the plates were mixed to increase homogeneity of the samples.

When it came to the XTT assay, the prepared XTT labelling mixture (37.5 µl) was added to each well. The plates were then incubated at 37 °C for 4 h and absorbance was measured at 450 nm. The background absorbance was measured at a wavelength of 690 nm.

E.2.5 XTT cytotoxicity assay in Matrigel®

Cells (A375) were seeded in Matrigel® as described in Section E.2.3. Twenty four hours after seeding, the cells were treated with 70 µl of the treatment dispersions. The treatment dispersions resulted in final treatment concentrations of 16.7, 33.3, 66.7 and 670.0 µg/ml of WSW, WSE and WS50. On the third day (24 h after treatment) 55 µl of XTT solution was added to each well and the plates were placed in the incubator (37 °C) for 4 h. Absorbance was measured at 450 nm

with a reference wavelength of 690 nm. In order to investigate the influence of the Matrigel®, blank controls were included which contained Matrigel® alone and Matrigel® plus the XTT solution.

E.2.6 Apoptosis assays – 2D

The apoptosis inducing capabilities of *W. somnifera* were examined by the use of various apoptosis assays. These assays focused on DNA damage, influence of caspases, membrane permeability and mitochondrial membrane potential.

E.2.6.1 APO-BrdU TUNEL assay

During apoptosis nucleases that degrade nuclear DNA into small fragments are activated and these DNA breaks expose 3`-hydroxyl ends which can be labelled using Terminal Deoxynucleotide Transferase dUTP Nick End Labelling (TUNEL). The TUNEL assay detects DNA fragmentation by incorporating 5-bromo-2`-deoxyuridine (BrdU) into DNA at these DNA breaks and detecting the BrdU using anti-BrdU antibodies (Haughland, 2005:280; Wlodkowic *et al.*, 2011:14). In this case an anti-BrdU antibody labelled with AlexaFluor® 488 dye was used. Therefore detection of AlexaFluor® 488 represented the presence of DNA fragmentation which is characteristic of apoptosis. Cells were also stained with propidium iodide (PI) in order to determine total DNA content.

E.2.6.1.1 Day one

On the first day, 2 ml of $5x10^5$ cells/ml were seeded into a 6 well plate and left overnight in a 37 °C incubator to attach to the plate and acclimatise to the conditions. This gave a total of $1x10^6$ cells per well. The experiment was conducted on both A375 and HaCaT cells.

E.2.6.1.2 Day two

On the following day both the A375 and HaCaT cells were treated with the following treatments. Each treatment was done in triplicate. The pure compound treatment concentrations were based on the respective IC_{50} values obtained from the MTT assay. In contrast, the treatment concentrations for the extracts were deduced from the WFA+WNA IC_{50} since the crude extracts contained both WFA and WNA. This was done in order to warrant uniformity with respect to the WFA concentrations and allow for comparison on that level.

- Withaferin A (≈26.25 μg/ml)
- Withanolide A (≈80.61 μg/ml)
- Withaferin A + withanolide A (≈13.87 μg/ml each)
- *W. somnifera* water extract (equivalent to ≈13.87 μg/ml WFA)
- *W. somnifera* ethanol extract (equivalent to ≈13.87 μg/ml WFA)
- *W. somnifera* 50% ethanol extract (equivalent to ≈13.87 μg/ml WFA)

Positive (methanol) and negative (complete media) control treatments were also set up so as to allow for correct interpretation of results.

E.2.6.1.3 Day three

After the 24 h treatment period floating cells were transferred to relevantly labelled FACS tubes and the remaining cells were lifted from the plates using 0.25% trypsin-EDTA. Subsequent to removing the trypsin cells were re-suspended in 500 µl PBS (pH 7.4) and fixed for 15 min using 1% (w/v) paraformaldehyde on ice. The paraformaldehyde was removed and the cells were rinsed twice prior to letting them stand in ice cold 70% ethanol for 45 min.

The cells were washed twice with wash buffer and suspended in DNA labelling solution. The DNA labelling solution was prepared using 10 µl reaction buffer, 0.75 µl TDT enzyme, 8 µl BrdUTP and 31.25 µl of UltraPure water to give 50 µl of DNA labelling solution per sample. Thereafter the cells were incubated at 37 °C for 60 min then rinsed twice with rinse buffer and suspended in 100 µl of antibody staining solution. The samples were then incubated for 30 min in the dark at room temperature prior to adding 500 µl of PI/RNAse staining buffer to each sample. The final sample preparation step was to incubate the cells at room temperature in the dark for a further 30 min. To detect fluorescence from AlexaFluor® 488 and PI, samples were then analysed using the blue filter on the BD FACSVerse™. In order to set up gates and compensation parameters on the flow cytometer samples were prepared in which cells were unstained or stained with only a single dye and these samples were analysed prior to analysing the experimental samples.

E.2.6.2 Membrane permeability/dead cell assay

In this assay cells were stained with green YO-PRO®-1 dye and PI. YO-PRO®-1 is able to enter apoptotic cells due to their increased membrane permeability while PI is only able to penetrate into dead cells (Haughland, 2005:749; Wlodkowic *et al.*, 2011:12). Therefore apoptotic cells fluoresced green while dead cells primarily had red fluorescence due to PI and some green fluorescence. The first and second days of sample preparation proceeded as described in Sections E.2.6.1.1 – E.2.6.1.2.

On the third day cells were harvested using 0.25% trypsin-EDTA and transferred to appropriately labelled FACS tubes. The cells were washed in cold PBS (pH 7.4) and suspended in 1 ml of PBS at a concentration of approximately $1x10^6$ cells/ml. YO-PRO®-1 stock solution (1 µl) and PI stock solution (1 µl) were added to each sample tube and incubated on ice for 30 min. The cells were immediately analysed using 488 nm excitation on the flow cytometer. Unstained and single stained cell samples were firstly analysed in order to set up gates and compensation parameters.

E.2.6.3 Caspase-3/7 green flow cytometry assay

In the early stages of apoptosis caspase enzymes are activated and they are involved in reactions that are triggered by various pro-apoptotic signals. These caspase regulated reactions result in the cleavage of protein substrates and the disassembly of cells leading to cell death (Kill & Faragher, 2000:293). The reagent used in this assay is able to detect activated caspases 3 and 7 that are present in apoptotic cells. Activated caspases 3 and 7 cleave the caspase recognition sequence present in the reagent thus allowing the reagent to bind to DNA of apoptotic cells (Haughland, 2005:756). The caspase detection reagent fluoresces bright green thus distinguishing it from live and dead cells. The samples were prepared as described in Sections E.2.6.1.1 – E.2.6.1.2, the only difference was in that the cells were only treated with WFA, WNA and WFA+WNA.

Twenty-four hours after the treatment, the cells were harvested using 0.25% trypsin-EDTA and the cell concentration was adjusted to ≈1x10^6 cells/ml using complete media. The cell suspension (1 ml) was transferred to labelled FACS tubes and 1 µl of CellEvent® Caspase-3/7 Green Detection Reagent was added to each sample. Thereafter the samples were incubated in the dark for 60 min at room temperature. In the last 5 min of the incubation 1 µl of 1 mM SYTOX® AADvanced dead cell stain was added. The samples were then analysed using 488 nm excitation on the flow cytometry. The absorption and emission spectra of CellEvent® caspase detection reagent are 511nm/533nm and of the SYTOX® AADvanced® stain are 546nm/647nm. In order to set up gates and compensation parameters unstained and single stained cells were initially analysed.

E.2.6.4 Mitochondrion membrane potential assay

The mitochondria in live cells generate an electric potential across their membranes due to the electron transport chain. When apoptosis occurs the mitochondrial membrane potential collapses and the mitochondrial permeability transition pores open. The opening of these pores leads to the release of cytochrome c which is responsible for downstream apoptotic events. In normal cells the mitochondrial potential dye accumulates in the mitochondria and is seen as an increase in fluorescence whereas in apoptotic cells the mitochondrial membrane potential collapses resulting in reduced accumulation of dye which is seen as decreased fluorescence (Wlodkowic *et al.*, 2011:5). The excitation and emission peaks of the dye are 540 and 590 nm respectively.

The first and second days of sample preparation were similar to those described in Sections E.2.6.1.1 – E.2.6.1.2 except that cells were seeded in black 96 well plates with a clear bottom. Cells were seeded at a cell density of 8x10^4 cells/well (100 µl).

On the third day (24 h after treatment) the cell medium was removed and 100 µl of the dye loading solution was added. The dye loading solution was prepared by mixing 50 µl of the 200x mitochondrial potential dye with 10 ml of assay buffer A. The plates were then placed in the incubator (37 °C) for 20 min. After the incubation period 50 µl of assay buffer B were added to each well and the samples incubated at 37 °C for a further 30 min. Thereafter fluorescence intensity was measured using a Spectramax® microplate reader (Molecular Devices, Sunnyvale, CA, USA).

E.2.7 Apoptosis determination in 3D

Apoptosis assays were also carried out on cells in the Matrigel® 3D environment. The aim was to determine whether the 3D environment had any impact on the effects of *W. somnifera* on A375 cells.

E.2.7.1 Annexin V FITC apoptosis assay

When cells go through apoptosis phosphatidylserine (PS) is translocated from the inner leaflet of the cell membrane to the outer surface of the cell. The PS therefore acts as a marker for apoptosis and it's detection on the outer cell surface correlates with occurrence of apoptosis. Annexin V, a phospholipid binding protein with high affinity for PS, is used to detect apoptosis when conjugated to fluorescein (FITC) (Haughland, 2005:753; Wlodkowic *et al.*, 2011:10). In this assay cells going through apoptosis fluoresce green due to fluorescein while dead cells are stained with PI and therefore show red and green fluorescence.

On day one A375 cells were seeded in Matrigel® as described in Section E.2.3 and it was ensured that each well had a cell concentration of $1.5x10^6$ cells/ml. On the second day the cells were treated with the water, ethanol or 50% ethanol extracts and the respective SLN formulations. It was ensured that each treatment resulted in a final WFA concentration of ≈13.87 µg/ml. A negative control of treatment with complete media was used in this assay.

The Annexin V binding buffer was prepared on the morning of day three and stored in the refrigerator till required. The Annexin V binding buffer consisted of 10 833 µl of 12mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4 (HEPES), 1 820 µl of 1 M NaCl, 325 µl of 100 mM $CaCl_2$ and 22 µl of double-distilled water. The following sample preparation procedure was followed on the third day:

- Media from each well was transferred to relevantly labelled Eppendorf® tubes as it may have contained some floating cells.
- To each well, 200 µl of 5 mg/ml dispase was added and the cell culture plates placed on a shaker for 20 min in order to lyse the Matrigel®.

- The dispase/Matrigel® cell suspension was then added to the respective Eppendorf® tube and centrifuged for 10 min at 1000 rpm.
- The supernatant was discarded and the pellet was re-suspended in 1 ml of PBS.
- The PBS cell suspension was centrifuged for 5 min at 3000 rpm and the supernatant discarded.
- Annexin V FITC binding buffer (300 µl) was added to each tube then the contents of the Eppendorf® tube were transferred to respectively labelled FACS tubes.
- Annexin working solution (4 µl) and 10 µl of 50 µg/ml PI were added to each tube.
- The samples were mixed using a pipette and left to stand for 15 min at room temperature.
- Single-stained and unstained cell samples were analysed in order to set up gates and compensation parameters.
- All samples were analysed using 488 nm excitation on the BD FACSVerse™.

E.2.7.2 Seeding cells in Matrigel® in 4-well dish for microscopy analysis

Cells (A375) were seeded in Matrigel® then viewed using the confocal microscope on day one and thereafter viewed again on day ten. The following method was followed for seeding the cells.

i. Media was aspirated from a 25 cm^2 T-flask and 1 ml of 2 µM CellTracker™ red solution was added.

ii. The flask was placed back in the incubator for 20 min after which the CellTracker™ red was removed and the cells were rinsed twice with PBS (pH 7.4)

iii. A375 cells were harvested from the flask as per the normal method using 0.25% trypsin-EDTA.

iv. Cells were suspended in 1ml of complete media and counted using a haemacytometer.

v. $2x10^5$ cells were placed in an Eppendorf® tube and centrifuged for 5 min at 1000 rpm. Then 1000 µl of Matrigel® were added to the tube.

vi. A pre-chilled 4-well dish was evenly coated with 100 µl of Matrigel® and incubated for 20 min at 37 °C.

vii. The stained A375 cells in Matrigel® (250 µl) were added to each well in the dish and incubation continued for 20min

viii. Complete media (150 µl) was gently added to each well and the dish was placed in the incubator.

ix. Every second day the media added in step (viii) was removed and replaced with 150 µl of the treatment dispersions in complete media. This was continued till day ten.

Complete media and the 50% ethanol extract are the treatments that were used for the confocal analysis. Prior to viewing the cells on the tenth day, the cells in Matrigel® were exposed to 2 µM CellTracker™ red (250 µl/well) for 20 min in order to label the live cells. The cells were then rinsed twice with PBS (pH 7.4) to remove any excess dye. Thereafter the fluorescence of the cells was viewed using confocal microscopy.

E.3 Results and Discussion

E.3.1 MTT and XTT

Data from the cytotoxicity assays was analysed to calculate the 50% inhibitory concentration (IC_{50}). An IC_{50} value is used to indicate how effectively a particular compound inhibits a process such as the growth of a cell population (Wilson, 2000:212). It represents the concentration of compound that kills 50% of the cell population. To calculate the IC_{50}, data were log transformed and fitted with non-linear regression. The data were presented as mean ± SD. Figures E.1 – E.2 show the mean % cell viability that was obtained after 24 h treatment with each treatment dispersion.

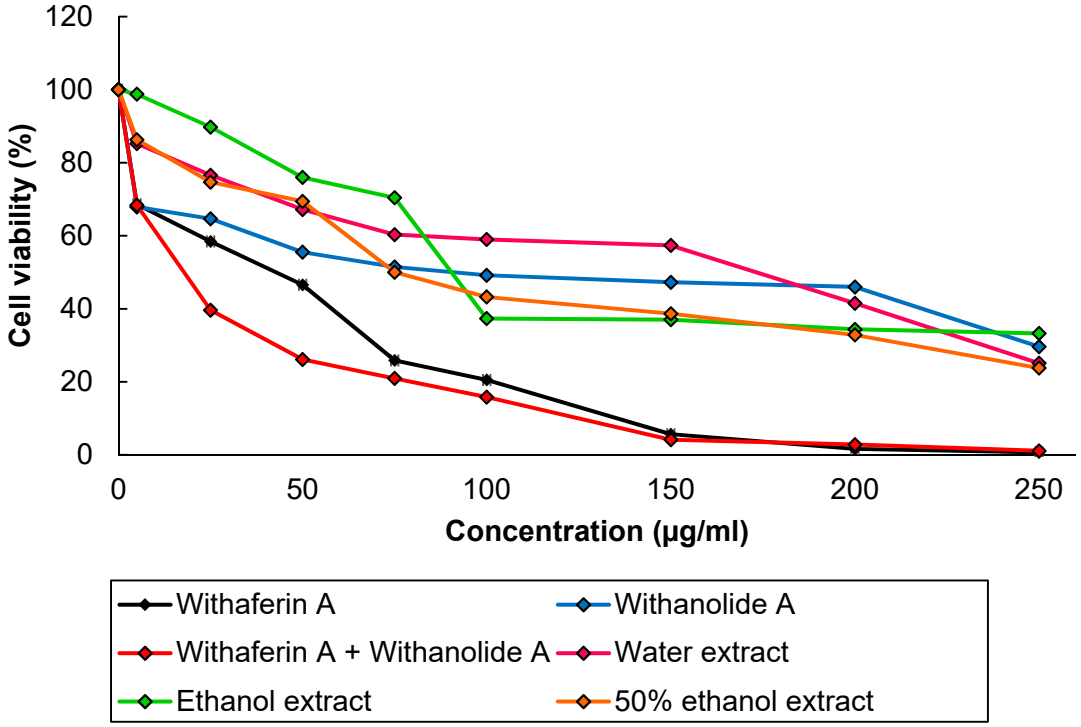


Figure E.1 Cytotoxic effects of pure compounds and crude plant extracts on A375 melanoma cells. The results are shown as the mean of 9 experiments. (n=9).

WFA and the WFA+WNA combination had the greatest inhibitory effect on A375 cells as compared to the other treatments. At the lower concentrations there was a synergistic effect when WFA and WNA were combined. WNA alone had a small effect on the A375 cell viability at

the low concentrations. The crude plant extracts showed less activity against the A375 cells as compared to pure withaferin A. This may have been due to the fact the crude plant extracts had a mixture of compounds and therefore had a lower constitution of the potent WFA as compared to the pure standard compound.

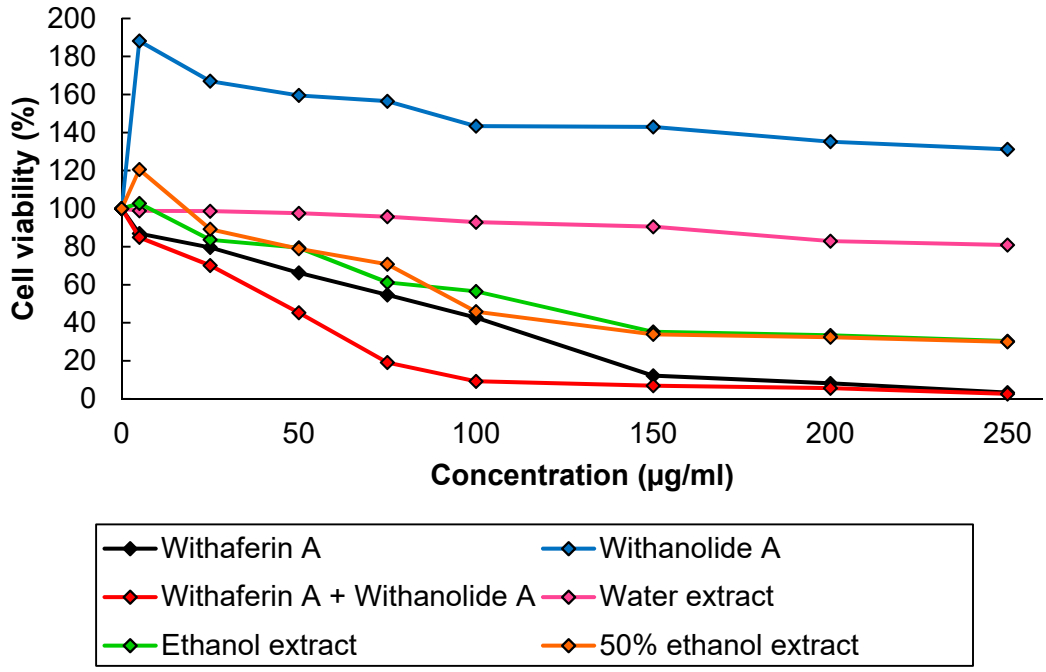


Figure E.2 Cytotoxic effects of pure compounds and crude plant extracts on HaCaT cells. The results are shown as the mean of 9 experiments (n=9).

As observed with the A375 cells, WFA and the WFA+WNA combination had the greatest inhibitory effect on HaCaT cells. Once again at the low concentrations the combination of WFA and WNA resulted in a synergistic inhibitory effect on cell viability. WNA treatment alone had no observed inhibitory effect on the viability of HaCaT cells, even at the high concentrations. A similar observation was made on the water extract treated HaCaT cells.

The cell viability results obtained were processed to calculate the IC_{50} values for each treatment on the two cell types. IC_{50} calculations were done using Graphpad Prism 7 (GraphPad Software, San Diego, CA, USA) on log transformed and normalised data. However, due to the minimal toxicity of WNA to HaCaT cells at the used concentrations an accurate IC_{50} could not be calculated for this treatment in HaCaT cells. Figure E.3 is a graphical representation of the IC_{50} values obtained. The IC_{50} values for all the treatments with crude plant extracts were calculated to give the WFA concentrations represented.

The pure compounds were found to have a cytotoxic effect which was relatively selective for A375 cells as reflected by the IC_{50} values. Selectivity for A375 versus HaCaT is shown by the HaCaT IC_{50} being greater than the respective A375 IC_{50}. This implies that a higher treatment

concentration in HaCaT cells is required to exert the same level of cytotoxicity in A375 cells as a low treatment concentration. Selectivity was calculated by dividing the HaCaT IC_{50} with the A375 IC_{50} with a high value indicating higher selectivity for A375 cells. In this case the selectivity index (x) value implied that the treatment was (x) times more selective for inducing cell death in melanoma cells vs keratinocytes. Selectivity indices (A375 vs. HaCaT) of each treatment have been tabulated in Table E.3.

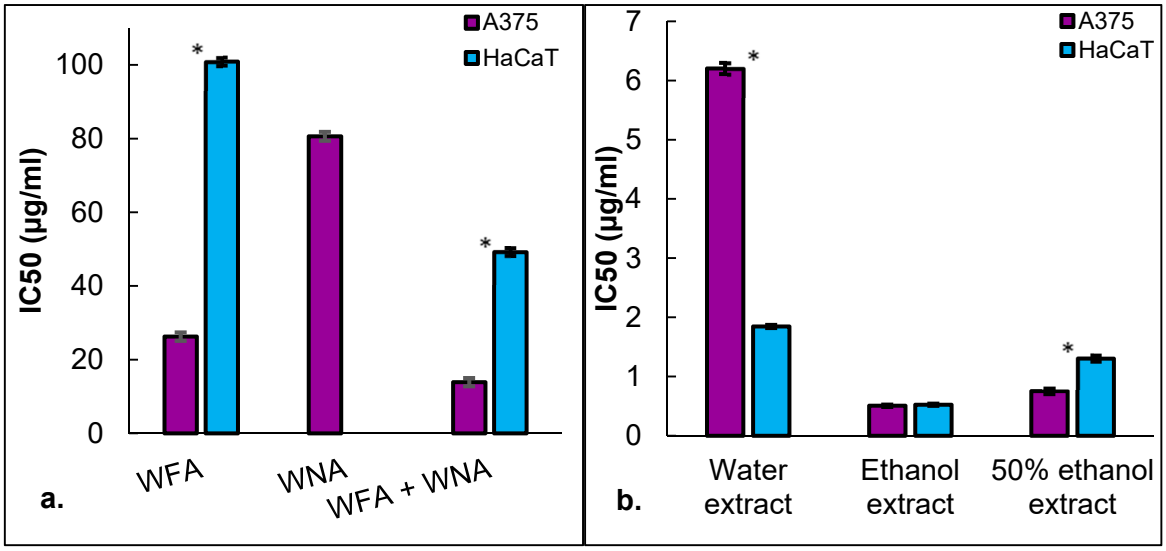


Figure E.3 IC_{50} va lues of the pure compounds (a) and IC_{50} values (WFA) of the plant extracts calculated using withaferin A content in extracts (b). * - A375 differed significantly from HaCaT. Values are shown as mean ± SD of experiments done on cells grown in complete media only (n=9).

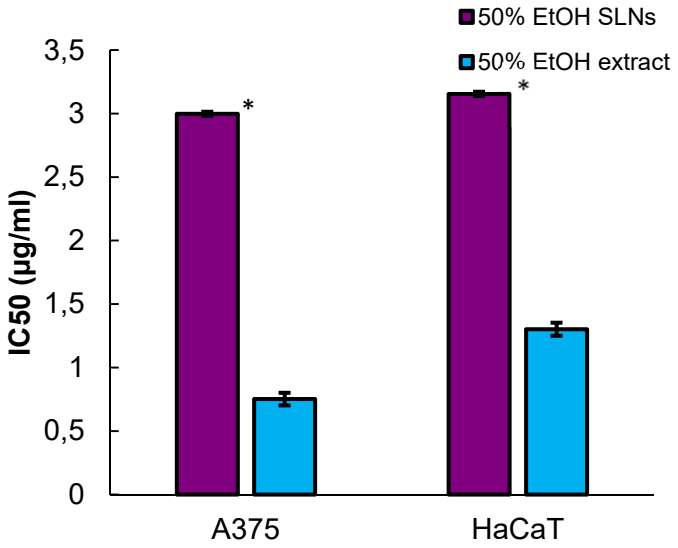


Figure E.4 Comparison of IC_{50} values of the 50% ethanol extract and its respective SLN formulation calculated using WFA content in the extracts. * - SLNs differed

significantly from crude extract. Values are shown as mean ± SD of experiments (n=9).

The plant extract treatments had somewhat inconsistent cytotoxicity results in that, the water and 50% ethanol extracts were selective for melanoma cells while the ethanol extract showed almost no selectivity. Generally the most potent extract was the ethanol extract. This implies that the ethanol extract comprised of compounds that are highly cytotoxic, to both A375 and HaCaT cells. Encapsulation of the 50% ethanol extract in SLNs resulted in a higher IC_{50} suggesting that the SLNs made the keratinocytes and melanoma cells more resistant to the cytotoxic effects of the plant extract. Contrary to most studies in which SLNs enhanced the efficacy of the cytotoxic agent the SLN formulation in this study shielded the cells from the harmful effects of the encapsulated extract.

Table E.3 Selectivity of treatments for A375 cells versus HaCaT cells calculated as ratio of HaCaT IC_{50} to A375 IC_{50}

Treatment	Selectivity Index (complete media – 2D)	Selectivity Index (Matrigel® – 3D)
Water extract	3.362	0.559
Ethanol extract	1.035	0.559
50% ethanol extract	1.731	1.341
Withaferin A	3.844	2.779
Withanolide A	*	*
Withaferin + Withanolide	3.545	2.846

*- selectivity not calculated due to lack of data on cytotoxicity of withanolide A to HaCaT cells.

The plant extract treatments did not show much selectivity for melanoma cells versus the normal keratinocytes except the water extract treatment which was selective for melanoma cells in 2D culture. On the other hand all the pure compounds had selectivity for the melanoma cells. Therefore the pure compounds have a better potential for use as anti-cancer agents as they would cause more damage to cancer cells versus normal skin cells. However the ideal cytotoxic agent should have a much higher selectivity for cancer cells versus normal cells so as to ensure minimal damage to normal cells and reduce side effects.

The pure compounds had IC_{50} values that reflected selectivity for inducing cytotoxicity in A375 cells in Matrigel® while the water and ethanol extracts were more selective for HaCaT cells. Only the 50% ethanol extract was slightly selective for A375 cells versus HaCaT cells. Presence of Matrigel® did not cause a major change in the WFA, WNA and WFA+WNA IC50 values in A375 cells. The concentration of WFA required to inhibit 50% growth (in 2D and 3D) was lower with the plant extracts as compared to pure WFA which reflected that WFA was not the only compound possessing anti-melanoma properties in the crude extracts. There may have been other highly

potent compounds besides WFA present in the extracts or the unidentified compounds worked synergistically with WFA resulting in a lower IC_{50}.

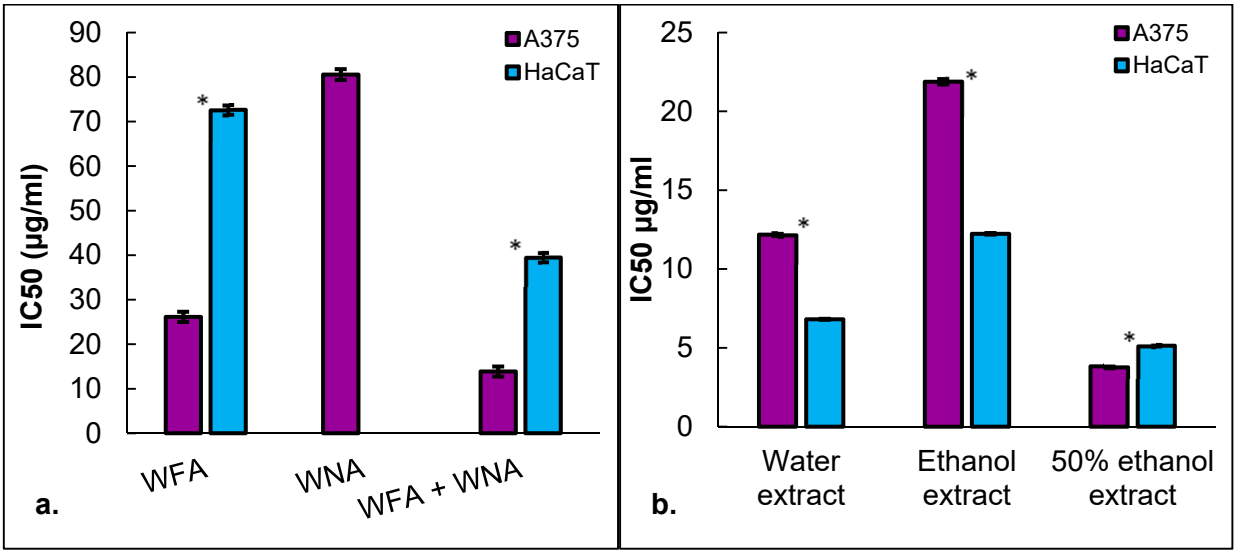


Figure E.5 IC_{50} values of the pure compounds (a) and withaferin A IC_{50} values of the plant extracts calculated using withaferin A content in extracts (b). * - A375 differed significantly from HaCaT. Values are shown as mean ± SD of experiments done on cells grown in Matrigel® (n=9).

When it came to the plant extracts, the IC_{50} values of all the treatments on cells in Matrigel® were much higher than those of cells in complete media only. This may have been due to a protective effect being rendered by the extracellular proteins present in Matrigel® that were absent in the complete media. This proposed protective effect of the Matrigel® coupled with the plant extracts seemed enhanced in A375 cells as shown by the marked decrease in selectivity indices as compared to those of cells grown in normal complete media.

E.3.2 Apo BrdU TUNEL assay

This assay was used to detect the occurrence of apoptosis by detecting DNA strand breaks and changes in light scattering properties. The light scattering properties of cells going through apoptosis are different from those of normal live cells due to changes in cell morphology. Cells going through apoptosis show a decrease in intensity of forward light scatter (FSC) due to dehydration which results in cell shrinkage. The side scatter signal (SSC) may remain unchanged or it may increase in early apoptosis due to increased complexity caused by condensation of nucleus and cytoplasm. As apoptosis proceeds cells become smaller and SSC also decreases. Conversely a decrease in FSC and an increase in SSC is an indicator of early apoptosis while a decrease in FSC accompanied by a decrease in SSC is an indicator of late apoptosis (Wlodkowic *et al.*, 2011:4). Light scatter parameters however, cannot be used alone to indicate whether apoptosis is occurring or not and are therefore used in conjunction with the assessment of other

markers of cell death. In this study it was observed that there was a decrease in both FSC and SSC for the melanoma cells after all the treatments (see Figure E.6) thus indicating late apoptosis or necrotic cell death.

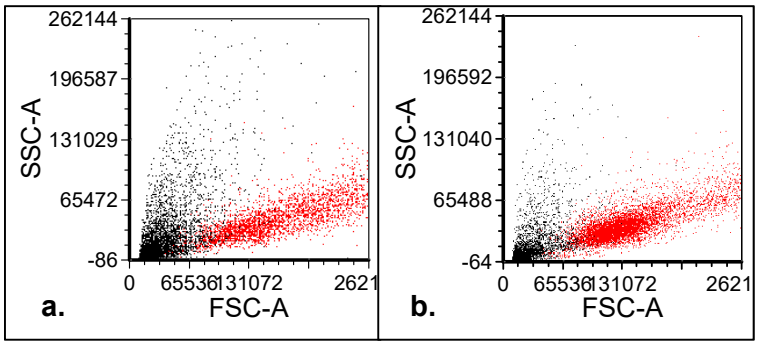


Figure E.6 Representa tive scatter plots showing forward scatter and side-scatter as indicators of cell death in a) live untreated cells and b) treated cells.

In Figures E.7 – E.9 total DNA amounts are represented by PI. The small amounts of black cells are known as singlets and were excluded from the analysis. Below is the gating strategy that was used for analysis of both the A375 and HaCaT cells.

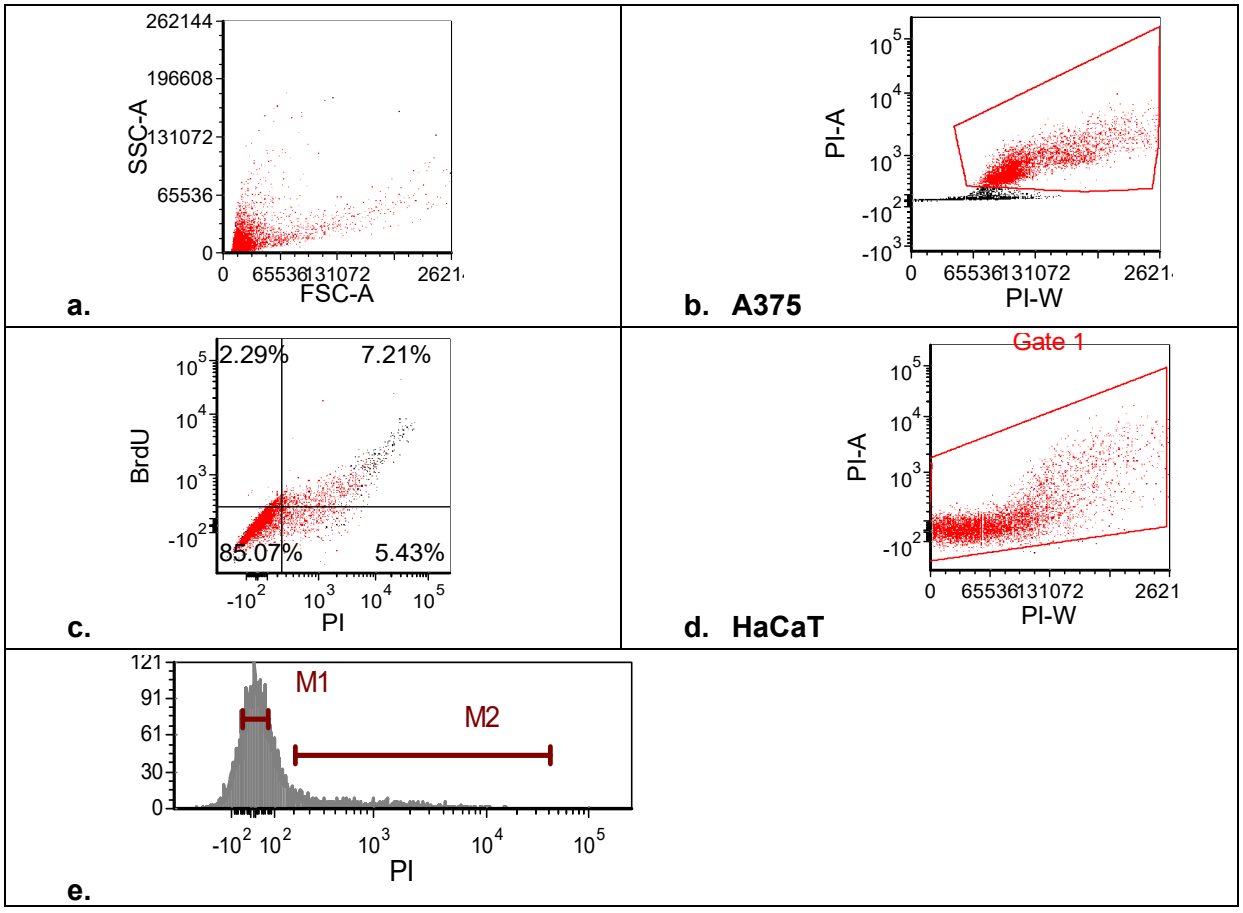


Figure E.7 Representative dot-plots (a-d) and histogram (e) indicating the gating strategy that was used to detect apoptotic cells and total DNA content in the TUNEL assay.

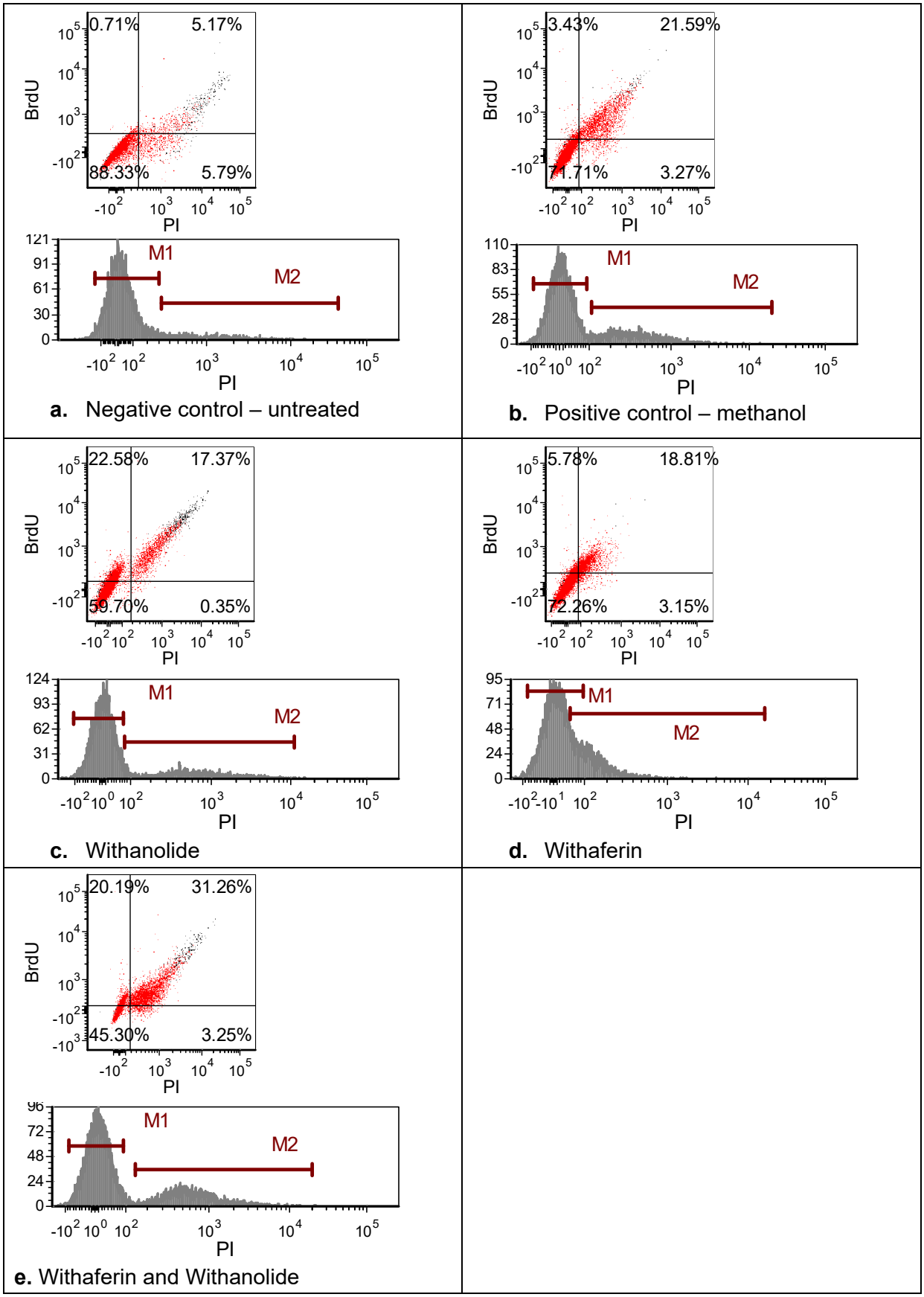


Figure E.8 Representative dot-plots and histograms of HaCaT cells treated with the different plant compounds showing the live and apoptotic cells as detected using a BrdU TUNEL assay.

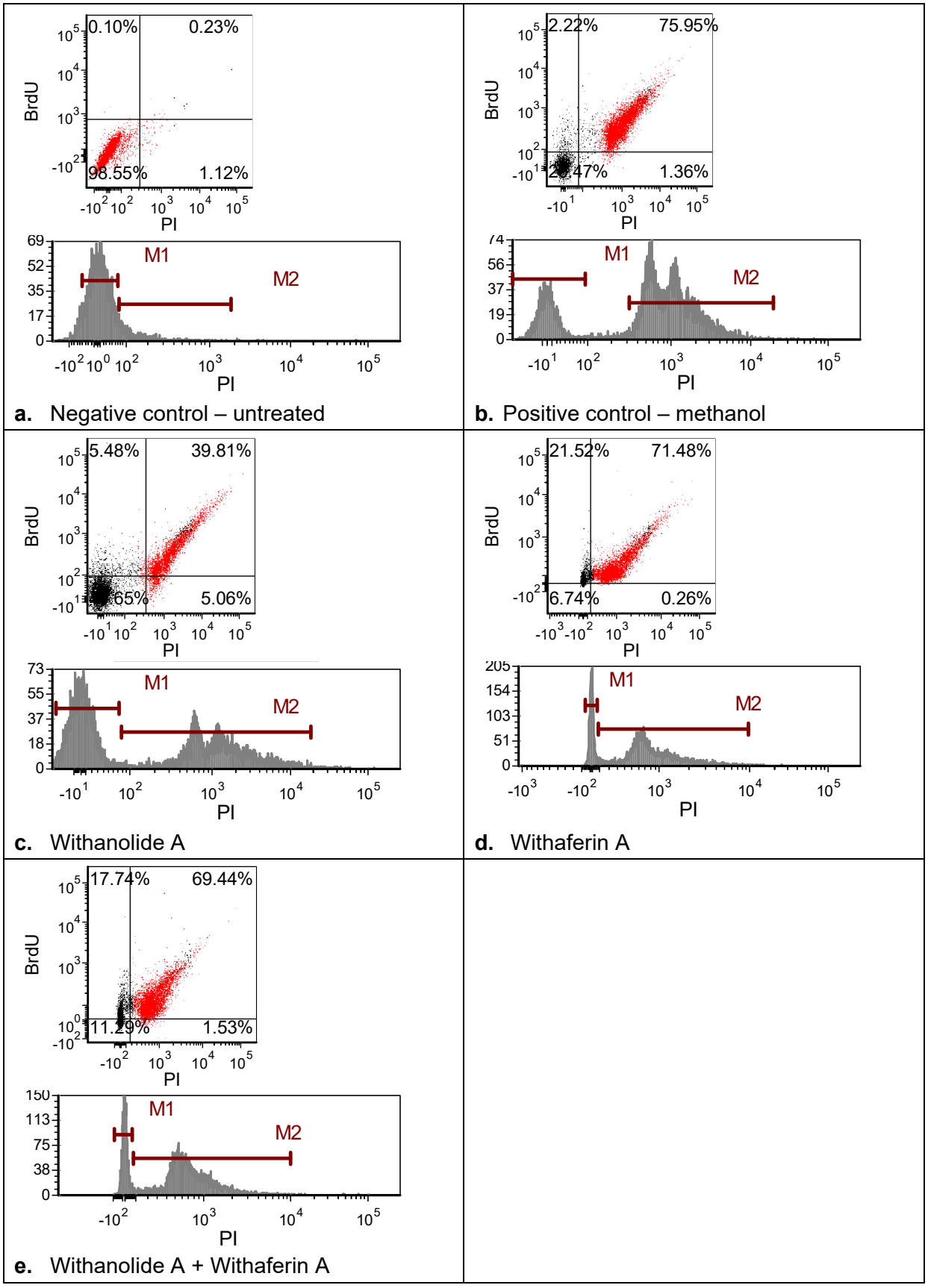


Figure E.9 Representative dot-plots and histograms of A375 cells treated with the different plant compounds showing the live and apoptotic cells as detected using a BrdU TUNEL assay.

The upper right quadrant in the BrdU/PI dot-plots denotes the percentage of cells that were positive for FITC-BrdU fluorescence which is characteristic of apoptotic cells in this assay. Whereas the histograms denote the total amount of DNA. The difference in the HaCaT and the A375 cells in terms of the total DNA content can be observed by taking a look at the PI histograms.

HaCaT cells showed very little apoptosis after treatment with the plant extracts. The greatest apoptosis was observed after treatment with the combination of WFA and WNA thus showing the synergistic effect of the combination. The pure compound treatments all resulted in a higher occurrence of apoptosis as compared to the crude plant extracts.

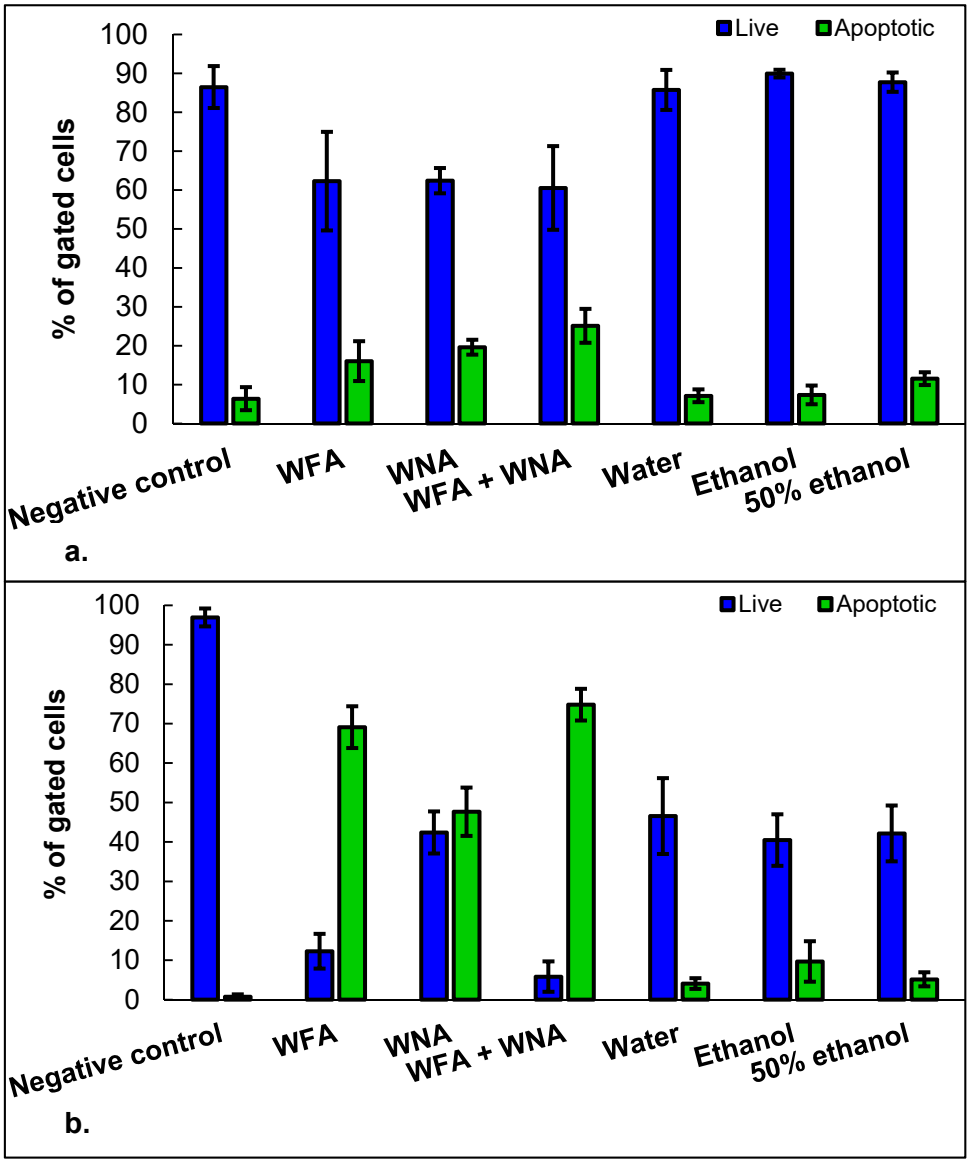


Figure E.10 Bar graph of average (%) live cells and apoptotic cells by detecting DNA fragmentation after treatment of HaCaT (a) and A375 (b) cells. Values are shown as mean ± SD.

The Apo-BrdU TUNEL assay showed that the pure compounds had a greater tendency to induce apoptosis in the A375 cells as compared to the plant extracts (shown in Figure E.10). Control

cells presented with a basal level of apoptosis which was lower than that in the treated cells. A375 cells treated with plant extracts also had a high percentage of dead cells (not shown on graph) which may have been due to necrosis or other cell death mechanisms.

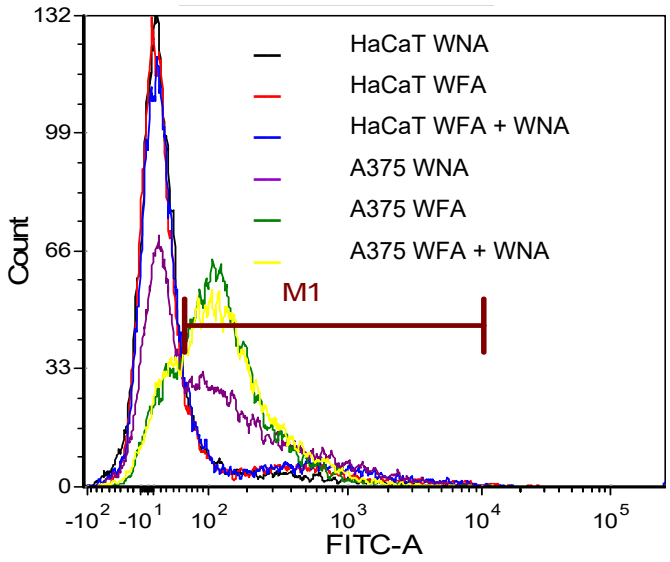
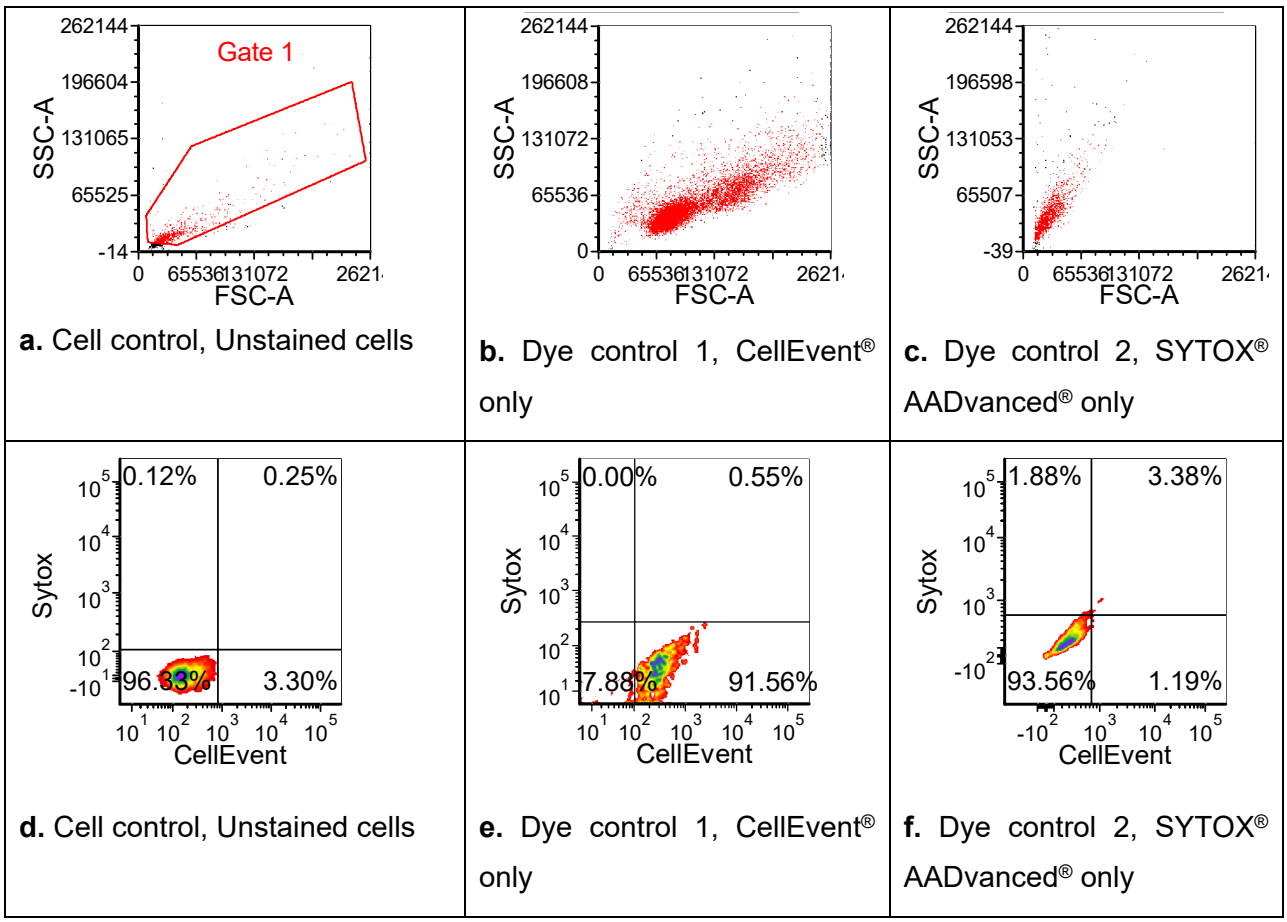


Figure E.11 Overlay histogram showing selectivity of the pure compounds to induce apoptosis in A375 cells vs HaCaT cells.

Apoptosis occurred after all the treatments but there was a greater occurrence of apoptosis in A375 cells as opposed to the HaCaT cells (Figure E.11). This confirms that most of the treatments had relative selectivity for inducing cell death in melanoma cells as shown by the results of the cytotoxicity assays. Results were also analysed using fluorescence intensity. In this assay the mean fluorescence intensities of WNA, WFA and WFA + WNA treated HaCaT cells were 742.82, 250.61 and 669.55 respectively. The respective fluorescence intensities of the A375 cells were 1228.45, 832.25 and 658.79. This revealed that the WNA and WFA treatments were selective for A375 while the WFA + WNA treatment did not have much selectivity.

E.3.3 Caspase 3/7 apoptosis assay

Representative dot-plots, density plots and histograms indicating the gating strategy used to distinguish apoptotic cells from necrotic cells using caspase-3/7 are shown in Figure E.12. The FSC/SSC dotplots gave an indication of the live cells that can be separated (gated) from debris in the lower left corner. The CellEvent®/SYTOX density plots discriminate between live, necrotic and apoptotic cells based on fluorescence.



a. Cell control, Unstained cells

b. Dye control 1, CellEvent® only

c. Dye control 2, SYTOX® AADvanced® only

d. Cell control, Unstained cells

e. Dye control 1, CellEvent® only

f. Dye control 2, SYTOX® AADvanced® only

Figure E.12 Representative (a-c) dotplots of FSC vs SSC and (d-f) density plots of CellEvent® vs SYTOX® indicating the gating strategy that was used to distinguish apoptotic cells from necrotic cells using caspase-3/7.

Gate 1 was set around a control population of cells, containing no dyes to locate the live population of cells unaffected by the staining procedure. The same gate is visible in all the other dot plots as the red cell population (although the gate itself is not shown in the figure). The SSC gives an indication of the cell viability of the cells and all cells in the upper half of the FSC/SSC plot are considered dead and therefore left out of the analysis. It is clearly visible in Figure E.12 (a-c) that the dyes had an effect on the morphology of the cells, but not on the viability. In the cell control density plots (d-f) the quadrants were placed to exclude the debris in the lower left corner. In this representative plot the cells contained 91.56% viable cells. Again it can be observed that the dyes influenced the morphology of the cell populations, but not the viability. For the experimental data only the density plots were used to discriminate between the populations of interest. The same gating strategy was used for analysing both the A375 and HaCaT cells.

The experimental samples from the caspase 3/7 assay were then analysed using the gate that was setup as described above. A comparison was made between live, dead and apoptotic cells for each treatment on A375 and HaCaT cells. Representative density plots and bar graphs of results are shown in Figure E.13.

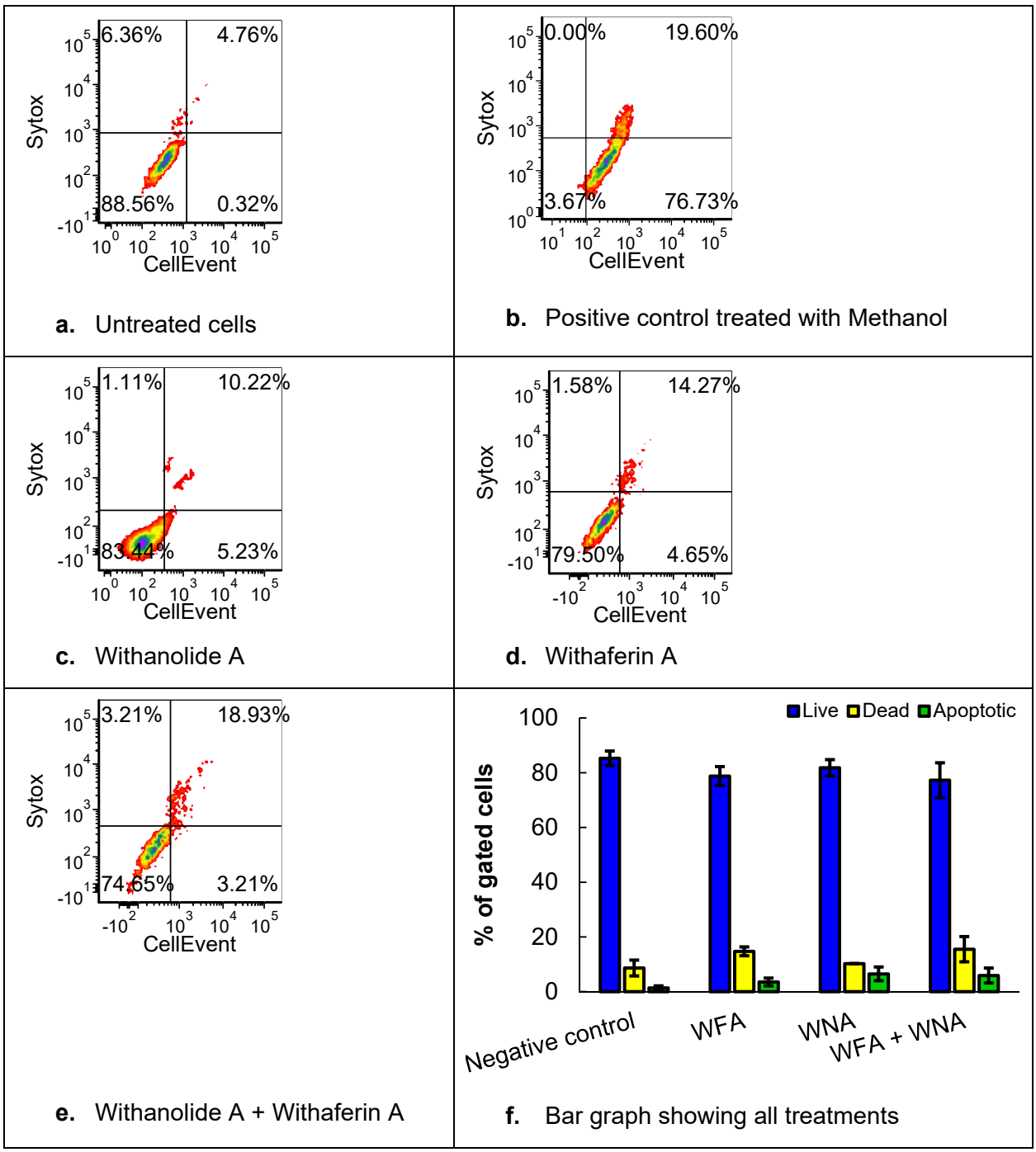


Figure E.13 Representative density plots of HaCaT cells treated with the different plant compounds (a-e) and bar graph showing the live, dead and apoptotic cells as reflected by activity of caspases 3/7 (f). Values are shown as mean ± SD (n=3).

The lower left quadrant represents the live cells (negative for both dyes), the lower right quadrant represents the apoptotic cells (positive for CellEvent® only) and the upper right quadrant represents the necrotic cells (positive for both CellEvent® and SYTOX® AAdvanced®. In Figure E.13 it is evident that the pure compounds resulted in minimal apoptosis or necrotic cell death in the HaCaT cells. This is in line with results that were obtained from the MTT assay. All the

experimental samples resulted in an average of more than 75% live cells after the 24 h treatment period. There was minimal caspase 3/7 activity that was detected from the treated keratinocytes.

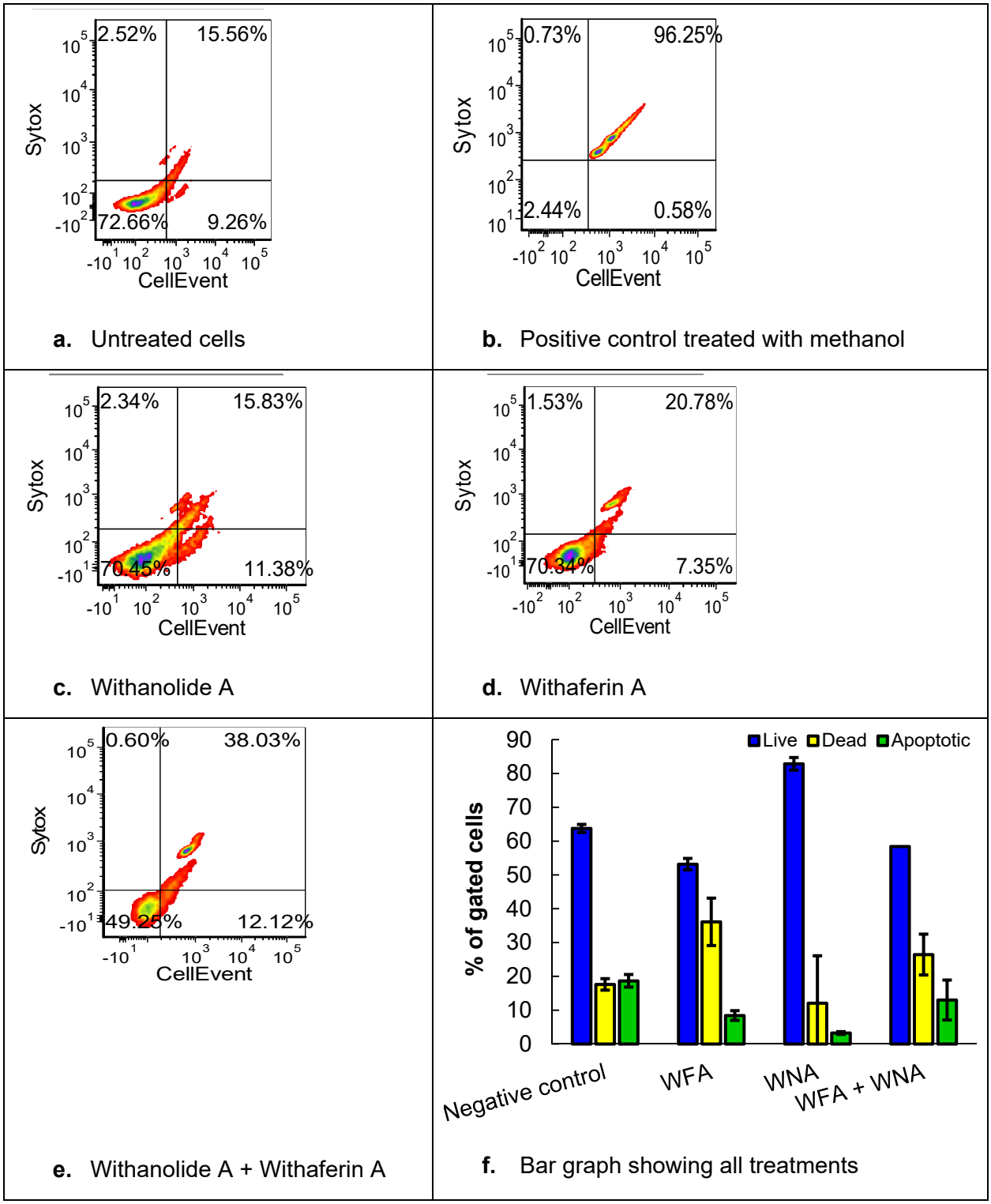


Figure E.14 Representative density plots of A375 cells treated with the different plant compounds (a-e) and bar graph showing the live, dead and apoptotic cells as reflected by activity of caspases 3/7 (f). Values are shown as mean ± SD (n=3).

The WFA and WFA+WNA treatment resulted in the highest average percentage of dead A375 cells. All the treatments resulted in a higher percentage of dead and apoptotic cells in melanoma cells as compared to keratinocytes.

E.3.4 Membrane permeability

The same gating strategy as that which was used for the caspase assay was used for the membrane permeability assay. A gate was set around the viable cell population (HaCaT cells) and the fluorescence was plotted on a density plot, indicating the percentage cells. YoPro® dye enters apoptotic cells and a large increase in green fluorescence separates the apoptotic cells (lower right quadrant) from the live cells (lower left quadrant). PI is unable to enter the apoptotic cells and is only able to bind to the DNA once the cell membrane has been damaged. With this particular assay the necrotic cells form a distinct diagonal population due to the loss of membrane structure. Addition of the dye did not change the cell population in the FSC/SSC plot.

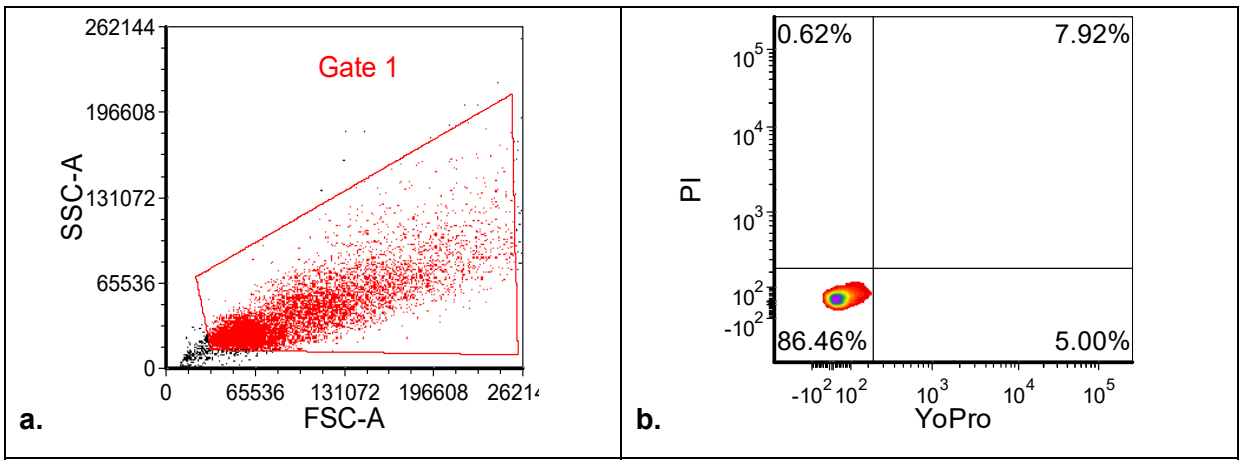


Figure E.15 Representative dotplots of FSC vs SSC (a) and a density plot of YOPRO® vs PI (b) indicating the gating strategy that was used to distinguish apoptotic cells from necrotic cells using differences in membrane permeability.

Figures E.16 – E.17 give a graphical presentation of the results that were obtained after evaluating the membrane permeability of keratinocytes and melanoma cells treated with pure compounds and plant extracts. It is evident that the plant extracts (contained withaferin A equivalent to that in the combination (WFA + WNA) treatment) had a greater effect on the membrane permeability of the cells.

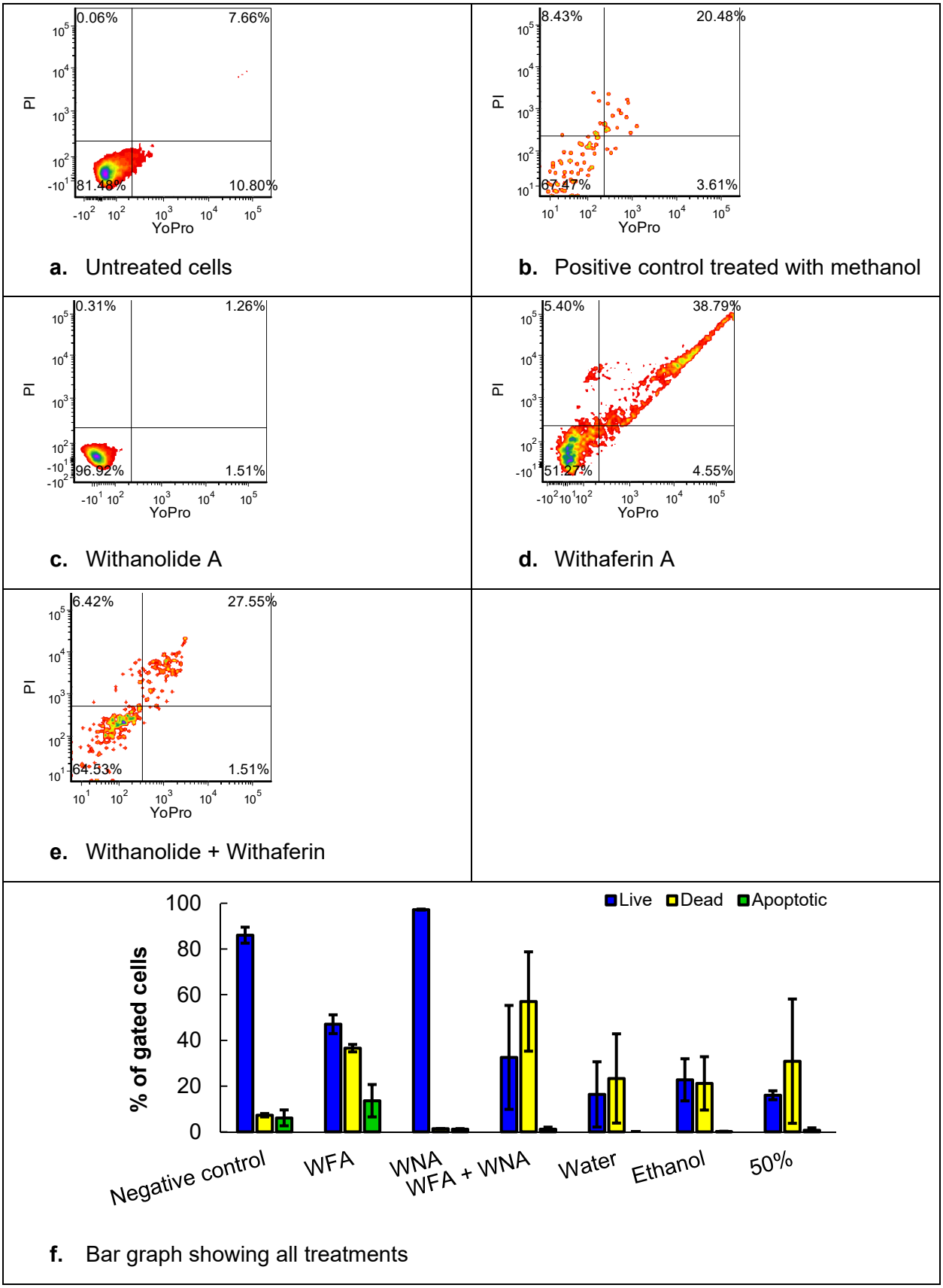


Figure E.16 Representative density plots of HaCaT cells treated with the different plant compounds (a-e) and bar graph showing the live, dead and apoptotic cells according to membrane permeability (f). Values are shown as mean ± SD (n=3).

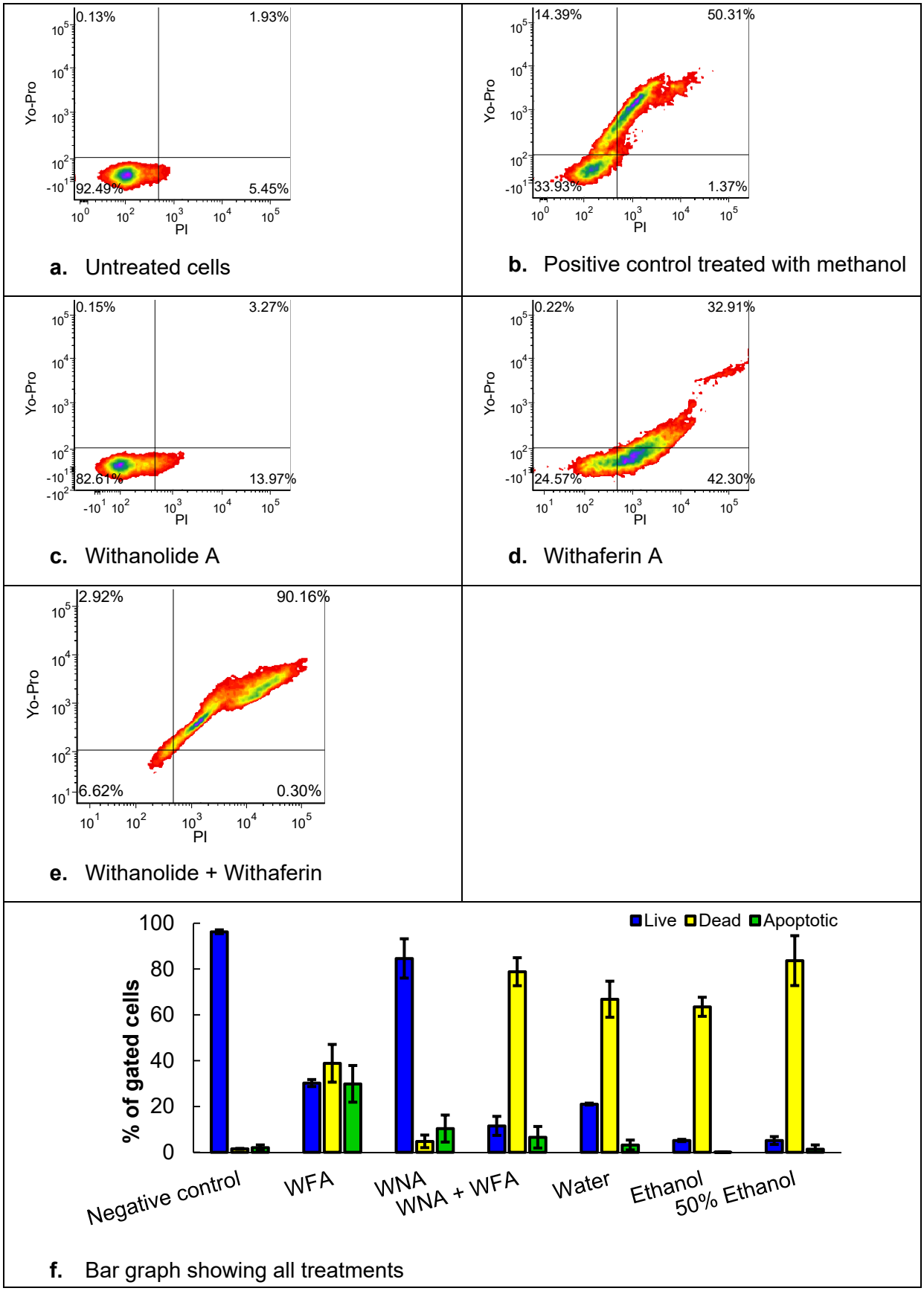


Figure E.17 Representative density plots of A375 cells treated with the different plant compounds (a-e) and bar graph showing the live, dead and apoptotic cells according to membrane permeability (f). Values are shown as mean ± SD (n=3).

The plant extracts and the WFA+WNA treatments resulted in a very high percentage of apoptotic and dead cells. However, the results do not clearly distinguish between cell death due to apoptosis and necrotic cell death. To overcome this challenge different treatment durations could have been used in a preliminary experiment in order to determine the optimum time frame for use in the assays. The results do show that although the treatments were cytotoxic to both A375 and HaCaT cells the effect on membrane permeability was relatively selective for A375 cells.

E.3.5 Mitochondrion Membrane Potential

The lowering of the mitochondrion membrane potential is taken as an apoptosis marker. In this assay as the mitochondrion membrane potential decreases the ability of the JC-1 dye to accumulate within cells reduces and this is seen as a decrease in mean fluorescence intensity (MFI). Therefore live cells were expected to have a high MFI while cells going through apoptosis where expected to have a low MFI. Percentage MFI was calculated as a function of the live cells MFI which was taken to be 100%. The results are graphically presented in Figure E.18

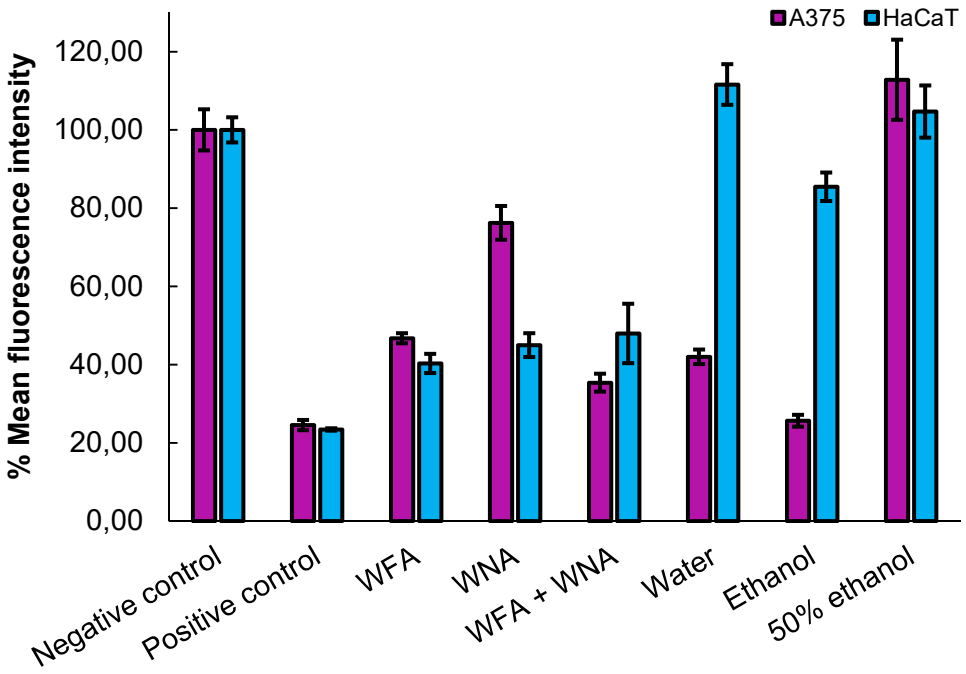


Figure E.18 Bar graph showing the percentage mean fluorescence intensity of JC-1 as a measure of mitochondrion membrane potential. Values are shown as mean ± SD for all the treatments (n=3).

The mitochondrion membrane potential reflected that apoptosis did occur in all the cell treatments, with the least occurring in the plant extract treatments on the HaCaT cells. The 50% ethanol treatment on the A375 cells also reflected minimal occurrence of apoptosis. WFA, WFA+WNA, water extract and ethanol extract treatments were selective for inducing a decrease in the mitochondrion membrane potential of A375 cells as compared to HaCaT cells.

E.3.6 Annexin V FITC apoptosis assay

Single stained cells (Annexin V only or PI only) and unstained cells were used for setting up compensation parameters and gating of the cell population. Gated cells are represented by the red dots in the dot-plots. Untreated cells were used as the negative control. The results showed that the untreated cells had a high percentage of dead (31.5%) and apoptotic (25.3%) cells after 24 h in Matrigel®. This may have been due to the cells not thriving in the Matrigel® because of the high density of cells that were in each well. Another concern is the dispase that was required to lyse the Matrigel®. The melanoma cells were in direct contact with dispase for 20 min and dispase can be slightly toxic to cells at high concentrations. This contact time may have affected the cells and further damage may have occurred to the already vulnerable cells during the 10 min centrifuge. The combined effect could have caused the reduced cell viability as the cells did not have time to recover from the dispase exposure. The cell recovery method from Matrigel® may need improvement so as to avoid unnecessary damage to cells.

Treated cell samples however presented with a greater occurrence of necrotic and apoptotic cell death as compared to the untreated cells thus showing that the treatments were effective. With all the treatments there was a higher occurrence of necrosis as compared to apoptotic cell death. The untreated cells (41.59%) had the highest percentage of live cells followed by cells treated with water extract SLNs (30.43%), ethanol extract SLNs (27.14%), water extract (16.70%), 50% ethanol extract SLNs (11.62%), 50% ethanol extract (7.31%) and lastly the ethanol extract (5.64%). These results reflected that SLNs may have once again proffered a somewhat protective effect on the melanoma cells as the SLN encapsulated extracts resulted in a higher percentage of live cells as compared to the respective crude extract alone. This is in line with the results that were obtained for 50% ethanol and 50% ethanol SLNs XTT results, whereby the crude extract had a greater toxicity than the SLN encapsulated extract.

With respect to the percentage apoptotic cells the SLN encapsulated extracts resulted in a lower percentage of cells going through apoptosis as compared to the crude extracts with the exception of the water extract. The 50% ethanol extract (39.58%) had the highest percentage of apoptotic cells followed by 50% ethanol extract SLNs (35.95%), ethanol extract (35.55%), untreated (25.34%), water extract SLNs (24.66%), ethanol extract SLNs (22.83%) and lastly water extract (13.98%). There however was a high percentage of dead cells in addition to the apoptotic cells as shown in Figure E.19.

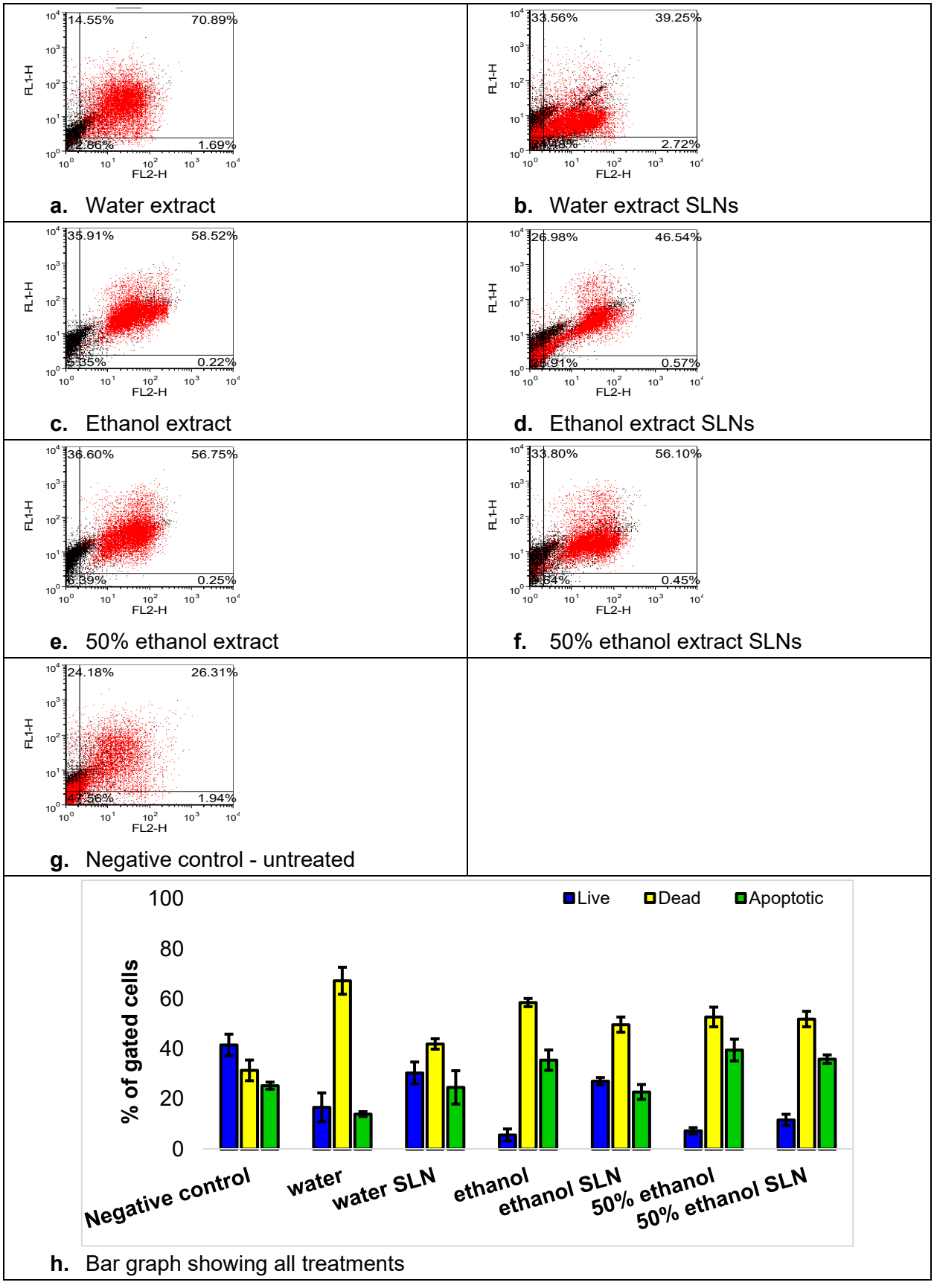


Figure E.19 Representative dot plots of A375 cells in Matrigel® treated with the crude extracts and SLN encapsulated crude extracts (a-e) and bar graph showing the live, dead and apoptotic cells (f). Values are shown as mean ± SD (n=3).

E.3.7 Confocal microscopy imaging

Melanoma cells were seeded in Matrigel® and treated with the 50% ethanol extract. Images were taken using a confocal microscope a day after seeding and 10 days after seeding. There are differences that can be seen between untreated and treated cell populations after the 10 day treatment period.

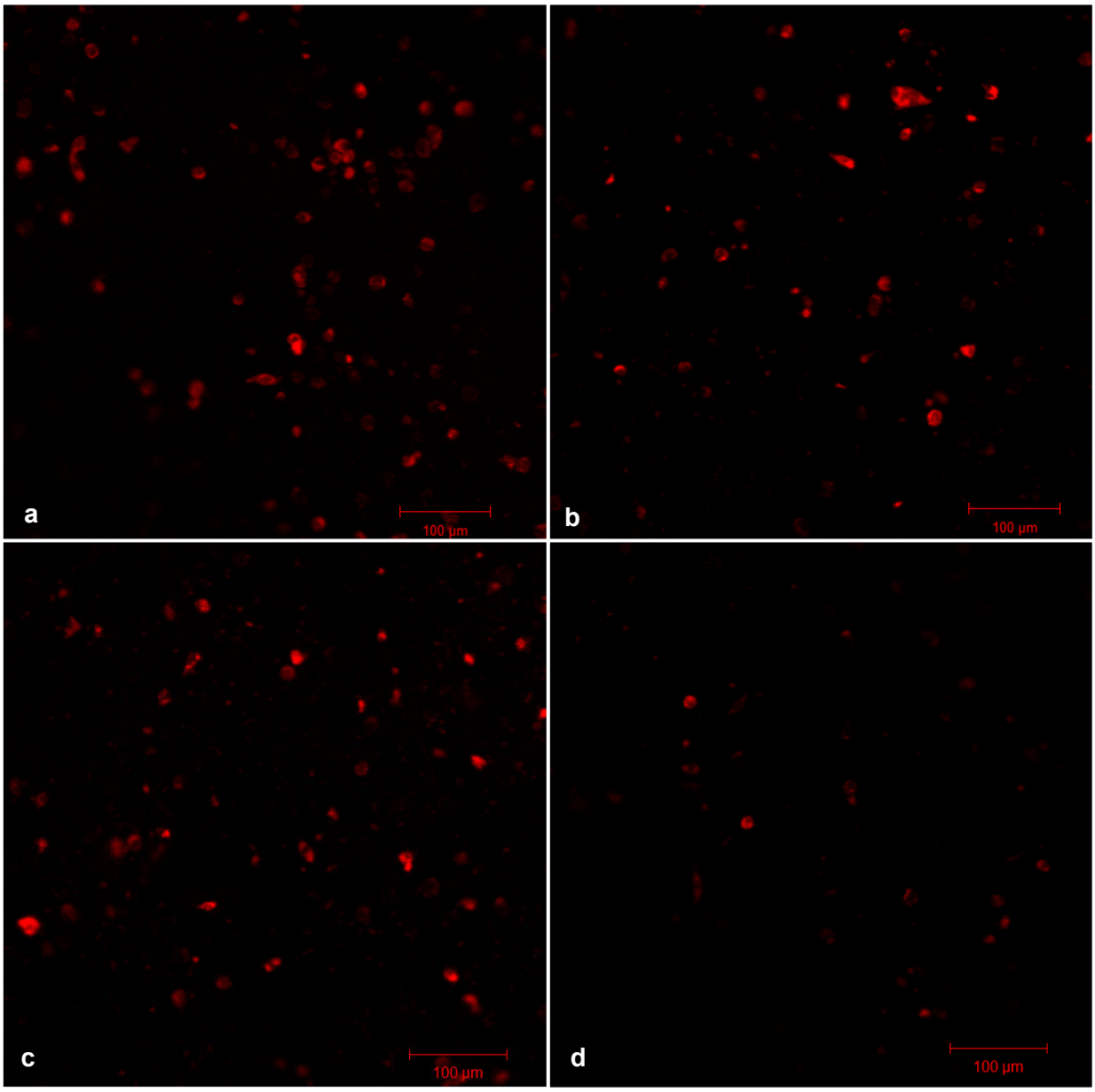


Figure E.20 Confocal images of A375 cells; untreated on day 1 (a), treated on day 1 (b), untreated on day 10 (c) and treated on day 10 (d). Treated cells were treated with 50% ethanol extract every 2nd day.

CellTracker™ Red is a vital dye which works by freely diffusing into live cells and converting into membrane impermeant glutathione fluorescent dye adducts through the actions of glutathione *S*-transferase. Therefore the CellTracker™ dyes can be used as measures of metabolic activity in

cells (Plaetse & Schoeters, 1995:1907; Haughland, 2005:644). Reduced fluorescence is taken to represent reduced metabolic activity of the cells in question. After one day, the treated cells presented with reduced fluorescence as compared to the untreated cells. This phenomenon may be attributed to increased porosity of the cell membrane during cell death which may have allowed the membrane impermeant dye adduct to exit the cells. The reduced fluorescence intensity in the treated cells after one day of treatment shows that the cell death process had already begun. In figure E.20(d) the treated cells had reduced fluorescence intensity on day ten as compared to the untreated cells. This test also served to show that the A375 cells were able to thrive in the Matrigel® environment for up to ten days as high fluorescence is observed for the untreated cells on day ten. These results are in support of the outcome of the XTT cytotoxicity and Annexin V FITC apoptosis assays which showed that the 50% ethanol extract was cytotoxic to the cells in Matrigel®.

E.4 Statistical analysis

The statistical analysis of the cell culture data was done using IBM SPSS Statistics 23, (IBM Corporation, Armonk, New York, USA). A one-way analysis of variance (ANOVA) was done to test for differences between groups and a Games-Howell post-hoc test was done to test for within group differences. A p value below 0.05 was indicative of a significant difference between the means.

The cytotoxicity results were processed in order to make comparisons between A375 and HaCaT IC_{50} values. Table E.4 shows the p numbers that were obtained for each comparison. An asterisk (*) marks comparisons in which there was a significant difference between the IC_{50} values of the two cell types.

Table E.4 Statistical significance of differences in IC_{50} values for A375 and HaCaT cells

Comparison	p-value
A375 vs. HaCaT (WFA)	0.000291*
A375 vs. HaCaT (WFA + WNA)	0.000424*
A375 vs. HaCaT (Water)	0.004090*
A375 vs. HaCaT (Ethanol)	0.496967
A375 vs. HaCaT (50% ethanol)	0.015211*
A375 vs. HaCaT (WFA – Matrigel®)	0.000291*
A375 vs. HaCaT (WFA + WNA – Matrigel®)	0.000297*
A375 vs. HaCaT (Water – Matrigel®)	0.000961*
A375 vs. HaCaT (Ethanol – Matrigel®)	0.002128*
A375 vs. HaCaT (50% ethanol – Matrigel®)	0.010333*
50% ethanol extract vs. 50% ethanol SLNs (A375)	0.000394*
50% ethanol extract vs. 50% ethanol SLNs (HaCaT)	0.003394*

The TUNEL assay results revealed that there was no significant difference between the apoptotic cells in the negative control and all the treatments for the HaCaT cells showing that the basal apoptosis that occurred in the control cells was not significantly different from that which occurred in treated cells. Whereas, for the A375 cells there was a significant difference between the negative control and the WFA ($p=0.023$) and the WFA+WNA ($p=0.011$) treatments. This shows that apoptosis occurred to a significantly higher extent in these treated cells versus the untreated cells. There was also no significant difference between the A375 and HaCaT control. A comparison between A375 and HaCaT respective treatments revealed statistical significance between the WFA ($p=0.013$) and the WFA+WNA ($p=0.007$) treatments only.

For the caspase assay there was a significant difference in the mean % apoptotic cells between the groups. There was no significant difference between the HaCaT control and the experimental HaCaT samples. However, there was a significant difference between the control and WNA apoptotic cells for the A375 cells ($p=0.046$). A comparison of A375 and HaCaT cells showed that there were no significant differences between the % apoptotic cells from the identical treatments.

An ANOVA analysis of the membrane permeability results revealed that there were significant differences between groups ($p<0.05$) with respect to the live, dead and apoptotic cells. There however, was no statistical significance regarding apoptotic cells in the comparisons of interest (negative control versus individual treatments for both cell types and A375 versus HaCaT for all treatments).

The %MFI that was detected for the mitochondrial membrane potential was used in the statistical analysis. A comparison was made between the live control and the treatments for both cell types and comparisons were also made between the A375 and HaCaT cells for each treatment. The Games-Howell test revealed significant differences between the HaCaT live control and the WFA ($p=0.001$), WNA ($p=0.001$) and the WFA+WNA ($p=0.041$) treatments on HaCaT cells. Significant differences were also found between the A375 live control and the WFA ($p=0.024$), WFA+WNA ($p=0.009$) and the EtOH extract ($p=0.010$) treatments on A375 cells. The WNA treatment had a significant selectivity for HaCaT cells versus A375 cells with a p value of 0.023. On the other hand, the water and ethanol extract treatments caused a significant lowering of the mitochondrion membrane potential in A375 cells as compared to HaCaT cells ($p=0.009$ and 0.040 respectively) thus showing desired selectivity for A375 cells.

A one-way ANOVA revealed that there were significant differences in the results for the live ($p<0.000001$), dead ($p=0.000003$) and apoptotic cells ($p=0.000072$) after the Annexin V FITC assay. Further comparisons were made to detect differences within groups with a main focus on live cells. The focus was on live cells as this would give an indication of cells that failed to thrive

under the different treatments in Matrigel® in comparison to untreated cells. There were significant differences between the live control and the water extract (p=0.000233), ethanol extract (p=0.000174), ethanol extract SLNs (p=0.013710), 50% ethanol extract (p=0.000174) and lastly the 50% ethanol extract SLNs (p=0.000178). A comparison of crude extract vs respective SLN formulation live cells revealed the following statistically significant differences; water extract vs water extract SLNs (p=0.019969) and ethanol extract vs ethanol extract SLNs (p=0.000527). With respect to apoptotic cells there was a significant difference between untreated cells and the 50% ethanol extract (p=0.021776). Differences were also detected with the comparisons of ethanol extract vs ethanol extract SLNs (p=0.045747), water extract vs ethanol extract (p=0.000747), water extract vs 50% ethanol extract (p=0.000251), ethanol extract SLNs vs 50% ethanol extract SLNs (0.037768). These results show that the different extracts had apoptosis inducing effects that were significantly different from each other and that the presence of SLNs also resulted in a statistically significant change.

E.5 Conclusion

The TUNEL assay reflected that DNA fragmentation which is characteristic of apoptosis was rampant after treatment with the pure compounds as compared to the plant extracts. This was true for both the melanoma cells and keratinocytes. Nevertheless, some selectivity for melanoma cells was still observed. In previous studies *W. somnifera* was found to be a safe and non-toxic medicinal plant thus giving it the potential to be safely used in the treatment of cutaneous melanoma (Kulkarni & Dhir, 2008:1103). In this case, the plant extracts had minimal activity therefore it would be more advisable to pursue the pure compounds, specifically WFA for potential use in treatment of melanoma.

There was minimal caspase activity that was detected but the caspase assay detected a high percentage of necrotic A375 cells after treatment with pure compounds. A similar observation was made with the membrane permeability assay and the highest incidence of A375 apoptosis happened after exposure to WFA. The high composition of necrotic cells may be indicative of a treatment exposure time which was too long for the assays in question or the use of concentrations which were too high thus resulting in necrotic cell death. Plant extracts barely had an effect on the mitochondrial membrane potential of HaCaT cells but the ethanol and water extracts had an impact on the A375 cells. It must however be remembered that some cell lines may not exhibit certain fundamental characteristics of apoptosis *in vitro* due to loss of signal transduction pathways or metabolic components (Bortner *et al.*, 1995:25)

W. somnifera extract has been encapsulated in niosomes for cancer treatment by Sheena *et al.,* (1998:47) and it was found that niosomal encapsulation resulted in increased efficacy. In this study, the encapsulation of *W. somnifera* in SLNs resulted in the opposite effect which may have been due to the lipids of the vesicles working against the entry of the toxic molecules into the

cells. This was observed with the XTT cytotoxicity assay on melanoma cells in 2D and with the Annexin V FITC apoptosis assay on melanoma cells in 3D.

The results that were found in this study largely concur with previous studies that have been done on various melanoma cell lines (M14, Mel501, SK28 and Lu1205), in which WFA was toxic to cutaneous melanoma with 24 h IC_{50} values ranging from 1.8 – 6.1 μM (Mayola *et al.*, 2011) 1017. In this study the WFA IC_{50} value was much higher at 55.8 μM. The large difference may have been due to differences in the aggressive nature of the melanoma cell lines. A crude water extract of *W. somnifera* roots was tested on A375 cells and it was found that the crude aqueous extract was toxic to the human melanoma cells with a 24 h IC_{50} of 350 μg/ml (Halder *et al.*, 2015). In comparison the IC_{50} of the crude aqueous extract in this study was found to be 633 μg/ml. The differences in plant part and extraction method may have accounted for this discrepancy as the extraction method influences the content of the crude extracts and the roots are known to contain high concentrations of withanolides. Leaves however are more abundant and easily available and are commonly used for their medicinal properties which is why they were used in this study.

Throughout the study it was observed that the pure compounds and the crude plant extracts were toxic to the A375 melanoma cells and the HaCaT cells. There generally was selectivity for A375 cells especially with the WFA and WFA+WNA treatments, with the 2D IC_{50} results corresponding with the 3D IC_{50} results for the pure compounds. Table E.5 shows a summary of results for the melanoma cells whereby a cross (x) represents a statistically significant apoptotic effect as compared to untreated cells.

Table E.5 Summary of apoptosis results for the melanoma cells. (x) – Statistically significant occurrence of apoptosis; (-) – result not significant.

Assay	WFA	WNA	WFA+WNA	Water extract	EtOH extract	50% EtOH extract
APO BrdU apoptosis TUNEL	**x**	-	**x**	-	-	-
Caspase 3/7 apoptosis	-	**x**	-	-	-	-
Membrane permeability	-	-	-	-	-	-
Mitochondrial membrane potential	**x**	-	**x**	-	**x**	-

The assays that were used in this study assessed the occurrence of apoptosis via different apoptosis defining processes and the results obtained mainly showed that WFA is a potent anti-melanoma compound whose actions can be enhanced by the presence of WNA. Crude plant extracts do have anti-cancer activity but a better scenario would be in which the most potent compounds are used in their pure form for the treatment of cutaneous malignant melanoma.

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Appendix F: Molecules submission guidelines

F.1 Manuscript Submission Overview

F.1.1 Types of Publications

Molecules has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced by other groups. *Molecules* encourages authors to publish all experimental controls and full datasets as supplementary files (please read the guidelines about Supplementary Materials carefully and references to unpublished data).

The different types of articles published in *Molecules* are indicated in the first section of the Aims & Scope. The main types are:

Articles: research manuscripts report new evidence or new conclusions which have neither been published before nor are under consideration for publication in another journal. MDPI considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. We strongly recommend authors not to unnecessarily divide their work into several related manuscripts.

Short communications of preliminary, but significant, results will also be considered.

Reviews: review manuscripts provide concise and precise updates on the latest progress made in a given area of research.

Conference Papers: Expanded and high quality conference papers are also considered in *Molecules* if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright to the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper.

F.2 Submission Process

Manuscripts for *Molecules* should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting authors must ensure that all co-authors have been included in the author list (read the criteria to qualify for authorship) and that they all

have read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to this website. Once you are registered, click here to go to the submission form for *Molecules*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

F.2.1 Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. Accepted file formats are:

Microsoft Word: Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Molecules* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.

LaTeX: Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Molecules* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Molecules*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.

F.2.2 Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of your paper is significant, placing your findings in the context of existing work and why it fits the scope of the journal. Please confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

F.2.3 Note for Authors Funded by the National Institutes of Health (NIH)

The editors of this journal are able to deposit papers to the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/) on your behalf. If you are funded by NIH, please request this service from our editors after acceptance of your paper.

F.3 Preparation of a Manuscript

F.3.1 General Considerations

Research manuscripts should comprise:

Front matter: Title, Author list, Affiliations, Abstract, Keywords

Research manuscript sections: Introduction, Results, Discussion, Experimental Section, Conclusions (optional), Supplementary Materials

Back matter: Acknowledgments, Author Contributions, Conflicts of Interests, References.

Review manuscripts should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure.

Abstract Graphic: Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents, if you have not done so already. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.

"Data not shown" should be avoided in research manuscripts. We encourage our authors to publish all observations related to the submitted manuscript as Supplementary Materials. "Unpublished data" intended for publication in a different manuscript, *i.e.*, in a manuscript that is either planned, "in preparation" or that have been "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communications" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Abbreviations should be defined in parentheses the first time they appear in the abstract, main text and in figure captions.

SI Units (International System of Units) should be used for this journal. Imperial, US customary and other units should be converted to SI units whenever possible before submission of a manuscript to the journal.

Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided in the Experimental Section section. Please also read the Guidelines for Deposition of Sequences and of Expression Data.

Equations: If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on in your paper. Equations should be editable by the editorial office and not appear in a picture format.

Chemical Structures and Reaction Schemes: Chemical structures and reaction schemes should be drawn using an appropriate software package designed for this purpose. As a guideline, these

should be drawn to a scale such that all the details and text are clearly legible when placed in the manuscript (*i.e.*, text should be no smaller that 8-9 pt.). To facilitate editing we recommend the use of any of the software packages widely available for this purpose: MDL® Isis/Draw, ACD/ChemSketch®, CS ChemDraw®, ChemWindow®, *etc.*. Free versions of some of these products are available for personal or academic use from the respective publishers. If another less common structure drawing software is used, authors should ensure the figures are saved in a file format compatible with of one of these products.

Physical and Spectroscopic Data: Physical and spectroscopic data as well as tables for NMR data should be prepared according to the ACS's *Preparation and Submission of Manuscripts* standard (page 4).

Experimental Data: To allow for correct abstracting of the manuscripts all compounds should be mentioned by correct chemical name, followed by any numerals used to refer to them in the paper. The use of the IUPAC nomenclature conventions is preferred, although alternate naming systems (for example CAS rules) may be used provided that a single consistent naming system is used throughout a manuscript. For authors perhaps unfamiliar with chemical nomenclature in English we recommend the use of compound naming software such as AutoNom. Full experimental details must be provided, or, in the case of many compounds prepared by a similar method, a representative typical procedure should be given. The general style used in the Journal of Organic Chemistry is preferred. Complete characterization data must be given for all new compounds. For papers mentioning large numbers of compounds a tabular format is acceptable. For known compounds appropriate literature references must be given.

X-Ray Crystallographic Data: to avoid publication of extensive compilations of crystallographic data and facilitate the refereeing of manuscripts, *Molecules* asks authors to deposit the crystallographic data prior to the submission of the manuscript.

COD: Preferably, the data should be deposited with the Crystallography Open Database (COD). Please deposit as "pre-publication data" at http://www.crystallography.net/initiate_deposition.php prior to the submission of the manuscript. COD numbers for structures will be displayed immediately after the data is validated and deposited, and should be included in the manuscript, along with the following text: "COD ..... contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.crystallography.net/search.html". This text may be included in the experimental section or as a suitably referenced endnote.

CCDC: Alternatively, the data can be deposited to the Cambridge Crystallographic Data Centre (CCDC). For instructions on doing this, see: http://www.ccdc.cam.ac.uk/conts/depositing.html. The deposition numbers are usually provided by the CCDC within three working days and should

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Supplementary Materials and Research Data: To maintain the transparency and reproducibility of research results, authors are encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as "Supplementary Materials". Large datasets and files should be deposited in specialized data repositories. Small datasets, spreadsheets, images, video sequences, conference slides, software source code, *etc.* can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be made available to the referees during the peer-review process and be published online alongside the manuscript. Please read the information about Supplementary Materials and Data Deposit for additional guidelines.

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F.3.2 Front Matter

These sections should appear in all manuscript types

Title: The title of your manuscript should be concise, specific and relevant. When gene or protein names are included, the abbreviated name rather than full name should be used.

Author List and Affiliations: Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.

Abstract: The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

Keywords: Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

F.3.3 Research Manuscript Sections

Introduction: The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be reviewed carefully and key publications should be cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research.

Results: This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

Discussion: This section may be divided by subheadings. Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

Experimental Section: This section should be divided by subheadings. Materials and Methods should be described with sufficient details to allow others to replicate and build on published results. Please note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Give the name and version of any software used. Please disclose at the submission stage any restrictions on the availability of materials or information. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.

Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Conclusions: This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.

Supplementary Materials: This section should be included when supplementary information is published online alongside the manuscript. Please indicate the name and title of each supplementary file as follows Figure S1: title, Table S1: title, *etc.*

F.3.4 Back Matter

Acknowledgments: All sources of funding of the study should be disclosed. Please clearly indicate grants that you have received in support of your research work. Clearly state if you received funds for covering the costs to publish in open access. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published. Authors must have obtained specific permission from individuals and institutions to mention their names in the Acknowledgements.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "X and Y conceived and designed the experiments; X performed the experiments; Y analyzed the data; W contributed reagents/materials/analysis tools; Y wrote the paper." Authorship must be limited to those who have contributed substantially to the work reported. Please read the section concerning the criteria to qualify for authorship carefully.

Conflicts of Interest: Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state “The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results”.

References: References must be numbered in order of appearance in the text (including tables and legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references.

Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The Reference list should include the full title as recommended by the ACS style guide. The style file for endnote, MDPI.ens, can be found at http://endnote.com/downloads/style/mdpi

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1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* Year, *Volume*, page range, DOI or other identifier. Available online: URL (accessed on Day Month Year).

Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.

3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A.; Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

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5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).

6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year). Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

F.3.5 Preparing Figures, Schemes and Tables

All figure files should be separately uploaded during submission.

Figures and schemes must be provided at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). All Figure file formats are accepted. However, TIFF, JPEG, EPS and PDF files are preferred.

Molecules can publish multimedia files in articles or as supplementary materials. Please get in touch with the Editorial office for further information.

All Figures, Schemes and Tables should also be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).

All Figures, Schemes and Tables should have a short explanatory title and a caption.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but in no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.

For multi-panel figures, the file must contain all data in one file. For tips on creating multi-panel figures, please read the helpful advice provided by L2 Molecule.

Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). Full color graphics will be published free of charge.

F.4 Qualification for Authorship

Authorship must include and be strictly limited to researchers who substantially contributed to the design of the study, the production, analysis, or interpretation of the results, and/or preparation of the manuscript. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE). The journal also adheres to the standards of the

Committee on Publication Ethics (COPE) that "all authors should agree to be listed and should approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]

Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

F.5 Research Ethics Guidelines

F.5.1 Research Involving Animals

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any suffering endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

Replacement of animals by alternatives wherever possible,

Reduction in number of animals used, and

Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must be conducted in accordance with relevant national legislation on the use of animals for research. Authors should follow the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines (http://www.nc3rs.org.uk/page.asp?id=1357) for reporting experiments using live animals. Authors may use the ARRIVE guidelines as a checklist (www.nc3rs.org.uk/ARRIVEchecklist).

An approval from an ethics committee must be obtained before undertaking the research. The project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Committee (CEUA) of the Institute Pasteur Montevideo (Protocol 2009_1_3284). They are in accordance with FELASA guidelines and the National law for Laboratory Animal Experimentation (Law no. 18.611).

F.5.2 Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (http://www.wma.net/en/30publications/10policies/b3/), revised in 2008. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. As a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). A written informed consent for publication must be obtained from participating patients in this case.

Editors reserve the rights to reject any submission that does not meet these requirements.

Example of Ethical Statements:

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code).

F.5.3 Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter

plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

F.5.4 Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

F.6 Correct Identification of Components of Natural Products

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* 2006, *21*, 185. These recommendations may be summarized as follows:

Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different

methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.

F.7 Potential Conflicts of Interest

It is the authors' responsibility to identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of clinical research. If there is no conflict, please state here "The authors declare no conflict of interest." This should be conveyed in a separate "Conflicts of Interest" section preceding the "References" sections at the end of the manuscript.

F.8 Editorial Procedures and Peer-Review

F.8.1 Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether it is properly prepared and whether the manuscript follows the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journals ethical policy will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or the *Guest Editor* (or an *Editorial Board member* in case of a conflict of interest) to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the significance or potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the Editor-in-Chief.

F.8.2 Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. In the case of a special issue, the *Guest Editor* will

advise in the selection of reviewers. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with one of the institutes of the co-authors of the submitted manuscript.

F.8.3 Editorial Decision and Revision

Based on the comments and advice of the peer-reviewers, an external editor – usually the *Editor-in-Chief* or a *Guest Editor* – will make a decision to accept, reject, or to ask authors to revise the manuscript.

For *Minor Revisions* the authors will have one week to resubmit their revised manuscript. For *Major Revisions* the authors will have two weeks to resubmit their revised manuscript. However, authors should contact the editorial office if extended revision time is anticipated.

F.8.4 Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and relating information (including the identities of the referees) to an Editorial Board member who was not involved in the initial decision-making process. If no appropriate Editorial Board member is available, the editor will identify a suitable external scientist. The Editorial Board member will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be revoked.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and relating information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be revoked.

F.8.5 Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

F.9 Suggesting Reviewers

During the submission process, authors are pre encouraged to list five names of potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may also suggest reviewers from among the authors that you frequently cite in your paper.

F.10 English Corrections

This journal is published in English. To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. If you are not a native English speaker, we recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. MDPI can provide a language check via our English editing service. For additional information see the Article Processing Charges page.

F.11 Publication Ethics Statement

Molecules is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal take the responsibility to enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Molecules* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Molecules* are asked to abide to the following rules:

Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.

Authors should accurately present their research findings and include an objective discussion of the significance of their findings.

Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.

Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.

Simultaneous submission of manuscripts to more than one journal is not tolerated. Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).

If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.

Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. Plagiarism, data fabrication and image manipulation are not tolerated. Plagiarism is not acceptable in *Molecules* submissions. Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

Image files must not be manipulated or adjusted in any way that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate

action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

F.12 Supplementary Materials and Data Deposit

In order to maintain the integrity, transparency and reproducibility of research records, and to retain important chemical and structural information, authors are strongly encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal. Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process, although referees are not specifically asked to review these files. Accepted file formats include (but are not limited to):

- spectral data (NMR, IR, Raman, ESR, etc.) in JCAMP (JDX) format
- 3D coordinate structures (in PDB, MOL, XYZ or other common format)
- crystallographic information (in CIF format)
- data tables and spreadsheets (text files, MS Excel, OpenOffice, CSV, XML, *etc.*)
- text documents (text files, PDF, MS Word, OpenOffice, *etc.*; text documents will usually be converted to PDF files for publication)
- images (JPEG, PNG, GIF, TIFF, BMP, *etc.*)
- videos (AVI, MPG, QuickTime, *etc.*)
- executables (EXE, Java, *etc.*)
- software source code
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

Large data sets and files should be deposited to specialized service providers (such as Figshare) or institutional/subject repositories, preferably those that use the DataCite mechanism. For a list of specialized repositories for the deposit of scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal *Data* (ISSN 2306-5729) also accepts submissions of data set papers, and the publication of small data sets along with the paper, and/or software source codes is encouraged.

F.13 Guidelines for Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

New nucleic acid sequences must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.

New high throughput sequencing (HTS) datasets(RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.

New microarray data must be deposited either in GEO or ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed. New protein sequences obtained by protein sequencing must be submitted to UniProt (submission tool SPIN). All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

Appendix G: Pharmacognosy magazine submission guidelines

G.1 About Phcog.Net

Natural products are the most consistent and successful source of drugs. In India, Ayurveda remains one of the most ancient and living traditions, which is practiced for the treatment of various diseases and disorders. India has many number of plant species and medicinal properties have been assigned to several thousands. Many major institutes and research centres are currently involved in exploring this opportunity to investigate newer drugs from ancient principles of ayurveda. Further researchers believe in combining the strengths of ayurveda with modern scientific techniques such as NMR, MS and chromatographic techniques to provide new functional leads with high therapeutic value in a short span of time. But Natural products research often comes across many hurdles, which dims the drug development goals. We believe that natural products research information can potentially benefit many researchers involved in this area. Also we believe that the research on natural products is often staggered due to lack of required information available for medicinal plants. In order to combat these problems, Pharmacognosy Network Worldwide (Phcog.net) – A Platform for Natural Product Researchers was started, which makes innovative use of best tools for information dissemination and solve the hurdles in Natural Product Research. Pharmacognosy Network Worldwide is a non-profit network dedicated to Natural Products Research in order to develop promising drugs.

Journals from Phcog.Net

- Pharmacognosy Magazine
- Pharmacognosy Research
- Pharmacognosy Reviews

G.2 About Journal

Pharmacognosy Magazine [ISSN: Print -0973-1296, Online - 0976-4062] [http://www.phcog.com], a quarterly publication from Phcog.Net, Bangalore, INDIA and published by Wolters Kluwer - Medknow Publications and Media Pvt. Ltd, Mumbai, INDIA. It provides peer-reviewed original research articles from the field of Natural Products. The journal serves an international audience of scientists and researchers in a variety of research and academia by quickly disseminating research findings related to Medicinal Plants and Natural Products.

It is a peer reviewed journal aiming to publish high quality original research articles, methods, techniques and evaluation reports, short communications and editorials of all aspects of medicinal

plant research. The journal is aimed at a broad readership, publishing articles on all aspects of pharmacognosy, and related fields. The journal aims to increase understanding of pharmacognosy as well as to direct and foster further research through the dissemination of scientific information by the publication of manuscripts. The submissions of original contributions in all areas of pharmacognosy and Natural Products are welcome.

Further, to strengthen the journal, the corresponding authors are requested to take subscription to the journal either print/online or both.

NOTE: Articles clearing in peer review process or upon acceptance, Authors are required to pay Article Processing Charges.

G.3 Indexing Information

Pharmacognosy Magazine [ISSN: Print -0973-1296, Online - 0976-4062] is indexed with Ayush database, CAB Abstracts, Caspur, Chemical Abstracts, CSA databases, DOAJ, EBSCO Publishing's Electronic Databases, Excerpta Medica / EMBASE, Genamics JournalSeek, Google Scholar, Health & Wellness Research Center, Health Reference Center Academic, Hinari, Index Copernicus, Indian Science Abstracts, Journal Citation Reports, OpenJGate, PrimoCentral, ProQuest, PubMed, Pubmed Central, Science Citation Index Expanded, Scimago Journal Ranking, SCOLOAR, SCOPUS, SIIC databases, Summon by Serial Solutions, Ulrich's International Periodical Directory, Web of Science

Impact Factor® for 2014 : 1.256

5 year Impact Factor : 1.279

Immediacy Index : 0.081

Eigenfactor Score : 0.00106

Article Influence Score : 0.224

2015 Journal Citation Reports® (Thomson Reuters, 2015)

G.4 Scope of the journal

The journal will cover research studies/reviews related to Natural products including some of the allied subjects. Articles with timely interest and newer research concepts will be given more preference.

G.5 The Editorial Process

A manuscript will be reviewed for possible publication with the understanding that it is being submitted to Phcog Mag. alone at that point in time and has not been published anywhere, simultaneously submitted, or already accepted for publication elsewhere. The journal expects that authors would authorize one of them to correspond with the Journal for all matters related to the manuscript. All manuscripts received are duly acknowledged. On submission, editors review all submitted manuscripts initially for suitability for formal review. Manuscripts with insufficient originality, serious scientific or technical flaws, or lack of a significant message are rejected before proceeding for formal peer-review. Manuscripts that are unlikely to be of interest to the Phcog Mag. readers are also liable to be rejected at this stage itself.

Manuscripts that are found suitable for publication in Phcog Mag. are sent to two or more expert reviewers. During submission, the contributor is requested to provide names of two or three qualified reviewers who have had experience in the subject of the submitted manuscript, but this is not mandatory. The reviewers should not be affiliated with the same institutes as the contributor/s. However, the selection of these reviewers is at the sole discretion of the editor. The journal follows a double-blind review process, wherein the reviewers and authors are unaware of each other's identity. Every manuscript is also assigned to a member of the editorial team, who based on the comments from the reviewers takes a final decision on the manuscript. The comments and suggestions (acceptance/ rejection/ amendments in manuscript) received from reviewers are conveyed to the corresponding author. If required, the author is requested to provide a point by point response to reviewers' comments and submit a revised version of the manuscript. This process is repeated till reviewers and editors are satisfied with the manuscript. Manuscripts accepted for publication are copy edited for grammar, punctuation, print style, and format. Page proofs are sent to the corresponding author. The corresponding author is expected to return the corrected proofs within three days. It may not be possible to incorporate corrections received after that period. The whole process of submission of the manuscript to final decision and sending and receiving proofs is completed online. To achieve faster and greater dissemination of knowledge and information, the journal publishes articles online as 'Ahead of Print' immediately on acceptance.

G.6 Editorial Policy

Authors should prepare their manuscripts submitted to the journal exactly according to the instructions given here. Manuscripts which do not follow the format and style of the journal may be returned to the authors for revision or rejected. The journal reserves the right to make any further formal changes and language corrections necessary in a manuscript accepted for

publication so that it conforms to the formatting requirements of the journal. Manuscripts and figures are not returned to the authors, not even upon rejection of the paper.

G.7 Submission of Manuscripts

Manuscripts must conform to the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals” http://www.icmje.org/. Contributions and Manuscripts must be written in English and submitted exclusively to Pharmacogn Mag. Manuscripts must be typewritten (double-spaced) with liberal margins and space at the top and bottom of the page.

All manuscripts must be submitted on-line through the website www.journalonweb.com/pm. First time users will have to register at this site. Registration is free but mandatory. Registered authors can keep track of their articles after logging into the site using their user name and password. If you experience any problems, please contact the editorial office by e-mail at editor@phcog.com.

Download and fill the authorship form available at this LINK.

Kindly send the filled forms along scanned copyright form to email : journals@phcog.net

Phcog.Net Journals follows Uniform Requirements for Manuscripts Submitted to Biomedical Journals as per ICMJE.

Phcog.Net Journals follows the guidelines of the ICMJE (International Committee of Medical Journal Editors: Uniform requirements for manuscripts submitted to biomedical journals editors October 2008 update; www.icmje.org).

- Download: Authorship Forms
- Download: Uniform requirements for manuscripts
- Download: Reference Style

For Manuscript Preparation and Submission, follow the guidelines as per ICMJE

- Preparing a Manuscipt for Submission to Biomedical Journals
- Sending the Manuscript to the Journal
- Conflict of Interest Declaration - www.icmje.org/coi_disclosure.pdf. All authors must complete COI as prescribed by ICMJE and send it to corresponding author. It is mandatory to submit COI form along with Manuscript.
- ARRIVE 2010 checklist for animal studies (Papers dealing with Animal Studies)
- ARRIVE 2010 checklist for Clinical Trials (Clinical trial based papers)

G.7.1 Additional Guidelines

Authors reporting in vivo animal experiments and clinical trials are encouraged to refer to one of our associated journal "Journal of Pharmacology and Pharmacotherapeutics) about the ARRIVE guidelines (http://www.jpharmacol.com/text.asp?2010/1/2/94/72351) and the CONSORT statement (http://www.jpharmacol.com/text.asp?2010/1/2/100/72352) respectively and use the checklists given within them to improve the quality of the manuscripts. The checklists (Animal Experiments, Clinical Trial) must be downloaded, filled in and submitted along with the manuscripts. This would not only help the authors to include all the important information while preparing the manuscript but also speed up the peer review process.

G.7.2 Publication / processing fee

The journal does not charges for processing fee of articles at the time of submission.

G.7.3 Article Processing Charges

Upon acceptance, Authors are required to pay a nominal pre-press charges is to be paid to parent organization PHCOG.NET, Bangalore, India or Medknow Publications and Media Pvt. Ltd., Mumbai.

Article processing charges (APCs) are associated with only accepted articles to cover the costs of making the final version of the manuscript freely available via open access.

Article type	Authors from India (INR)	Foreign authors (USD)
Original Article (word limit - 3500 3 figures, 3 tables)	8500	400
Short Communication (word limit -2500 2 figures, 2 tables)	6500	300
Letter to Editor (word limit - 1500 1 figure, 1 table)	2500	100

Note : Above charges are applicable for accepted articles and minimum number of words allocated for different article types (Check Table above). For more than limit, additional charges of 1000 INR or 50 USD for every 500 words.

(As mandated by the Indian Government and based on the Service tax Law and procedures, Medknow Publications and Media Private Ltd/Phcog.Net, would be charging service tax @14%

on fees in INR collected from Indian authors with effect from 1st June 2015. The said tax will be in addition to the prices maintained on the website to be collected from the authors and will be paid to the Indian Government.)

G.7.4 Covering Letter

Disclose all possible conflicts of interest (e.g., funding sources for consultancies or studies of products). A brief indication of the importance of the paper to the field of Pharmacy is helpful in gaining appropriate peer review.

G.7.5 Copyright Form

The contributors' / copyright transfer form (template provided below) has to be submitted in original with the signatures of all the contributors within two weeks of submission via courier, fax or email (copyright AT medknow DOT com) as a scanned image. Print ready hard copies of the images (one set) or digital images should be sent to the journal office at the time of submitting revised manuscript. High resolution images (up to 5 MB each) can be sent by email on images AT medknow DOT com).

The hard copies of the Contributors' form / copyright transfer form may be sent to the following addresses or submitted online from the authors' area on www.journalonweb.com/pm

G.7.6 Authorship Criteria

Authorship credit should be based only on substantial contributions to each of the three components mentioned below:

1. Concept and design of study or acquisition of data or analysis and interpretation of data;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

Participation solely in the acquisition of funding or the collection of data does not justify authorship. General supervision of the research group is not sufficient for authorship. Each contributor should have participated sufficiently in the work to take public responsibility for appropriate portions of the content of the manuscript. The order of naming the contributors should be based on the relative contribution of the contributor towards the study and writing the manuscript. Once submitted the order cannot be changed without written consent of all the contributors. The journal prescribes a maximum number of authors for manuscripts depending upon the type of manuscript, its scope and number of institutions involved (vide infra). The authors should provide a justification, if the number of authors exceeds these limits.

G.7.7 Contribution Details

Contributors should provide a description of contributions made by each of them towards the manuscript. Description should be divided in following categories, as applicable: concept, design, definition of intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review. Authors' contributions will be printed along with the article. One or more author should take responsibility for the integrity of the work as a whole from inception to published article and should be designated as 'guarantor'.

G.7.8 Conflicts of Interest/ Competing Interests

All authors of must disclose any and all conflicts of interest they may have with publication of the manuscript or an institution or product that is mentioned in the manuscript and/or is important to the outcome of the study presented. Authors should also disclose conflict of interest with products that compete with those mentioned in their manuscript.

G.7.9 Author-Suggested Reviewers (Optional)

This field gives you the chance to suggest individuals who specialize in the topic(s) your paper covers to be used as reviewers for your paper. It is not necessary to suggest reviewers when you submit a paper, as the Journal maintains a large pool of reviewers drawn from all specialties in the arena of medicinal plants. However, the editors welcome your suggestions if you have some people in mind. You may submit up to 2 suggested reviewers, but all must be submitted with a valid email address. The Journal does not guarantee that the editors will choose to utilize all or even part of any suggested reviewer/s.

G.7.10 Copyright Form

All manuscripts are considered to be the property of Phcog.Net from the time of submission. If Phcog.Net is not publishing the paper, Phcog.net releases its rights therein at the time the manuscript is rejected following editorial/peer review or retracted by the authors. Manuscripts published in Phcog Mag. become the sole property of, with all rights in copyright reserved to Phcog.Net. The corresponding author, on behalf of all authors, signs a copyright transfer form.

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Kindly send the filled forms along scanned copyright form to email : journals@phcog.net

G.8 Preparation of Manuscript

Your Manuscript should be typed, double-spaced on standard-sized – paper (8.5" x 11") with 1" margins on all sides. You should use 12 pt Times New Roman font. Authors should take care over the fonts which are used in the document, including fonts within graphics. Fonts should be restricted to Times New Roman, Symbol and Zapf Dingbats.

Title : Should be in Title Case ; The first character in each word in the title have to be capitalized.

A research paper typically should include in the following order

- Abstract
- Keywords
- Introduction
- Materials and Methods
- Results
- Discussion
- Conclusion
- Acknowledgements (If any)
- References
- Tables and/or Figures
- Appendixes (if necessary)
- Abbreviations (if necessary)

G.8.1 Abstract – Limit of 250 Words

A brief summary of the research. The abstract should be in structured format include a brief introduction, a description of the hypothesis tested, the approach used to test the hypothesis, the results seen and the conclusions of the work.

G.8.1.1 Example

Background: *Sophora flavescens Aiton* is an important medicinal plant in China. Early in vitro researches of *S. flavescens* were focused on callus induction and cell suspension culture, only a few were concerned with in vitro multiplication. **Objective:** To establish and optimize the rapid propagation technology of *S. flavescens* and to generate and characterize polyploid plants of *S. flavescens*. **Materials and Methods**: The different concentrations of 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA) and kinetin (KT) were used to establish and screen the optimal rapid propagation technology of *S. flavescens* by orthogonal test; 0.2% colchicine solution was used to induce polyploid plants and the induced buds were identified by root-tip chromosome determination and stomatal apparatus observation. **Results**: A large number of buds could be

induced directly from epicotyl and hypocotyl explants on the Murashige and Skoog medium (MS; 1962) supplemented with 1.4–1.6 mg/l 6-benzylaminopurine (BAP) and 0.3 mg/l indole-3-acetic acid (IAA). More than 50 lines of autotetraploid plants were obtained. The chromosome number of the autotetraploid plantlet was 2n = 4x = 36. All tetraploid plants showed typical polyploid characteristics. **Conclusion**: Obtained autotetraploid lines will be of important genetic and breeding value and can be used for further selection and plant breeding.

G.8.2 Key words

Please, write no more than six keywords. Write specific keywords. They should be written left aligned, arranged alphabetically in 12pt Times Roman, and the line must begin with the words Keywords boldfaced. A 12pt space should separate the keywords from the affiliations.

G.8.3 Introduction

Description of the research area, pertinent background information, and the hypotheses tested in the study should be included under this section. The introduction should provide sufficient background information such that a scientifically literate reader can understand and appreciate the experiments to be described. The introduction MUST include in-text citations including references to pertinent reviews and primary scientific literature. The specific aims of the project should be identified along with a rationale for the specific experiments and other work performed.

G.8.4 Materials and Methods

Materials and/or subjects utilized in the study as well as the procedures undertaken to complete the work. The methods should be described in sufficient detail such that they could be repeated by a competent researcher. Please include the company sources for all uncommon reagents (kits, drugs, etc). Illustrations and/or tables may be helpful in describing complex equipment or elaborate procedures. The statistical tool used to analyze the data should be mentioned. All procedures involving experimental animals or human subjects must accompany with statement on necessary ethical approval from appropriate ethics committee.

G.8.5 Results

Data acquired from the research with appropriate statistical analysis described in the methods section should be included in this section. The results section should describe the rational for each experiment, the results obtained and its significance. Results should be organized into figures and tables with descriptive captions. The captions, although brief, should tell the reader the method used, explain any abbreviations included in the figure, and should end with a statement as to the conclusion of the figure. Qualitative as well as quantitative results should be included if applicable.

G.8.6 Discussion/Conclusion

This section should relate the results section to current understanding of the scientific problems being investigated in the field. Description of relevant references to other work/s in the field should be included here. This section also allows you to discuss the significance of your results - i.e. does the data support the hypotheses you set out to test? This section should end with new answers/questions that arise as a result of your work.

G.8.7 Tables and Figures

G.8.7.1 Tables

Each table must start on a separate sheet. They should be numbered with Roman numerals according to their sequence in the text, and have a short self-explanatory heading. Use SI units. Tables should include vertical rules, but horizontal rules should separate column headings from the content. Authors should keep in mind the page layout of the journal when designing tables. Tables that fit onto one printed page are preferred. Detailed explanations of symbols, units, and abbreviations should follow below the table.

G.8.7.2 Illustrations

Figures for final production should be submitted as electronic files and hard copy so that the editorial office can ensure that the output of electronic files matches the hardcopy. Please pay particular attention to the guidelines below. The editorial office cannot undertake preparation of manuscripts and illustrations not conforming to journal style. Manuscripts of insufficient quality will be returned immediately without refereeing. A high standard of illustration (both line and photo) is an editorial priority. All illustrations should be prepared for printing to fit 80 x 240 mm (column width) or 169 mm by up to 240 mm (full page) size. It is preferred that the full-page length is not used and that authors keep in mind that the caption will be placed underneath the figure. In the event that full-page length is necessary for plates, captions will have to appear on adjacent pages. Figure(s) must be numbered consecutively in the text. Compound figures with more than one micrograph or photo should be referred by a single figure reference (e.g. Figure 1), and individual parts should be labeled with capitalized letters in the lower left-hand corner. Lettering should be of a sans-serif type (i.e. fonts without serifs such as Arial) with a minimum published size of 4.2 mm (12 pt). Descriptive labeling in the figures should be clearly readable, and all lettering should have a minimum published size of 6 pt (2.1 mm) for labeling items on photographs or in line art is recommended and a maximum size of 10 pt is suggested. Use a scale bar to indicate magnifications and place in the lower right corner if possible. Computer prepared photographic images must be at a minimum of 350 dpi at the final publication size. Lower

resolution will result in pixilation and poor quality images. These should be submitted as JPEG, TIFF or PPT files, but encapsulated postscript (EPS) format is also acceptable.

Computer drawn figures are accepted provided they are of high quality. Please note that graphs produced by many statistic packages are rarely adequate. In particular, letter quality on axes and captions are often poor. Such figures should be exported into an accepted graphics package and lettering rendered using a text function. Authors should note that .dot, .bmp, and .pat fills should be avoided. Do not use postscript fill patterns as these are often based on bit map patterns that result in screening patterns during final reproduction. When filling illustrations, use fills such as lines, tints or solids. Line width minimum is 0.25 pt (0.09 mm). Also avoid the use of bitmap scans to render text and detail. Text should be saved as text at a minimum text size of 6 pt (2.1 mm). Please submit line art as Corel Draw, Adobe Illustrator, or EPS files. These must be at a minimum resolution of 800 DPI at publication size. High resolution may be necessary where fine line detail is present.

For graphs Excel graphs are also acceptable. Note that vertical axes must all be at the same scale especially where the paper compares between them. Otherwise they should be produced as separate figures. Avoid 3D plots when presenting 2D data. Where electronic figures are submitted, please submit a hard copy also at final acceptance stage so that it can be checked against the electronic files during proof preparation.

G.8.7.3 Table and Figure captions

Figure and table captions should be included at the end of the manuscript. Figure captions/legends should include a statement at the end of each caption/legends about reproduction size (e.g. at full page width, at column width). They should be double spaced and typed in the journal format. Explanations should be brief and authors should keep in mind that captions/legends will be placed below figures.

G.8.8 Acknowledgements – Limit of 100 Words

This is a brief section crediting the people who have helped make your manuscript possible and who aided you in your work but are not part of the authorship. Please mention all applicable grants and other funding that supported your work.

G.8.9 Formatting

- Page size Letter Portrait 8 ½ X 11
- Margins All Margins, 1cm
- Page number Numbered at bottom right
- Footer / Headers None

- Title 14 pt Times New Roman, bold, centered.
- Author and co-authors 12 pt Times New Roman centered, bold - author and all co-authors names in one line. The corresponding author should include an asterisk*.
- Authors address 12 pt Times New roman centered - giving each authors' affiliation (i.e. Department/Organization/Address/Place/Country/email). Followed by single line spacing.
- Author for Correspondence: 10pt Times New roman centered - giving a valid e-mail of the corresponding (main) author is a must. It should be indicated as* followed by two line spacing.
- Abstract 12 pt Times New roman, full justification Normal - maximum 250 words
- Text 12 pt Times New roman, full justification – 1.5 line spacing between paragraphs. No indentation
- Headings and numbering Major headings (ABSTRACT, KEYWORDS, INTRODUCTION, MATERIALS AND METHODS, RESULTS NAD DISCUSSION, ACKNOWLEDGEMENTS, REFERENCES) in upper case left-justified, 12 pt bold, Intermediate headings should be in italics, sentence case, left justified, 12 pt
- Tables To be incorporated at the end of Manuscript

Correct

“Table 1 : Serum enzyme levels…….”

Incorrect

“Table No. 1 : Serum enzyme levels…….”

Figures /Graphs Figures may be embedded in your word document but they should be created with a program that allows you to save them as gif, jpg or tiff format. For any figures or other materials directly extracted from previously published materials, you must have written permission from the publisher of that material for reprint use. A copy of that permission release must be submitted with your article.

It is the individual author's responsibility to attain this permission.

To be incorporated at the end of the manuscript with proper labeling

Correct

“Figure 1 : Serum enzyme levels…….”

Incorrect

“Figure No. 1 : Serum enzyme levels…….”

Graphs: To be included from excel, it should be editable.

Non – editable graphs will not be accepted.

All text should be fully justified. Please put all primary section titles in UPPER CASE letters and subheading in both Upper and Lower Case letters. Do not number your titles (for example, 1.0 Introduction; 2.0 Background). Do not use the tab key to indent blocks of text such as paragraphs of quotes or lists because the page layout program overrides your left margin with its own, and the tabs end up in mid-sentence.

G.8.10 Reference List: Author/Authors

References should be numbered consecutively in the order in which they are first mentioned in the text (not in alphabetic order). Identify references in text, tables, and legends by Arabic numerals in superscript with square bracket after the punctuation marks. References cited only in tables or figure legends should be numbered in accordance with the sequence established by the first identification in the text of the particular table or figure. Use the style of the examples below, which are based on the formats used by the NLM in Index Medicus. The titles of journals should be abbreviated according to the style used in Index Medicus. Use complete name of the journal for non-indexed journals. Avoid using abstracts as references. Information from manuscripts submitted but not accepted should be cited in the text as "unpublished observations" with written permission from the source. Avoid citing a "personal communication" unless it provides essential information not available from a public source, in which case the name of the person and date of communication should be cited in parentheses in the text. The commonly cited types of references are shown here, for other types of references such as newspaper items please refer to ICMJE Guidelines (http://www.icmje.org or http://www.nlm.nih.gov/bsd/uniform_requirements.html).

G.8.10.1 Correct / Acceptable Format

Natural products have proven to be a great source of new biologically active compounds. Thus, in an effort to discover new lead anti-malarial compounds, several research group screen plant extracts to detect secondary metabolites with relevant biological activities that could served as templates for the development of new drugs. Flavonoids have been isolated and characterized from many medicinal plants used in malaria endemic areas.[10] However, controversial data have been obtained regarding their antiplasmodial activity, probably because of their structural diversity.[11-13] More recently, several flavonoids have been isolated from Artemisia afra[14] and Artemisia indica,[15] two plants related to Artemisia annua, the famous traditional Chinese medicinal plant from which artemisinin is isolated.

1. Single/Multiple Authors

Halpern SD, Ubel PA, Caplan AL. Solid-organ transplantation in HIV-infected patients. N Engl J Med. 2002 Jul 25;347(4): 284-7.

2. More than six authors

Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res. 2002; 935(1-2): 40-6.

3. Organization as Author

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension. 2002; 40(5): 679-86.

4. Unknown Author

21st century heart solution may have a sting in the tail. BMJ. 2002; 325(7357): 184-5.

5. Journal article on the Internet

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

Note: Plant/Micro organisms, in-vivo, in-vitro should be in italics.

6. Personal author(s)

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

7. ditor(s), compiler(s) as author

Gilstrap LC 3rd, Cunningham FG, VanDorsten JP, editors. Operative obstetrics. 2nd ed. New York: McGraw-Hill; 2002.

8. Author(s) and editor(s)

Breedlove GK, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services; 2001.

9. Organization(s) as author

Royal Adelaide Hospital; University of Adelaide, Department of Clinical Nursing. Compendium of nursing research and practice development, 1999-2000. Adelaide (Australia): Adelaide University; 2001.

10. Chapter in a book

Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93-113.

11. Conference proceedings

Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.

12. Thesis

N. Khoshakhlagh. The compositions of volatile fractions of Peganum harmala seeds and its smoke. Pharm. D. Thesis, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. (2002).

13. WEBSITES

Website information

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/

G.8.11 Acknowledgements

All messages and reviews sent electronically will be acknowledged electronically upon receipt.

G.9 Submission of manuscript

Manuscripts always be submitted only by Web based Manuscript Submission Systems[WBMS] PREFERABLY. Visit www.journalonweb.com/pm

Note : Do not send hard copies/CDs, until you receive e-mail request from Editorial office.

A timely submission, however, is not a guarantee that your work will be accepted for forthcoming publication. All submissions are peer reviewed by the editorial board and a select group of reviewers. Please make sure that all guidelines are followed carefully. All the accepted articles

will be queued for publication and will appear in the futures issues based on the priorities set by the editorial board.

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Other correspondence : journals@phcog.net

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Ph: 080-65650760

E: journals@phcog.net (link sends e-mail)

G.10 HARD COPY SUBMISSION

Hard Copy of Manuscripts are not being accepted, Submissions should be made by Web Based Manuscript Submission Systems ONLY

Visit : www.phcog.com ; www.journalonweb.com/pm

G.11 Checklist before Submitting Manuscript

1. Covering letter
2. Copyright Forms (Scanned)
3. Manuscript
4. Illustrations (if any)

G.11.1 AUTHOR CHECKLIST FOR SENDING PROOFS TO EDITORIAL OFFICE

In order to maintain quality and consistency in Phcog.Net Journals, we ask you to perform the following checklist prior to submitting your final proof for publication:

1. Include the original, hard copy of Author's Transfer of Copyright signed by each author

2. Thoroughly check the reference style as mentioned above.
3. Thoroughly check the article for correct grammar, in particular: spelling of names, affiliations, any symbols, equations, etc.
4. Provide laser printed hard copies of all figures and graphics in black and white or colour (If any)
5. Submit a proof corrected with RED INK ONLY or as directed by the editor handling your manuscript.
6. Send the Corrected Proof (scanned), Copyright Transfer Form, Subscriptions (If any) with covering letter in a single envelope to the given address.

Note : e-mails with the forcibly/influential consideration, multiple queries on status, vicious language, offensive writings to Editor/Associate Editors will lead to rejection of the Manuscript.

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Appendix H: PLoS-ONE submission guidelines

H.1 Style and Format

File format: Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. icrosoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.

Length: Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.

Font: Use any standard font and a standard font size.

Headings: Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.

Layout: Manuscript text should be double-spaced. Do not format text in multiple columns.

Numbers: Include page numbers and line numbers in the manuscript file.

Footnotes: Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

Language: Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.

Abbreviations: Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.

Referencing: PLOS uses “Vancouver” style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.

Equations: We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor is acceptable. Avoid using MathType or Equation Editor to insert single variables (e.g., “$a^2 + b^2 = c^2$”), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values. Do not use MathType or Equation Editor for only a portion of an equation. Rather, ensure that the entire equation is included. Avoid “hybrid” inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.

Nomenclature: Use correct and established nomenclature wherever possible.

Units: Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Drugs: Provide the Recommended International Non-Proprietary Name (rINN).

Species: Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).

Genes, mutations, genotypes, and alleles: Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

H.2 Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service."

Submissions are not copyedited before publication. Submissions that do not meet the *PLOS ONE* publication criterion for language standards may be rejected.

H.3 Manuscript Organization

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* Title page: List title, authors, and affiliations as first page of manuscript Abstract Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* Materials and Methods Results Discussion Conclusions (optional)
Ending section	*The following elements are required, in order:* Acknowledgments

	References Supporting information captions (if applicable)
Other elements	Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. Tables are inserted immediately after the first paragraph in which they are cited. Supporting information files are uploaded separately.

Please refer to our downloadable sample files to make sure that your submission meets our formatting requirements:

- Download sample title, author list, and affiliations page (PDF)
- Download sample manuscript body (PDF)

Viewing Figures and Supporting Information in the compiled submission PDF:
The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

H.4 Parts of a Submission

H.4.1 Title

Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Example
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial
Short title	70 characters	State the topic of the study	Cigarette Smoke Exposure and Innate Immunity SODIS and Childhood Diarrhoea

Titles should be written in title case (all words capitalized except articles, prepositions, and conjunctions). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

H.4.2 Author List

Who belongs on the author list. All authors must meet the criteria for authorship as outlined in the authorship policy. Read the policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments. Read more about Acknowledgments.

H.4.2.1 Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system. On the title page, write author names in the following order:

- First name (or initials, if used)
- Middle name (or initials, if used)
- Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation. Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

H.4.2.2 Corresponding author

One corresponding author should be designated in the submission system as well as on the title page. One corresponding author should be designated in the submission system. However, this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

H.4.2.3 Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and include the full list of members in the Acknowledgments or in a supporting information file. Read the group authorship policy.

H.4.2.4 Author Contributions

Enter all author contributions in the submission system during submission. The contributions of all authors must be described using the CRediT Taxonomy of author roles. Read the policy. Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at

submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

H.4.3 Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page. The cover letter should include the following information:

- Summarize the study's contribution to the scientific literature
- Relate the study to previously published work
- Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- Describe any prior interactions with PLOS regarding the submitted manuscript
- Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- List any opposed reviewers

IMPORTANT: Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

H.4.4 Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

H.4.5 Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system. The Abstract should:

Describe the main objective(s) of the study

- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

H.4.6 Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

H.4.7 Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as supporting information. Read the supporting information guidelines.

H.4.7.1 Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

H.4.7.2 Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data). For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

H.4.7.3 Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

H.4.7.4 New taxon names

Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

H.4.8 Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

H.4.9 Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution. Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named. Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

H.4.10 References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, if the manuscript is submitted to a journal and also publicly available as a preprint

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)
- References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries. Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

H.5 Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial. PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support. Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	**Format**
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005 *Note: A DOI number for the full-text article is acceptable as*

	an alternative to or in addition to traditional volume and page numbers.
Accepted, unpublished articles	Same as published articles, but substitute “In press” for page numbers or DOI.
Web sites or online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14.
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Accessed 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09

Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

H.6 Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size. Authors may use almost any description as the item name for a supporting information file as long as it contains an "S" and number. For example, "S1 Appendix" and "S2 Appendix," "S1 Table" and "S2 Table," and so forth. Supporting information files are published exactly as provided, and are not copyedited.

H.6.1 Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file. The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order. Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

H.7 Figures and Tables

H.7.1 Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file. Cite figures in ascending numeric order upon first appearance in the manuscript file. Read the guidelines for figures.

H.7.2 Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document. At a minimum, include the following in your figure captions:

A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”). The figure must have a concise, descriptive title and the caption may also include a legend as needed. Read more about figure captions.

H.7.3 Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file. Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files. Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table. Read the guidelines for tables.

H.8 Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article. Read our policy on data availability. Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones. See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.
- If you have any questions, please email us.

H.8.1 Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories. Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

H.8.2 Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

H.9 Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results. The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image. Striking images should not contain potentially identifying images of people. Read our policy on identifying information. The PLOS content license also applies to striking images. Read more about the content license.

H.10 Additional Information Requested at Submission

H.10.1 Funding statement

This information should not be in your manuscript file; you will provide it via our submission system. This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission.

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role. Read our policy on disclosure of funding sources.

H.10.2 Competing interests

This information should not be in your manuscript file; you will provide it via our submission system. All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full. Read our policy on competing interests.

H.10.3 Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite input from the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process. If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

H.10.4 Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

H.11 Guidelines for Specific Study Types

H.11.1 Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained. Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee. All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines. Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME

Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed

Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:

- Why written consent could not be obtained
- That the Institutional Review Board (IRB) approved use of oral consent
- How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

H.11.2 Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. Where trials were not publicly registered before participant recruitment began, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form. The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

H.11.3 Animal research

We work in consultation with the *PLOS ONE* Animal Research Advisory Group to develop policies. Animal Research Advisory Group members may also be consulted on individual submissions. All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research. If we note differences between an IACUC-approved protocol and the methods reported in a submitted manuscript, we may report these discrepancies to the relevant institution or committee. Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s). Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why.
- Relevant details for efforts taken to ameliorate animal suffering

Example ethics statement: *This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.*

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title. Where unregulated animals are used or ethics approval is not required, authors should make this clear in submitted articles and explain why ethical approval was not required. Relevant regulations that grant exemptions should be cited in full. It is the authors' responsibility to understand and comply with all relevant regulations. We reserve the right to reject work that the editors believe has not been conducted to a high ethical standard, even if authors have obtained formal approval or approval is not required under local regulations. We encourage authors to follow the Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that inclusion of a completed ARRIVE Checklist may be a formal requirement for publication at a later date.

H.11.3.1 Non-human primates

Manuscripts describing research involving non-human primates must include details of animal welfare, including information about housing, feeding, and environmental enrichment, and steps taken to minimize suffering, including use of anesthesia and method of sacrifice if appropriate, in

accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF).

H.11.3.2 Humane endpoints

Manuscripts describing studies that use death as an endpoint will be subject to additional ethical considerations, and may be rejected if they lack appropriate justification for the study or consideration of humane endpoints.

H.11.4 Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

H.11.5 Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement: *All necessary permits were obtained for the described study, which complied with all relevant regulations.* If no permits were required, please include the following statement: *No permits were required for the described study, which complied with all relevant regulations.*

Manuscripts describing paleontology and archaeology research are subject to the following policies: Sharing of data and materials. Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.

Ethics. *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported

H.11.6 Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available.

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract. If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

H.11.7 Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

H.11.8 Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section. Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent. For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research. See our reporting guidelines for human subjects research.

H.11.9 Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate. Authors must also include the following information for each cell line:

- For *de novo* (new) cell lines, including those given to the researchers a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:
- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin
- For established cell lines, the Methods section should include:
- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line

authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

H.11.10 Blots and gels

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

H.11.11 Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.
- The manuscript should also report the following experimental details:
- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

H.11.12 Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

H.11.12.1 Utility

The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

H.11.12.2 Validation

Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

H.11.12.3 Availability

Software should be open source, deposited in an appropriate archive, and conform to the Open Source Definition. Databases must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. Authors should provide a direct link to the deposited software or the database hosting site from within the paper.

H.11.13 Software submissions

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

H.11.14 Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

H.11.15 New taxon names

H.11.15.1 Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni Fisher sp. nov.urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the Methods section, in a sub-section to be called “Nomenclatural Acts”:

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

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H.11.15.2 Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the Methods section, include a sub-section called “Nomenclature” using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies. In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories:

[INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

H.11.15.3 Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies. Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the Methods section, include a sub-section called “Nomenclature” using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for

algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies. In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

H.11.16 Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.