In vitro cytotoxicity of *Siphonochilus aethiopicus* in combination with selected fillers for tableting

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PREFACE

True character is built during tough and enduring times. You only learn your true strength when you are at your lowest, when you have no other option than to pick up the pieces and start all over again.

God blessed me, yes blessed me, with such a predicament. No words can describe the pain of losing a sibling, let alone two. However, it remains your choice how you want to process it. If you think you will be the same after it, you are wrong. You do not get rid of the pain, you learn to live with it. A loss that significant creates a hole in your humanity, a hole that I tried to fill with work and achievements. But I soon learnt the truth: it can only be filled by memories; beautiful and painful memories as well as life lessons you endured together.

Your names were Giverny and Corné. Yes, you were not of my blood, but you were my sister and brother, whom I loved unconditionally. If it were not for you, I don't know what type of person I would have become. You taught me the value of choice. You taught me that you can choose to feel sorry for yourself, or you can rise above the hand you were dealt. Your lives were legendary and impactful, you always had smiles on your faces and constantly saw splendour in the small things. You taught me that God's grace is always enough, not matter what the situation. Through your example I learnt to be thankful for my weaknesses, as it is there through God's true power and mercy is glorified (2 Corinthians 12:9-10).

God called you home when the time was right. At that moment it felt like my world fell apart, but in retrospective I realise that your purpose on this earth was fulfilled. Yes, I miss you every day and not a day goes by that I do not long for one more hug, but I remain thankful for the privilege to have called you family. I find solace in knowing that one day we will be united again.

I dedicate this thesis to you. You inspired me to never settle and to always challenge myself to do greater things.

I love you always and forever.

"Whatever you do, work at it wholeheartedly as though you were doing for the Lord and not merely for people."

Colossians 3:23

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ABSTRACT

The use of herbal medicines is currently experiencing a revival of sorts. However, this resurgence in popularity is casting a much needed light on safety issues relevant to the use thereof, since many herbal products remain untested with regards to their toxicological properties. *Siphonochilus aethiopicus*, or African ginger, is one of the most desired medicinal plants at South African muthi markets. Traditional uses vary from malaria to various inflammatory related conditions such as asthma and dysmenorrhoea. Though African ginger has shown auspicious potential in several pharmacological studies - possessing anti-inflammatory, anti-trypanosomal and antimalarial activity (to name only a few) – information relating to its cytotoxicity is limited. When considering the wide range of commercially available products containing this plant, this raises concern for consumer safety.

The main aim of the current study is to investigate the *in vitro* cytotoxicity of several *S. aethiopicus* extracts, alone and in combination with chitosan and Pharmacel® 101 fillers for tableting, on human hepatocellular liver carcinoma (HepG2) and human epithelial colorectal adenocarcinoma (Caco-2) cell lines since they represent the oral route of administration. Organic extracts were prepared by solvent extraction and compared with an aqueous extract and traditional infusion. These extracts were, subsequently, characterised with ultra-performance liquid chromatography quadruple time of flight mass spectrometry (UPLC-Q-TOF/MS) through the identification of AG 1–4, previously identified marker compounds. Standard cytotoxicity assays with different endpoints were selected and included the tetrazolium reduction (MTT) and lactate dehydrogenase (LDH) assays, as well as flow cytometry with fluorescent annexin V / propidium iodide (PI).

UPLC chromatograms of extracts revealed organic extracts (ethanol and diethyl ether) to contain all four isolated African ginger compounds (AG 1–4). Aqueous extracts only contained AG 1 and AG 2 in small amounts and did not contain AG 3 or AG 4. MTT assays proved organic extracts to reduce cell viability, despite interference, whereas aqueous extracts did not cause interference; nor did it reduce cell viability. LDH data also indicated only organic extracts to cause LDH release on both cell lines. During both assays Caco-2 cells proved to be less sensitive to the effects of the extracts, compared to HepG2 cells, since cell viability only decreased at considerably higher concentrations. Fillers seldom, if at all, caused a significant ($p \leq 0.05$) alteration of the effects caused by extracts. Subsequently, flow cytometric analysis was performed on organic extracts alone. Results indicated a definite decrease in cell viability of both cell lines following exposure, with a concomitant increase in apoptotic and necrotic cell populations.

Therefore, it can be concluded that aqueous extracts do not possess cytotoxic properties, whereas organic extracts caused apoptotic and necrotic cell death. Considering the difference in

phytochemical composition of these extracts, one cannot help to consider the possibility of AG 3 and AG 4, compounds only present in organic extracts, to be partly responsible for the observed cytotoxicity. Consequently, it is suggested to further isolate the major compounds present in *S. aethiopicus* and investigate their individual cytotoxicity. Selectivity of crude extracts and isolated compounds should, moreover, be investigated. Finally, development of polymer nanoparticle formulations are furthermore recommended as it might reduce toxicity.

KEYWORDS: *Siphonochilus aethiopicus*; African ginger; Cytotoxicity; Apoptosis; Necrosis; Cell viability; Traditional medicine

UITTREKSEL

Die gebruik van natuurlike medisinale produkte ondervind tans ‘n herlewing. Nietemin werp die toename in gebruik ‘n behoeftige lig op die veiligheidsaspekte verwant aan die gebruik daarvan, aangesien talle produkte ongetoets is in terme van hulle toksikologiese eienskappe. *Siphonochilus aethiopicus*, of Afrika gemmer, is een van die mees gesogte medisinale plante op Suid-Afrikaanse muti-markte. Tradisionele gebruike varieer van malaria tot verskeie inflammatories-verwante toestande soos asma en dismenoree. Alhoewel Afrika gemmer reeds belowende potensiaal getoon het in verskeie farmakologiese studies – waar dit anti-inflammatoriese, anti-tripanosomiale en anti-malariële eienskappe getoon het (om ‘n paar te noem) – is inligting aangaande die sitotoksisiteit daarvan beperk. Wanneer die wye reeks beskikbare kommersiële produkte wat die plant bevat in ag geneem word, wek dit kommer vir die verbruiker se veiligheid.

Die hoof-doelwit van die studie iss om die sitotoksisiteit van verskeie *S. aethiopicus* ekstrakte, alleen en in kombinasie met chitosan en Pharmacel® 101 vulstowwe vir tabletering, op menslike lewerkarsinoma (HepG2) en menslike epiteel kolorektale adenokarsinoma (Caco-2) sellyne te toets, aangesien dit die orale toedieningsroete verteenwoordig. Organiese ekstrakte is berei deur oplosmiddelekstraksie en vergelyk met ‘n water ekstrak asook ‘n traditionele infusie. Daarbenewens was ekstrakte gekarakteriseer deur ultra-prestasie vloeistofchromatografie gekoppel aan quadrupool tyd van vlug massaspektrometrie (UPLC-Q-TOF/MS). AG 1-4, voormalig geïdentifiseerde verbindings, was gevolglik bespeur. Standaard sitotoksiese toetse met verskillende eindpunte is geselekteer insluitend die tetrasolium reduksie (MTT) en die laktaat dehidrogenase (LDH) toetse asook vloeisitometriese analisering met fluoresserende annexin V/propidiumjodied.

UPLC chromatogramme van die ekstrakte het aangedui dat organiese ekstrakte (etanol en diëtieleter) al vier verbindings bevat (AG1–4). Waterige ekstrakte het slegs AG 1 en AG 2 in klein hoeveelhede en glad nie AG 3 en AG 4 bevat nie. MTT toetse het bewys dat organiese ekstrakte sellewensvatbaarheid verminder, ondanks ekstraksteuring. Waterige ekstrakte het nóg steuring, nóg vermindering in sellewensvatbaarheid veroorsaak. LDH-data het ook geïmpliseer dat slegs organiese ekstrakte LDH-vrystelling veroorsaak het op beide sellyne. Tydens beide toetse was Caco-2 selle minder vatbaar vir die effekte van die ekstrakte, vergeleke met HepG2 selle, aangesien sellewensvatbaarheid eers by baie hoër konsentrasies verminder het. Vulstowwe het ook selde, indien ooit, ‘n beduidende invloed op die effekte van ekstrakte gehad. Gevolglik is vloeisitometriese analise slegs uitgevoer op organiese ekstrakte. Resultate het ‘n definitiewe afname in beide sellyne se lewensvatbaarheid getoon, met ‘n gelyktydige toename in die apoptotiese en nekrotiese selpopulasies.

Daarom kan die gevolgtrekking gemaak word dat waterige ekstrakte nie sitotoksiese eienskappe besit nie. Daarteenoor het organiese ekstrakte apoptotiese en nekrotiese seldood veroorsaak. Met die verskil in fitochemiese samestelling in ag genome, kan mens nie help om die moontlikheid te oorweeg dat AG 3 en AG 4, verbindings wat slegs in organiese ekstrakte teenwoordig is, deels verantwoordelik kan wees vir die waargenome sitotoksisiteit. Gevolglik word isolasie van die onderskeie hoofverbindings van *S aethiopicus* en 'n ondersoek na hul individuele sitotoksisiteit voorgestel. Die moontlikheid van selektiwiteit van ru-ekstrakte sowel as individuele verbindings moet ook ondersoek word. Laastens, word die ontwikkeling van polimeriese nanopartikel formulerings aanbeveel, aangesien dit toksisiteit kan verlaag.

SLEUTELWOORDE: *Siphonochilus aethiopicus*; Afrika gemmer; Sitotoksisiteit; Apoptose; Nekrose; Sellewensvatbaarheid; Tradisionele medisyne

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Firstly, I want to thank my heavenly Father for blessing me throughout the progression of my study, for providing me with all my capabilities and for helping me to grow as a human being. I have learnt the value of patience, compassion and dedication.

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To my parents, family and soon to be family, thank you for your support in my search for knowledge. Thank you for your unconditional love and support. Without you I would not be the person I am today. Without you I would not have succeeded. You are special beyond words and I will always be grateful for your guidance. I love you all very much. To my other friends, you are few but you have become family; thank you for always being willing to provide me with some much needed distraction when it was due.

Riaan Smit, my fiancé, it has been a challenging two years for us both, but soon we will start a new chapter in our lives. You were slow to complain when I had little time for you, but you were quick to pick me up when life knocked me down. With a phone call, a cup of coffee or a long hug, you always knew what I needed to be able to carry on. It is my prayer to now be able to support you in pursuing your dreams, as you did me in mine. I love you endlessly.

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LIST OF ABBREVIATIONS

% w/w	Percentage weight-in-weight (g per 100 g)
µg	Microgram(s)
µM	Micromole(s)
µm	Micrometre(s)
ADME	Absorption, distribution, metabolism and elimination
AIDS	Acquired immune deficiency syndrome
ANOVA	One-way analysis of variance
API	Active pharmaceutical ingredient
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Before Christ
BCOP	Bovine corneal opacity and permeability
BEH	Ethylene bridged hybrid
BP	Base peak
BPI	Base peak intensity
Caco-2	Human epithelial colorectal adenocarcinoma
CDKs	Cyclin dependant kinases
COX	Cyclooxygenase
CQR	Chloroquine-resistant strain
CSIR	Council for Scientific and Industrial Research
CYP	Cytochrome
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia / for example
EtOH	Ethanol
Et_2O or DiEt	Diethyl ether

FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward-scatter light
g	Gram(s)
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices
GSP	Good Sourcing Practices
G_0-phase	Quiescence of the cell cycle
G_1-phase	First cell growth phase of the cell cycle
G_2-phase	Second cell growth and error control phase of cell cycle
h	Hour(s)
HepG2	Human hepatocellular liver carcinoma cells
HIV	Human immunodeficiency virus
HPMC	Hydroxypropylmethylcellulose
HS	Hillslope
HTS	High throughput screening
IC_{50}	Half maximum inhibitory concentration
IL	Interleukin
INT	2-(4'-Iodophenyl)-3-(4'-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride
Inter alia	Amongst other things
In situ	Within the original or natural position
In vitro	Biological process made to occur inside a laboratory vessel or controlled experimental conditions
In vivo	Biological process made to occur within a living organism
IUCN	International Union for Conservation of Nature
IV	Intravenous
kg	Kilogram(s)
LD_{50}	Lethal dose 50%
LDH	Lactate dehydrogenase

LOX	Lipoxygenase
MCC	Microcrystalline cellulose
MeOH	Methanol
mg	Milligram(s)
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre(s)
M-phase	Cell division phase of cell cycle
MS^E	Fragmentation spectra, MS to E
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADH	Reduced nicotinamide adenine dinucleatide
NAD+	Nicotinamide adenine dinucleatide
NF-κB	Nuclear factor-κB
ºC	Degree Celsius
nm	Nanometre(s)
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDA	Photodiode array
PDE	Phosphodiesterase
PI	Propidium iodide
PS	Phosphatidylserine
PSD	Particle size distribution
RAW 264.7	Mouse Abelson murine leukaemia virus-induced macrophage cells
RIPK1/3	Receptor-interacting protein kinase 1 or 3
rpm	Rotations per minute
RRI	Relative retention indices
SEM	Standard error of mean
S-phase	DNA replication phase of cell cycle
SANBI	South African National Biodiversity Institute

SFM	Serum free media
SPE	Solid phase extraction
SSC	Side-scattered light
TB	Tuberculosis
TNF-α	Tumour necrosis factor-α
TOF/MS	Time of flight mass spectrometry
UPLC	Ultra-performance liquid chromatography
UPLC-Q-TOF/MS	Ultra-performance liquid chromatography quadruple time of flight mass spectrometry
UV-DAD	Ultraviolet-diode array detection
Vice versa	In the opposite order that something has been stated
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION TO STUDY

1.1 BACKGROUND AND JUSTIFICATION

1.1.1 Popularity and safety of phytomedicines

Herbal medicines have enjoyed a resurgence in their public acceptability as alternative treatments for diseases like arthritis, diabetes and cancer in developed and developing countries during the past decade (Kunle *et al.*, 2012; Chawla *et al.*, 2013; Ju *et al.*, 2016). This revived popularity has also projected much needed attention on issues relating to the safety of herbal preparations and how it might affect public health (Ekor, 2014).

Too often, it is argued that the longstanding consumption of a medicinal plant is evident of its safety, when used at recommended therapeutic doses. This is worrying, as almost 60% of the rural South African population and an estimated 80% of the global population frequently utilise traditional medicine, obtained from traditional healers (Taylor *et al.*, 2003; Ifeoma & Oluwakanyinsola, 2013; Ekor, 2014; Moreira *et al.*, 2014). The safety of a medicinal plant cannot be assumed in the absence of acute evidential toxicity. While acute toxic effects are easily recognised, chronic, or long term side effects, such as chemically induced cancer, are more challenging to identify. Most likely, endemic cultures merely do not have the required reporting systems to accurately document any such observed adverse effects (Fabricant & Farnsworth, 2001; Moreira *et al.*, 2014). Although many herbal medicines may have auspicious potential, numerous products remain untested and unregulated with regards to toxicological, or safety evaluations, resulting in a limited awareness of their rational use, adverse effects and contra-indications (WHO, 2002; Ekor, 2014).

1.1.2 Siphonochilus aethiopicus

Siphonochilus aethiopicus or African ginger*,* is a member of the Zingiberaceae family. The roots and rhizomes are widely used as traditional medicine for coughs, colds, influenza, mild asthma, sharp pains, hysteria, malaria and dysmenorrhoea (Watt & Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; Steenkamp *et al.,* 2005; Van Wyk *et al.*, 2009; Fouche *et al*., 2011). It is indigenous to the tropical areas of Southern Africa, including South Africa, Malawi, Zimbabwe and Zambia, however its distribution is significantly restricted (Department of Agriculture, Forestry and Fisheries, 2014). For years, it has been listed as critically endangered on the South African Biodiversity Institute (SANBI) red list and has for some time been considered as the most sought after medicinal plant on the traditional medicines, or so called ‘muthi’, markets in South Africa (Lötter *et al*., 2006).

Various studies have investigated the diverse pharmacological effects of *S. aethiopicus* extracts, which range from anti-asthmatic to anti-trypanosomal activities. Some of the most prevalent and proven efficacies include anti-inflammatory, anti-allergic, and anti-asthmatic properties (Lindsey *et al.*, 1999; Light *et al.*, 2002; Fennell *et al.*, 2004; Jäger & Van Staden, 2005; Stafford *et al.*, 2005; Fouche *et al*., 2008; Fouche *et al.,* 2013). It has been established that *S. aethiopicus* also possesses antiplasmodial (Lategan *et al.*, 2009), anti-trypanosomal, antimycobacterial (Igoli *et al.*, 2012), antifungal (Motsei *et al*., 2003; Coopoosamy *et al.,* 2010), as well as antibacterial activity against Gram-positive and to a lesser extent, against Gram-negative bacteria (Fennell *et al.,* 2004; Stafford *et al*., 2005). These diverse pharmacological effects are possibly attributed to synergism amongst the different compounds present within the different extracts (Jäger & Van Staden, 2005; Lategan *et al.*, 2009).

Some studies have also indicated that *S. aethiopicus* extracts may possess genotoxic and cytotoxic effects. By using a cytotoxic assay, Lategan *et al.* (2009) determined that ethyl acetate extracts possessed a half maximum inhibitory concentration (IC_{50}) of 73.9 µg/ml (±12.8). Taylor *et al.* (2003) and Steenkamp *et al*. (2005) conducted assays during which deoxyribonucleic acid (DNA) damage was investigated, and obtained positive results with regards to some of the extracts tested. Steenkamp *et al.* (2005) furthermore observed contradictory results, as some extracts depicted both pro-oxidant, as well as anti-oxidant capacities in separate assays, when measuring different endpoints in oxidative stress. Moreover, a study by Light *et al*. (2002) showed aqueous leaf extracts to only exhibit minor cytotoxic effects after 7 days of exposure at concentrations higher than 250 µg/ml, whereas rhizome extracts exhibited cytotoxicity at lower concentrations. This was an interesting discovery, since traditionally, the rhizomes are often administered as a hot infusion, or steamed for inhaling the vapours (Fouche *et al.,* 2013).

1.1.3 Formulation factors of herbal preparations

S. aethiopicus is estimated as one of the most popular medical plants in South Africa and subsequently possesses a pronounced potential for commercial production (Street & Prinsloo, 2013). However, the formulation of pharmaceutical dosage forms by utilising plant materials involves stability and technology difficulties far greater than those experienced with isolated single compounds in conventional medicines (Lockwood, 2013). Together with the intricate mixtures of active phytochemicals contained within different extracts (Ogaji *et al.,* 2012), several other factors also influence the composition of herbal extracts, including environmental, genetic, harvesting, agricultural and manufacturing parameters. All of these parameters contribute towards the variability of constituents, the lack of reproducibility, and the difficulty to standardise such products (Ahmad *et al.*, 2006; Kunle *et al.*, 2012). The formulation of tablets that contain herbal materials presents further problems, due to the intrinsic poor rheological and compactability properties of dry herbal extracts, or powdered plant materials. The lack of information regarding the influence

of extracts on the physical-mechanical characteristics of regularly used excipients complicates formulation considerations even more (Palma *et al.*, 2002; Qusaj *et al.*, 2012). In addition, pharmaceutical excipients may also undergo chemical and/or physical interactions with active drug compounds, although they are generally deemed pharmacologically inert. Such interactions may result in altered stability, bioavailability, therapeutic efficacy and safety of a product (Crowley & Martini, 2001; Bharate *et al.,* 2010; Fathima *et al*., 2011; Hotha *et al*., 2016).

The solubility of compounds is another aspect that must be examined, because only compounds in solution are able to be absorbed across cell membranes (Ashford, 2013). Although the solubility of different compounds will determine the most suitable solvent to be employed in the manufacturing processes, formulation constraints may often result in partial insolubility, as extracts contain various compounds with ranges of differing solubilities (Lockwood, 2013). Solvents may also be used as a separation technique for extracting compounds selectively by utilising the constituents' polarities (Coopoosamy *et al.,* 2010; Dhanani *et al*., 2017). The outcomes from the investigations into these formulation factors may in future assist with choices with regards to the fillers to incorporate and the extraction procedures to be used for pharmaceutical formulations containing *S. aethiopicus*. This study further aimed at contributing towards the standardisation of formulations containing *S. aethiopicus*.

1.1.4 Cell based *in vitro* toxicity assays

Cell based cytotoxicity assays are often employed to determine the ability of test substances to affect cell viability and cell proliferation or to exhibit genotoxic and carcinogenic effects (Ifeoma & Oluwakanyinsola, 2013). They are particularly employed during early drug discovery and help to identify compounds with potential toxicity, by providing information relating to their mechanisms of toxicity (McKim, 2010; Zang *et al.*, 2012). Despite their limitations, cell based *in vitro* cytotoxicity assays provide a relevant biological microenvironment and accordingly represent an acceptable compromise between biochemical assays and living, whole organisms (Zang *et al.*, 2012). Several cell viability markers are used to determine cytotoxicity, including morphologic and intracellular differentiation markers, the inhibition of proliferation, as well as membrane and metabolic markers (Ifeoma & Oluwakanyinsola, 2013).

1.2 RESEARCH PROBLEM

A common misperception exists that traditional medicines or herbal products are safe for human consumption when used at therapeutic dosages (WHO, 2003; Ekor, 2014; Moreira *et al.*, 2014). Consequently, our knowledge of acute and chronic cytotoxic effects that are related to medicinal plants is at an undeveloped stage. This is problematic and a serious cause for concern, as many poverty stricken communities rely on the use of these plants to satisfy their health care needs

(Bagla, 2012). For example, the herbal product, 'Ma Huang', or *Ephedra*, is traditionally used in Chinese culture to treat respiratory ailments, while being marketed as a weight loss supplement in the USA. Many consumers thereof have reportedly suffered from heart attacks and strokes, while some even died in severe cases of overdosing (WHO, 2003). Fouche *et al.* (2013) state that continuous research into novel, herbal therapies is necessary, as they often contains numerous active compounds that act on multiple biological receptors, which may result in therapeutic successes. This implies validating the pharmacological efficacy and safety of herbal remedies. Despite its possible medicinal applications and popularity, no in-depth cytotoxicity studies have yet been conducted on African ginger extracts to verify its safety for human consumption. These extracts are traditionally used to treat chronic inflammatory diseases, such as asthma, which implies long term consumption and treatment. Considering the reported genotoxic, anti-oxidant and superficial cytotoxic results of some research projects (Light *et al*, 2002; Taylor *et al*., 2003; Steenkamp *et al*., 2005; Lategan *et al.*, 2009; Igoli *et al.*, 2012), concern for patient safety is raised. It is therefore vital to investigate the possibility of any cytotoxic effects of different extracts of *S. aethiopicus*, alone and in combination with selected fillers, when extracted with solvents of varying polarities; correlate these effects with the absence or presence of previously identified biomarker molecules (Bergh, 2016); and elucidate the mechanism of cell death evoked by these extracts. This study aimed at assisting in the future development of safe, oral pharmaceutical formulations of African ginger and to contribute towards its safety profile.

1.3 AIMS AND OBJECTIVES

1.3.1 Aims

This study aimed at investigating the cytotoxic activities of numerous *S. aethiopicus* extracts within their individual capacity, but also when compounded with selected excipients for tableting. In doing so, baseline cytotoxic profiles could be determined for the selected extracts and it could furthermore be established whether the particular excipients altered the effects induced by the respective extracts. Consequently, possible physicochemical interactions could be identified and aid future prospects of oral pharmaceutical formulation. Moreover, the chemical compositions of the extracts were determined, since cytotoxic properties might be directly related to the presence, or absence of specific compounds.

1.3.2 Objectives

The main objectives of this study were to:

- Prepare crude aqueous, diethyl ether and ethanol extracts of the rhizomes of *S. aethiopicus.* Extracts containing a commercially available product (Phyto nova African Ginger® tablets) and a traditional infusion were also prepared.

- Chemically profile the prepared crude extracts by means of ultra-performance liquid chromatography quadruple time of flight mass spectrometry (UPLC-Q-TOF/MS).
- Determine the cytotoxic activities of the various extracts on human hepatocellular liver carcinoma (HepG2) and human epithelial colorectal adenocarcinoma (Caco-2) cell lines, by using the following three assays: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) assay and flow cytometry using fluorescein isothiocyanate (FITC) labelled annexin V/propidium iodide (PI) double staining.
- Prepare combinations of plant extracts with two selected excipients: chitosan and Pharmacel® 101.
- Conduct the above mentioned cytotoxicity assays with plant extract-excipient combinations.
- Determine the respective half maximal inhibitory concentrations (IC_{50}) of the various *S. aethiopicus* extracts on both cell lines.
- Distinguish between apoptosis or necrosis as the mechanism of action of the different *S. aethiopicus* extracts on the two different cell lines.

1.4 EXPERIMENTAL LAYOUT

As illustrated in Figure 1.1, this study consisted of four main phases. Firstly, numeroues crude extracts were prepared from dry plant powder. Secondly, optimisation studies were conducted to determine an appropriate solvent system which could be applied to cell cultures and to optimise the time and concentration ranges to which the cells would be exposed to. Thirdly, *in vitro* viability and cytotoxicity assays were performed to investigate whether extracts possessed any anti-proliferative or cytotoxic properties. Through measuring multiple viability and cytotoxicity parameters, false positive and negative results would be minimalised, since each assay has its own limitations. Lastly, specific extracts were characterised chemically and according to particle size, aimed at elucidating the influence of these factors on the observed *in vitro* effects.

Figure 1.1: *A schematic illustration of the experimental design for this study*

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CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION TO MEDICINAL PLANTS

2.1.1 Historical value of medicinal plants and the place of traditional therapy in medicine today

Nature has produced magnificent and complex compounds over millions of years that synthetic production has yet to deliver. The first records about the utilisation of plant materials for medicinal purposes, dated back to 2 600 BC, were written in cuneiform on clay tablets and originated from Mesopotamia, whereas the well-known Ebers Papyrus, an Egyptian pharmaceutical record, dates back to 1 500 BC (Cragg & Newman, 2005; Cragg & Newman, 2013; Atanasov *et al.*, 2015). It was only after Friedrich Sertürner had isolated morphine from the opium poppy plant in 1804, that scientists realised that the therapeutic properties of plants were as a result of the bioactive compounds contained within them (Atanasov *et al.*, 2015; Rungsung *et al*., 2015).

A detailed definition of traditional medicine can be described as: "the sum total of the knowledge, skills and practices, based on the theories, beliefs and experiences, indigenous to different cultures, whether explicable or not, and their usage in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses" (WHO, 2000; Nethathe *et al*., 2016). Traditional medicine is an essential part of a community's identity, as it is a vital source of health care and an important source of income (Abbott, 2014). The conventional practice has demonstrated its contribution towards the reduction of extreme disability, morbidity and mortality due to diseases, such as sickle cell anaemia, human immunodeficiency virus (HIV), or acquired immune deficiency syndrome (AIDS), malaria, tuberculosis and diabetes (Elujoba *et al*., 2005; Ahmad *et al*., 2016; Bello *et al.*, 2016; Ramadani *et al.*, 2017).

Increased popularity of traditional medicines is a global phenomenon, with up to 70% of American and 80% of the African and Asian populations being estimated as using herbal remedies as complementary medication. In the past ten years, 65% of drugs that were approved for public marketing had been derived from natural products (Ifeoma & Oluwakanyinsola, 2011; Robotin, 2012). Within the South African traditional health care system, medicinal plants also fulfil a profound role. In 2007, statistics indicated that trade in medicinal plants represented 5.6% of the National Health budget, had an estimate worth of R 2.9 billion per year, comprised of 27 million consumers and employed at least 133 000 people, most of which were rural women (more recent statistics were unavailable) (Mander *et al*., 2007). Annual trade in medicinal plants was estimated at an R 2.7 billion in South Africa in 2016, indicating a slight decrease in use compared to the

statistics of 2007 (Xego *et al.,* 2016). The average consumer uses 750 g of medicinal plants a year – with an average of 128 million courses of traditional remedies prescribed and approximately 20 000 tonnes of indigenous plant material consumed annually; and typically purchased at traditional medicine markets. Many consumers of traditional medicine indicated that they use these medicines by choice, since the consultation process consists of a dual spiritual and physiological treatment, with rituals combining herbal medicines, prophecy and ancestral worship (Mander *et al*., 2007). These consumers view conventional drug therapy as a "reductionist approach", often focussing on a single therapeutic target, while traditional herbal preparations are regarded as a more holistic approach to the disease process and health states alike (Moreira *et al.*, 2014; Heafner & Buchanan, 2016).

2.1.2 Pharmacognosy: drug discovery and investigation of herbal remedies

Pharmacognosy is a term that has been used for nearly two centuries and refers to studies on natural drug products. The term has been described by Sarker (2012) as "the science of biogenic or naturally derived drugs, pharmaceuticals and poisons that incorporates various modern analytical techniques for authentication and quality control of crude drugs, as well as purified active extracts, fractions and components". In the modern age, it involves the investigation of plants, fungi, bacteria and marine living organisms (Sarker, 2012). Research areas embraced by pharmacognosy are continuously expanding, but may include aspects of bioactive compound discovery, analytical chemistry, cultivation of medicinal plants, ethnobotany, pharmacology, phytochemistry, phytotherapy, and the standardisation of traditional medicines, among others; consequently being a multidisciplinary and translational science of sorts (Kinghorn, 2001; Sarker, 2012). According to Popović *et al*. (2016), the interest in medicinal flora has grown dramatically in both scientific and public domains; as is evident in the growing number of articles reporting on the possible efficacy and safety of herbal medicines (Davids *et al.*, 2016; Mongalo *et al.*, 2016; Moteetee & Seleteng Kose, 2016; Seebaluck-Sandoram *et al.*, 2017; Sharma *et al.*, 2017).

The importance of natural products has been well established, with many of them serving as sources of diverse and complex bioactive compounds for direct use as medication, or as lead compounds for semi-synthetic manipulation to produce entities of higher activity or lower toxicity, or as templates for total synthetic modification (Fabricant & Farnsworth, 2001; Cragg & Newman, 2013, Dash & Nivsarkar, 2016). The importance of continuous natural drug discovery is emphasised, due to its exceptional success (Brower, 2008). A gradual shift from mono-drug therapy in orthodox medicine, to multi-targeted and multi-drug therapy is also promoting traditional medicine practices, as observed in the treatment of multiple diseases, for example malaria, hypertension and tuberculosis. It is argued that several low dosage bioactive compounds (as are found in plant extracts) can influence a multi-faceted pathophysiological process more

efficiently and with less adverse effects than an isolated, large dosage compound (Obodozie, 2012). The multiplex nature of pharmacodynamic and pharmacokinetic characteristics of herbal preparations should accordingly be kept in mind, since many attributes of active compounds, for example their bioavailability, are often influenced by other chemical entities also contained within the preparation (Dash & Nivsarkar, 2016). Hence, development of effective and safe traditional therapies is a critical tool to increase access to health care, particularly in developing countries (WHO, 2003).

2.2 IMPORTANCE OF *SIPHONOCHILUS AETHIOPICUS*

2.2.1 Botanical description

Siphonochilus aethiopicus, also known as “Natal (or wild) ginger”, as well as “African ginger” in English and “wilde gemmer” in Afrikaans, is a member of the Zingiberaceae family (Hutchings *et al*., 1996; Fouche *et al*., 2011). Wild ginger is indigenous to tropical areas of Southern Africa, including South Africa, Malawi, Zambia and Zimbabwe (Department of Agriculture, Forestry and Fisheries, 2014). In Swazi culture, it is traditionally known as “sithungula” or “isithungulu” (Crouch *et al.,* 2005). *S. aethiopicus* is a deciduous plant with large leaves, which develop annually from small cone-shaped rhizomes (Figure 2.1). Berry-like fruits are borne below or above ground, while the leaves and rhizomes are somewhat aromatic, with a smell similar to *Zingiber officinale* (ginger) (Van Wyk *et al.,* 2009).

Their flowers are often a combination of bright pink and white, with a yellow centre, but can vary in colour, as presented in Figure 2.2. Most plants are bisexual (Van Wyk & Gericke, 2000; Van Wyk *et al.,* 2009). Erect leaves only grow after flowering is complete, usually in mid-December, possibly to ensure that the flowers are prominently visible to pollinators (Nichols *et al.*, 1989). Like most members of the Zingerberaceae family, *S. aethiopicus* has a dormant phase (from June to November), during which the plants are leafless and no roots are present on the rhizomes. The roots and rhizomes, as observed in Figure 2.1, are normally utilised for medicinal purposes (Nichols *et al.,* 1989; Van Wyk *et al*., 2009).

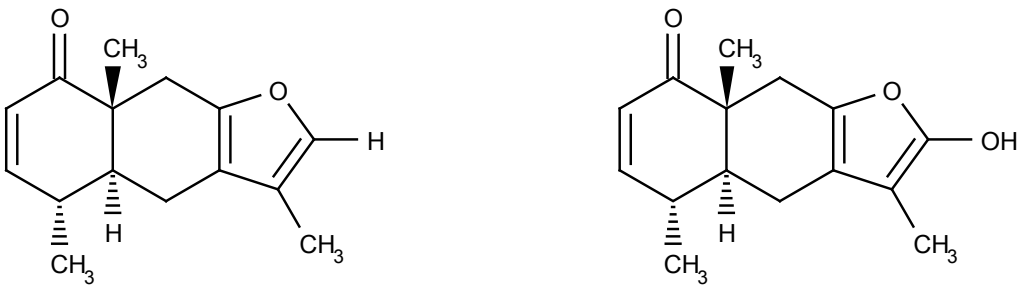
Figure 2.1: *Images of characteristic rhizomes of S. aethiopicus, (a) a whole and halved rhizome, (b) a collection of unwashed rhizomes, and (c) a close-up image of the distinct, cone-shaped S. aethiopicus rhizomes (photographed by the author)*

Figure 2.2: *The picturesque flowers of wild ginger or S. aethiopicus; photographs **(a)**, **(b)** and **(c)** were captured by Peter Vos on 28 Nov. 2013, unsure location (used with the permission of the photographer, refer to Annexure A) (Vos, 2014). Photographs **(d)**, **(e)** and **(f)** were taken by Rogan B. Roth at the University of KwaZulu-Natal Botanical Garden (Pietermaritzburg) on 26 Nov. 2010 (used with permission of the photographer, refer to Annexure A). These photographs clearly demonstrate the varying colours of the flowers of S. aethiopicus*

2.2.2 Chemical profiling and volatile oils

The chemical composition of *S. aethiopicus* has only been partially clarified, with limited information currently available in the literature, due to inter-individual phytochemical variations observed among raw plant material samples. Phytoconstituent variability is known to be present in different plant individuals (inter-individual), but also within the same individual (intra-individual) of the species. It may furthermore be influenced by harvesting practices and geographic distribution (Seasotiya *et al.*, 2014).

Two eudesmane sesquiterpenoids, furanoterpenoid derivates, namely 4aαH-3,5α,9-trimethyl-4,4a,8a,9-tetrahydronaphtho[2,3*b*]-furan-8-one and 2-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydronaphtho[2,3-*b*]-furan-8-one (Figure 2.3) were isolated by Holzapfel *et al.* (2002) as the two main constituents of *S. aethiopicus*, sourced in South Africa, during a chemotaxonomic investigation. In that same year, Viljoen *et al.* (2002) identified a total of seventy and sixty compounds in the essential oils of the roots and rhizomes, respectively. As listed in Table 2.1, these essential oils display a near identical composition and mostly only differ with regards to trivial quantitative aspects. Viljoen *et al.* (2002) furthermore suggested the name, siphonochilone, for the novel furanoterpenoid (compound 1, Figure 2.3) , as identified by Holzapfel *et al.* (2002), which was also identified as being the main compound present in the essential oil of *S. aethiopicus.* Similarly, it was later identified as the major constituent present in a diethyl ether extract of African ginger (Fouche *et al.*, 2011). Other minor compounds identified by Viljoen *et al.* (2002) included: 1,8-cineole, (*E*)-*β*-ocimene and *cis*-alloocimene. 1,8-Cineole is also known as eucalyptol, a monoterpene commonly used in the treatment of rhinitis and sinusitis, and it has accordingly been stated to be an effective bronchodilator for the treatment of asthma and bronchitis (Nikolić *et al.*, 2011).



(a) Compound 1 **(b) Compound 2**

Figure 2.3: *Chemical structure of two compounds found in the essential oil of S. aethiopicus – **(a)** 4aαH-3,5α,9-trimethyl-4,4a,8a,9-tetrahydro-naphtho[2,3b]-furan-8-one (compound 1 or siphonochilone) and **(b)** 2-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one (compound 2) - adapted from Holzapfel et al. (2002)*

Table 2.1: *Constituents of the essential oil of the rhizomes and roots of S. aethiopicus as identified by Viljoen et al. (2002)*

RRI'	Compound	Plant part	
		Roots (%)	Rhizome (%)
1020	Methyl 2-methyl butyrate	-	0.03
1014	Tricyclene	0.04	-
1032	α-pinene	0.84	0.72
1035	α -thujene	0.22	0.15
1076	Camphene	1.85	0.02
1118	β-pinene	1.96	1.86
1132	Sabinene	5.52	4.52
1159	δ-3-carene	2.42	0.96
1174	Myrcene	1.80	1.15
1176	α -phellandrene	-	-
1183	*p*-mentha-1(7),8-diene *(=pseudolimonene)*	0.06	-
1188	α -terpinene	0.44	0.25
1195	Dehydro-1,8-cineole	-	0.01
1203	Limonene	1.68	1.17
1213	1 ,8-cineole	9.63	16.11
1218	β-phellandrene	2.00	-
1246	(*Z*)-β-ocimene	1.03	0.75
1255	γ-terpinene	0.91	0.47
1266	(*E*)-β-ocimene	7.61	6.11
1278	*m*-cymene	0.05	0.02
1280	*p*-cymene	0.43	0.22
1286	Isoterpinolene	0.02	0.01
1290	Terpinolene	0.58	0.22
1382	*Cis*-alloocimene	10.27	7.63
1409	*Trans*-alloocimene	1.29	0.83
1460	2,6-dimethyl-1 3(*E*),5(*E*),7-octatetraene	0.02	0.02
1474	*Trans*-sabinene hydrate	0.31	0.26
1479	δ-elemene	0.08	-
1498	(*E*)-β-ocimene epoxide	0.01	-

Table 2.1: *Constituents of the essential oil of the rhizomes and roots of S. aethiopicus as identified by Viljoen et al. (2002) (continued)*

RRI'	Compound	Plant part	
		Roots (%)	Rhizome (%)
1532	Camphor	0.03	-
1553	Linalool	0.40	0.84
1556	*Cis*-sabinene hydrate	0.28	0.26
1562	Isopinocamphone	0.01	0.02
1571	*Trans*-*p*-menth-2-en-1-ol	0.16	0.16
1594	*Trans*-β-bergamotene	0.13	-
1597	Bornyl acetate	0.08	-
1600	β-elemene	0.69	0.25
1602	6-methyl-3,5-heptadien-2-one	0.21	-
1611	Terpinen-4-ol	3.05	3.47
1638	*Cis*-*p*-menth-2-en-1-ol	0.10	0.11
1650	γ-elemene	0.65	-
1668	(*Z*)-β-farnesene	0.07	0.01
1682	δ-terpineol	0.06	0.12
1687	α-humulene	0.05	0.01
1689	*Trans*-piperitol	0.08	0.06
1706	α-terpineol	1 01	1.71
1709	α-terpinyl acetate	0.55	0.50
1726	Germacrene-D	0.28	0.12
1733	Neryl acetate	-	0.04
1744	α-selinene	0.06	
1748	Piperitone	0.04	0.01
1758	*Cis*-piperitol	0.04	0.07
1758	(*E,E*)- α -farnesene	0.06	-
1773	δ-cadinene	0.03	-
1783	β-sesquiphellandrene	0.20	-
1786	Kessane	2.98	1.86
1796	Selina-3,7(11)-diene	0.25	0.07

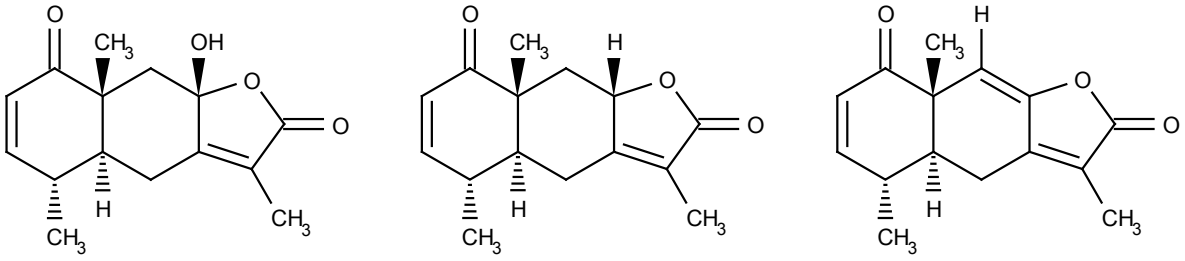
Table 2.1: *Constituents of the essential oil of the rhizomes and roots of S. aethiopicus as identified by Viljoen et al. (2002) (continued)*

1804	Isokessane	0.23	0.13
1830	2,6-dimethyi-3(*E*),5(*E*),7-octatriene-2-ol	0.04	0.07
1854	Germacrene-B	2.31	0.77
1864	*p*-cymen-8-ol	0.03	0.01
1878	2,5-dimethoxy-*p*-cymene	0.03	-
1886	Isofuranogermacrene	0.18	0.08
2008	Caryophyllene oxide	0.10	0.03
2033	Epiglobulol	-	0.01
2050	(*E*)-nerolidol	0.09	0.05
2073	*p*-mentha-1,4-diene-7-ol	-	0.01
2096	Elemol	0.56	0.38
2104	Dimethyl anthranilate	0.09	0.65
2185	γ-eudesmol	0.08	0.21
2250	α-eudesmol	0.04	0.01
2257	β-eudesmol	0.34	0.24
2273	Selin-11-en-4-α-ol	0.24	-
2320	Juniper camphor	0.16	0.05
2373	*Trans*-isoeugenol	-	0.08
2471	Indole	-	0.04
2698	(4aα,5,8α)-3,5,8a-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8(5H)-one (siphonochilone)	21.23	32.37

**RRI' Relative retention indices calculated against n-alkanes*

Lategan *et al.* (2009) isolated the three compounds illustrated in Figure 2.4 from ethyl acetate *S. aethiopicus* extracts. It is important to emphasise the structural relation between compounds 2 and 4 (Figures 2.3 and 2.4, respectively), as they are tautomers, meaning they are interconvertible (see Annexure A, Figure A2). Bergh (2016) furthermore isolated four biomarker molecules from methanol African ginger extracts for the purpose of establishing a chemical fingerprint for *S. aethiopicus*. These compounds, illustrated in Table 2.2, depicted noteworthy stereoisomeric properties, being enentiomers (mirror images) of previously identified compounds. Congruent to the findings of Viljoen *et al.* (2002), Bergh (2016) also identified eucalyptol and *cis*-

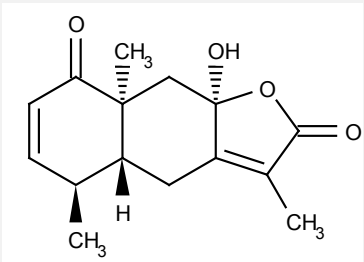
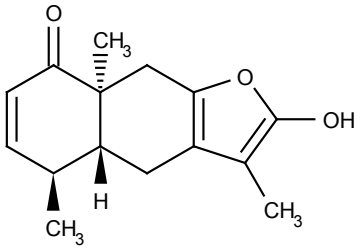
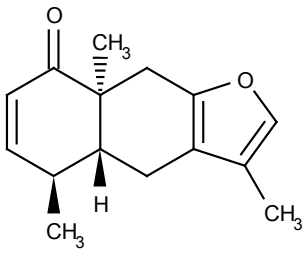
alloocimene as other major constituents present in the essential oil of *S. aethiopicus*, along with sabinene and β-phellandrene.



(a) Compound 3 **(b) Compound 4** **(c) Compound 5**

Figure 2.4: *Three novel furanoterpenoid compounds isolated by Lategan et al. (2009)*

Table 2.2: *Four biomarker molecules identified by Bergh (2016) aimed at developing a chemical fingerprint for S. aethiopicus*

Compound	Chemical structure	Chemical name	Enantiomeric compound
Compound 6		(4aR,5S,8aS,9aR)-9a-hydroxy-3,5,8a-trimethyl-4a,5,9,9a-tetrahydro-naphtho[2,3-b]furan-2,8(4H,8aH)-dione	Compound 3
Compound 7		(4aR,5S,8aS)-2-hydroxy-3,5,8a-trimethyl-4a,5,8a,9-tetrahydro-naphtho[2,3-b]furan-8(4H)-one	Compound 2
Compound 8	Not established	Not established	-
Compound 9		(4aR,5S,8aS)-3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one	Compound 1

In a recent study, Katele Zongwe (2015) reported that the major compound, siphonochilone (compound 9, Table 2.2; compound 1 in Figure 2.3 represents the stereo-isomer thereof), was extremely unstable – it was prone to autoxidation. During this reaction, siphonochilone (compound 9) was oxidised two compounds, namely compound 6 (hydroxyfuranoterpenoid;

Table 2.2) and compound 7 (hydroxylactone; Table 2.2) as had been individually identified by Holzapfel *et al.* (2002) and Lategan *et al*. (2009). In contrast to the findings of Holzapfel *et al.* (2002) and Lategan *et al*. (2009), who initially concluded that the autoxidation products had occurred naturally in *S. aethiopicus*, Katele Zongwe (2015) later identified that these compounds were only present in the dried plant material following the degradation of siphonochilone during storage and that they had not originally been present in fresh material. Characterisation of crude hexane extracts by Igoli *et al.* (2012) revealed five new compounds, not reported heretofore, to be present in the Nigerian variety of *S. aethiopicus*: an elemane sesquiterpenoid (*epi*-curzerenone), a germacrane sesquiterpenoid called furanodienone (8,12-epoxy-1(10),4,7,11-germacratetraen-6-one) and the labdane diterpenoids, 8(17),12E-labdadiene-15,16-dial, 15-hydroxy-8(17),12E-labdadiene-16-al and 16-oxo-8(17),12E-labdadiene-15-oic acid (Zerumin A).

It has therefore become evident that the chemical constituents present in those extracts had been influenced by a wide range of factors, including the plant parts being used, their geographic harvest location, storage duration, and very importantly, the solvent system utilised to extract the target components. Commonly, polar or hydrophilic compounds are extracted using polar solvents, whereas apolar solvents are utilised to extract lipophilic constituents (Sasidharan *et al.*, 2011). Evidently, the possibility therefore exists that different compounds would be present in different extracts, each of which would be responsible for various clinical effects (Stafford *et al.,* 2005; Fouche *et al.*, 2011; Złotek *et al.*, 2015).

2.2.3 Traditional uses

In Zulu culture *S. aethiopicus* is known as “indungulo”, “ithole” or “isiphephetho” (Hutchings *et al*., 1996; Crouch *et al.,* 2005). Traditionally, the roots and rhizomes are used for coughs, colds, influenza, mild asthma, sharp pains, as well as hysteria and epilepsy (Watt & Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; Van Wyk & Gericke, 2000; Steenkamp *et al.,* 2005; Van Wyk *et al.*, 2009; Igoli *et al.*, 2012). Rhizomes are further used for phlegm and planted for protection against lightning and snakes in the Zulu culture. Other cultural beliefs include keeping away drought and extreme heat if taken with mealie meal; and guarding the “inyanga” (traditional healer or herbalist) against any hazardous effects harvested plants (Watt & Breyer-Brandwijk, 1962; Hutchings *et al*., 1996). It is used for malaria by the Swazi, while women chew it during menstruation (Hutchings *et al*., 1996). Interestingly, rhizome infusions are administered to horses in the belief that it would cause horse sickness to present only very mildly, although not considered a cure, nor prophylaxis (Watt & Breyer-Brandwijk, 1962). Other dosage forms include hot and cold infusions, steam inhalations or teas (Hartzell, 2011; Fouche *et al.,* 2013).

A 200 mg dose of dried herb or powder is reportedly frequently taken three times (occasionally more) per day for traditional use (Van Wyk & Wink, 2009). Dr Gericke, as stated by Hartzell (2011), recommends a mixture of 50 mg of dried plant powder with caster sugar to reduce a child’s

fever from 38.5ºC to approximately 37.5ºC, whereas he also observed additional sedative effects. A mixture containing 30 mg dried plant powder and caster sugar, taken via the sublingual route 8 hourly, had effectively reduced his 20 month old daughter's acute asthma within 15 min. He further reported on the effective treatment of oral, oesophageal and vaginal thrush in acquired immune deficiency syndrome (AIDS) patients, following an oral dosage of 100 mg taken three times daily, with recovery within 3 days. Fascinatingly, Manzini (2005) established that 76% of surveyed traditional healers did not associate an age restriction with the use of African ginger; and it was deemed safe to administer to the elderly as well as infants, provided that the correct dosage was taken.

2.2.4 Medicinal uses and pharmacological effects

2.2.4.1 Cyclooxygenase enzyme inhibition

Possible medicinal uses for *S. aethiopicus* are very diverse, as indicated by its various traditional applications. Inhibition of prostaglandin synthesis activity by *S. aethiopicus* (evidence of its possible effectiveness against pain, inflammation and inflammatory related diseases) has been demonstrated during several studies (Lindsey *et al.*, 1999; Light *et al.*, 2002; Fennell *et al.*, 2004; Jäger & Van Staden, 2005). The synthesis of prostaglandins (pro-inflammatory molecules) from arachidonic acid is prevented through the inhibition of cyclooxygenase (COX) enzymes. Non-steroidal anti-inflammatory drugs (NSAIDS) mostly bind to COX 1 (constitutive enzymes) and COX 2 enzymes (inducible, facilitates inflammation) in a non-selective manner and are frequently used to treat pain and inflammation (Koneti & Jones, 2016).

Light *et al.* (2002) conducted COX 1 and COX 2 assays using aqueous, ethanol and ethyl acetate extracts having a final concentration of 250 µg/ml, prepared from the leaves, roots and rhizomes. In both the COX 1 and COX 2 assays, the ethanol and ethyl acetate leaf extracts exhibited the highest levels of inhibition, with the ethyl acetate extract displaying slightly higher activity than the ethanol extract, confirming results found by Zschocke *et al.* (2000). Extracts of the roots and rhizomes also depicted lower levels of inhibition, compared to the leaf extracts. These findings served as proof of the prospective anti-inflammatory activity of *S. aethiopicus.* Conversely, aqueous rhizome extracts exhibited reasonable levels of cytotoxicity and little COX 1 inhibition. Fascinatingly, a study by Jäger and Van Staden (2005), however, found that the aqueous rhizome extract revealed a slight COX 2 selectivity, although aqueous extracts had generally not been as active as non-polar solvent extracts. Agents with COX 2 selectivity have the therapeutic advantage of having anti-inflammatory properties without being ulcerogenic (Fennell *et al.*, 2004). Lindsey *et al.* (1999) further reported on the use of *S. aethiopicus* for dysmenorrhoea and revealed that the effectiveness of the extracts had been as a result of prostaglandin synthesis inhibition and its resulting anti-inflammatory effects, with ethanolic extracts again showing higher inhibitory activity, compared to their aqueous counterparts. These extracts did not cause

relaxation of uterine muscles as had initially been expected, but instead initiated mild contraction of the relaxed uterus.

In another anti-inflammatory application, Fouche *et al*. (2008) conducted studies to investigate the value of *S. aethiopicus* extracts on the specific treatment of asthma and allergies. Since phosphodiesterase IV (PDE IV) and 5-lipoxygenase (5-LOX) enzymes play key roles in the production of pro-inflammatory mediators, their inhibition would be beneficial in many inflammation related diseases. PDE IV inhibition causes activation of a second messenger pathway that results in the suppression of tumour necrosis factor-α (TNF-α) (Kumar *et al*., 2013), whereas 5-LOX inhibition results in decreased leukotriene production (Wisastra & Dekker, 2014). Diethyl ether extracts (100 µg/ml) showed significant activity: 57% inhibition of the PDE IV enzyme, 77% inhibition of glucocorticoid receptor binding and a 101% inhibition of the 5-LOX enzymes were obtained. Significant activity at the glucocorticoid binding site indicated a similar mechanism of action as corticosteroids. Fouche *et al*. (2008) concluded that an isolated sesquiterpene, compound 1 (Figure 2.3), had most likely been responsible for the biological activity of the extract, as it depicted a half maximum inhibitory concentration (IC_{50}) value quite similar to the diethyl ether extract (12.9 µg/ml). These findings were later confirmed by Fouche *et al.* (2011). Lower activity observed in the PDE IV assay may have suggested that the extract and compound were incapable of significant bronchodilatory activity.

In vivo studies conducted by Fouche *et al.* (2008) and Fouche *et al.* (2011) revealed that organic extracts (ethanol and diethyl ether), either administered via oral or intra-peritoneal routes, resulted in a remarkable reduction in lung inflammation and immune cell infiltration, particularly of eosinophils. The isolated sesquiterpene did not possess an equally noteworthy effect on lung inflammation, compared to the diethyl ether extract, possibly due to insufficient availability of the compound as a result of its poor solubility in biologically acceptable carriers (Fouche *et al.,* 2011). Fouche *et al.* (2013) furthermore, investigated the potential anti-inflammatory properties of *S. aethiopicus* regarding its nuclear factor-κB (NF-κB) inhibitory activity. The diethyl ether extract showed noteworthy activity. Inhibition of NF-κB, a dominant regulator of inflammation, could be beneficial for asthma treatment, as well as other inflammation related diseases, as it inhibits the release of many inflammatory and pro-inflammatory mediators, such as TNF-α, interleukin-1,-2 and -8 (IL-1, -2 & -8).

2.2.4.2 Antibacterial activity

In a study conducted by Light *et al.* (2002) aqueous, ethanol and ethyl acetate extracts were prepared from the leaves, roots and rhizomes of *S. aethiopicus*. Their antibacterial activities were tested against Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*). Aqueous extracts did not display any significant antibacterial activity, whereas ethanol and ethyl acetate extracts demonstrated higher

antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria (Light *et al.*, 2002), possibly due to it being more difficult to penetrate the cell walls of Gram-negative bacteria (Fennell *et al.*, 2004). A comprehensive study by Coopoosamy *et al*. (2010) *inter alia* investigated the antibacterial properties of ethyl acetate, acetone and aqueous extracts prepared from leaf and rhizome material on five Gram-positive bacteria (*B. subtilis, S. aureus, Staphylococcus epidermidis, Micrococcus kristinae and Bacillus cereus)* and four Gram-negative bacteria *(E. coli, Proteus vulgaris, Enterobacter aerogenes* and *Shigella sonnei).* Again, the aqueous extract did not demonstrate any antibacterial activity, whilst leaf and rhizome ethyl acetate and acetone extracts exhibited antibacterial effects against all Gram-positive bacteria (except *S. epidermidis* and *S. aureus* in some instances). Of the Gram-negative bacteria, only *P. vulgaris* and *E. coli* were inhibited by acetone rhizome extracts. Leaf extracts subsequently displayed antibacterial activity similar to those of the rhizomes, although not relatively as high. Granting furanoeremophil-2-en-1-one (Figure 2.5), a furanosesquiterpene structurally similar to compound 1 (Figure 2.3), was identified as one of the foremost antibacterial compounds, the antibacterial activity could not be attributed to a single compound; an indication of synergistic interactions between compounds (Jäger & Van Staden, 2005). Similar results were later obtained by Lategan *et al.* (2009), as SPE fractions showed increased activity compared to isolated compounds.

Furthermore, antimycobacterial activity, although not significant, was recorded against *Mycobacterium tuberculosis*, the primary organism responsible for causing human tuberculosis (TB) (Augenstreich *et al*., 2017). It demonstrated a minimum inhibitory concentration (MIC) of 250 µg/ml, but no antibacterial, nor antifungal activities were observed at this concentration (Lategan *et al.*, 2009). Igoli *et al.* (2012) also found evidence of potential antimycobacterial activity, as the diterpenes, 8(17),12E-labdadiene-15,16-dial and 15-hydroxy-8(17),12E-labdadiene-16-al referred to under section 1.2.2., displayed moderate levels of activity against *Mycobacterium aurum*, whereas the sesquiterpenoids depicted no such effects.

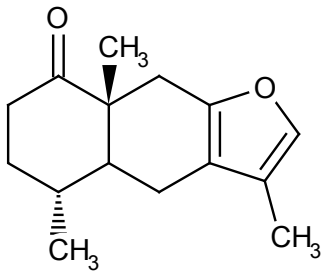


Figure 2.5: *The chemical structure of furanoeremophil-2-en-1-one - adapted from Jäger & Van Staden (2005)*

2.2.4.3 Antifungal activity

Motsei *et al*. (2003) investigated the antifungal properties of *S. aethiopicus* extracts (100 mg/ml). All three organic extracts tested (ethanol, ethyl acetate and hexane) were active against three different strains of *Candida albicans* (clinical isolates from a 5-month-old baby, an adult and a standard strain ATCC 10231) at concentrations between 1.03–2.09 mg/ml. The aqueous extract did not possess this property. Motsei *et al*. (2003) hence suggested that organic extracts should be further investigated for possible development of a hospital prepared product, but they concluded that these extracts would be unsuitable as a home care remedy. Coopoosamy *et al*. (2010) additionally found that the ethanol extract of the bulbs and leaves possessed effective antifungal activity against *Candida tropicalis, Aspergillus flavus, Trichophyton mentagrophytes* and *Trichophyton rubrum*.

2.2.4.4 Antiviral, anthelmintic and antischistosomal activities

Light *et al.* (2002) explored the potential antiviral, anthelmintic and antischistosomal activity of *S. aethiopicus* extracts. They concluded that neither the aqueous extracts of the leaves, nor the rhizomes, possessed any noteworthy activity against the Herpes simplex virus types 1 and 2 (3.9–500 µg/ml) or the influenza A virus (3.9–1000 µg/ml). No anthelminthic activity was detected against the nematode *Caenorhabditis elegans*, nor antischistosomal effects against schistosoma in *Bulinus africanus* snails.

2.2.4.5 Antiplasmodial and anti-trypanosomal activities

S. aethiopicus extracts had illustrated modest antiplasmodial activity in a study conducted by Lategan *at al*. (2009). Interestingly, an ethyl acetate extract exhibited antiplasmodial selectivity against a chloroquine-resistant strain (CQR) of *Plasmodium falciparum*, whilst compound 5 (Figure 2.4) presented comparable activity. It is possible that the furan moiety within the structure was responsible its antiplasmodial properties. Since these compounds had a significant structure-activity relationship, specifically towards the CQR strain, their basic structure could be used to synthesise more potent derivatives (Lategan *et al.*, 2009). These findings are of particular interest, as *P. falciparum* is the major cause of malaria related deaths worldwide due to its inherent ability to develop drug resistance. Continuous discovery of new drug treatments and strategies are of utmost importance, since many tropical parasite populations are starting to exhibit increased resistance to all currently used antimalarials (Hanboonkunuparkarn & White, 2016).

Igoli *et al.* (2012) explored the possible anti-trypanosomal activity of hexane *S. aethiopicus* extracts. *Trypanosoma brucei*, a parasite transmitted by the tsetse fly, causes human African trypanosomiasis, also known as African sleeping sickness. It is a tropical illness which is often neglected, but virtually always fatal if left untreated (Trindade *et al*., 2016). During the

investigation conducted by Igoli *et al.* (2012), the sesquiterpenes, e*pi*-curzerenone and furanodienone (section 2.2.2) demonstrated notable anti-trypanosomal activity against *T. brucei* blood forms, with an MIC of 6.9 µM, without any detrimental cytotoxic side effects. It also exhibited increased activity, compared to the crude extract. The diterpene, 8(17),12E-labdadiene-15,16-dial, on the other hand, displayed an MIC of 5.3 µM. Remarkably, the sesquiterpenoid compounds, as well as the diterpene compound, displayed MIC values higher than that of suramin (10 µM), a conventional anti-trypanosomal drug, known for its nephrotoxicity.

2.2.5 Commercial availability, trade and conservation

Traditional medicines are currently a major source of novel drug discovery with the purpose being further commercial product development (Igoli *et al.*, 2012; Street & Prinsloo, 2013; Bergh, 2016). Despite being classified as critically endangered in South Africa, *S. aethiopicus* is estimated as being one of the most popular medical plants and subsequently possesses a pronounced potential for commercial production (Street & Prinsloo, 2013). Numerous commercial products claiming to contain African ginger are readily available, specifically: Phyto nova African Ginger® tablets, Hot Toddy® sachets and effervescent tablets containing African ginger, Nature's health products African Ginger® capsules, Healing Earth® spa products, Big Tree African ginger® tablets and Bioharmony bio-African ginger® tablets (Bergh, 2016).

Herbal markets are expanding worldwide and have the potential of large commercial benefit. Nonetheless, it poses a possible threat to biodiversity due to the risk of overharvesting of raw material. Natural habitats and resources face the danger of being destroyed and entire species going extinct if these practices are not meticulously controlled (WHO, 2003). Large-scale exploitation of wild populations to supply the demand of Southern African herbal medicine markets remains the largest threat to *S. aethiopicus*, although habitat loss should also be considered (CITES, 2016). Southern African genetic reservoirs are not adequately protected, with 65% of the remaining natural populations situated outside of conservation areas and therefore vulnerable to exploitation. *S. aethiopicus* has for long been listed as critically endangered on the South African National Biodiversity Institute (SANBI) red list (Lötter *et al*., 2006), but has not been assessed for the International Union for Conservation of Nature (IUCN) Red List (CITES, 2016). Despite this, a study performed in 1998 found that 1.9 tonnes of *S. aethiopicus* were sold annually in South Africa alone. At an average trading price as high as R 450/kg, 10–30 times the average price of other popular plants, it has been one of the most expensive, yet still most sought after, medicinal plants available (Mander *et al.*, 1998; Xego *et al.*, 2016). A more recent investigation found vendors to cut the rhizomes into smaller pieces and sell them at R 5–R 7 each (Moeng, 2010). In order to supply the high demand of local traditional markets, cross-border trade from neighbouring Zimbabwe, Mozambique and Swaziland has increased as a result of the scarcity of African ginger in South Africa (CITES, 2016).

It has therefore become increasingly crucial to explore various ways to prevent overexploitation and extinction of *S. aethiopicus*. The WHO (2003) found that the legislative control of medicinal plants does not evolve around a structured model, as medicinal products are defined differently in different countries and various methods have been adopted with regards to the licensing, manufacturing, dispensing and trading thereof. Currently, no certification of indigenous medicines traded in South Africa is required, but products traded as medicine must be registered in accordance with legislation. Unfortunately, due to the informal nature of the traditional medicine market, the said legislation is not applicable. This limits investment in the formal cultivation of not only *S. aethiopicus*, but all plants used as traditional medicine. Other consequences include that very little standardisation is being applied to product quality, for example: shockingly, recycled newspaper and re-used liquor bottles are used as packaging. Hence, the stability and hygiene of these products are of immense concern, not only to consumers, but traditional healers, alike (Mander, 1998; Mander *et al*., 2007).

Besides legislative methods, more sustainable harvesting practices should also be explored to protect the preferred underground parts of the *S. aethiopicus* plants against destructive harvesting practices, such as substituting the trade in only certain parts of the plant to all parts, based on scientific evidence that no viable medicinal differences exist among the leafs, stems, rhizomes and roots. Chromatographic results of root and rhizome extracts narrowly resembled each other, whereas those of the leaf and stem extracts were also relatively similar (Zschocke *et al.*, 2000). A study by Viljoen *et al*. (2002) confirmed these findings, as the essential oil constituents of the rhizomes and roots were almost identical. Studies by Zschocke *et al.* (2000) and Coopoosamy *et al.* (2010) moreover reported aerial plant parts to respectively display COX-1 inhibition and antimicrobial activities fairly similar, or slightly less, compared to extracts of underground parts. These outcomes thus support the concept of plant part substitution and traditional healers should be made aware of the possibility to adjust their harvesting practices. Subsequently, collaborative research practices involving the researcher, traditional healer and community are imperative to ensure benefit to all. The education of traditional healers with regards to the findings from pharmacological studies, would not only enable them to improve their prescription and preparation methods, but also advance conservation and benefit the general health of consumers alike (Zschocke *et al.*, 2000; Viljoen *et al*., 2002; Stafford *et al.*, 2005).

2.2.6 Cultivation and propagation

S. aethiopicus was one of the first medicinal plants Geoff Nichols propagated via tissue culture; he was furthermore involved with the first efforts to clone the plant material at Kirstenbosch Botanical Gardens in Cape Town, South Africa. Growing clones were uncomplicated and the plants grown from one or two clones were gathered for the traditional medicine market. Cloning was, however, not the answer to reintroduce the plant into the wild and save it from complete

extinction, as it lacked the wide range of genetic material to be able to seed freely. Pollen incompatibility problems also arose (Nichols, 2005).

As the seeds of *S. aethiopicus* develop underground and are difficult to locate, vegetative dissemination remains the preferred method of propagation (Street & Prinsloo, 2013). Rhizome splitting and tissue culturing are the most popular vegetative means by which *S. aethiopicus* has been successfully propagated and are some of the most efficient means of doing so. During the dormant phase in June to November rhizomes can be dug up, divided, and replanted (Nichols *et al*., 1989). The commercial advantage of cultivation lies therein that it provides the opportunity to ensure a regular supply of plant produce, even at times when it would be scarce, whereas general dependence on wild populations for supply results in seasonal and scarcity related shortages. Indigenous medicinal plants require relatively little attention and are well suited to low input agricultural systems (Mander, 1998). *S. aethiopicus* is easily cultivated in the warmer regions of Southern Africa, especially in the warm Lowveld and sub-tropical East coast regions. Loose, friable soil that is well drained and rich in organic material provides optimal growing conditions (Nichols *et al*., 1989). Consequently, cultivation of *S. aethiopicus* should be economically viable, as it is high in demand and the profits received from these high value crops will justify the effort (Street & Prinsloo, 2013).

Several cultivation initiatives have been implemented in South Africa. In particular, Silverglen nursery in KwaZulu-Natal initiated an admirable training program to educate traditional healers in cultivation procedures to reduce the pressure on remnant wild populations. Intriguingly, a study conducted by Manzini (2005) revealed that 60% of participating traditional healers confirmed they would be interested to learn more about various cultivation processes pertaining to *S. aethiopicus*, should further research be conducted; and 17% voiced that research pertaining to other aspects of African ginger would also be of value. Though commercial production and cultivation of the species could benefit conservation and be economically viable to the community, care should be taken to avoid biopiracy. Biopiracy describes the actions of pharmaceutical companies that patent traditional remedies or medicine, sell them at a significant profit, and do not ensure that a share of that profit is ploughed back into the native community (Roberson, 2008).

2.3 FORMULATION FACTORS TO CONSIDER WHEN UTILISING HERBAL MEDICINES

2.3.1 Common constraints encountered with the standardisation of herbal medicine

Herbal medicines have in recent years enjoyed an increase in their acceptability as alternative treatments for diseases like arthritis and diabetes (Chawla *et al.*, 2013). However, the standardisation of herbal medicines is a major concern and an impediment to its universal acceptance (Kunle *et al.*, 2012). More than 40% of herbal medicines lack reproducibility of pharmacological activities when extracts are re-sampled or re-extracted (Ahmad *et al.*, 2006). Herbal preparations are affected by a range of variable factors that are not relevant to synthetic, or allopathic drugs. Environmental, genetic and cultural factors all affect the quantity and quality of active compounds in herbal medicines. For example, the quality and obtainability of raw plant materials are often challenging, multiple active constituents are contained in extracts and are often unknown (not even to mention the further complexity of poly-herbal formulations), reference compounds and analytical techniques are not commercially available, whereas the existence of chemo-varieties and chemo-cultivars complicate authentication. Furthermore, agricultural and manufacturing practices such as the use of fresh or dried plants, the plant parts used, accessibility to water and nutrients, light exposure and harvesting practices, storage, packaging and processing methods employed (polarity of extracting solvent, extraction method and stability of target compounds); all profoundly impact the chemical composition of a plant extract. All of these factors contribute towards variability in the constituents and batch-to-batch inconsistencies (Kunle *et al.*, 2012). Further challenges include contamination and adulteration of herbal ingredients with substances, for example: heavy metals, pesticide residues, fumigants and pathogenic contamination (Ahmad *et al.*, 2006; Kunle *et al.*, 2012).

Chromatographic techniques and identification of marker compounds assist in developing chemical fingerprints for herbal products, which may contribute to improving batch-to-batch uniformity, but they do not ensure consistent chemical stability, nor biological activity. To ensure the safe use and efficacy of any pharmaceutical dosage form, consistent composition and pharmacological activity are absolute requirements. Nonetheless, the lack of scientific research on active constituents, variances in compounds and their concentrations, together with the immense number of extraction and processing techniques used by various manufactures, deliver commercially available products that prominently fluctuate in their quality and content. In order to advance the possibility of integrating herbal remedies with modern medicine, herbal materials and their active constituents need to be correctly identified and standardised, their pharmacological efficacies and toxicities investigated, clinical and non-clinical trials be conducted,

Good Agricultural Practices (GAP), Good Sourcing Practices (GSP) and Good Manufacturing Practices (GMP), as well as strict regulations with regards thereto be implemented (Ahmad *et al.*, 2006).

In summary, the standardisation and development of herbal remedies are intricate phenomena that require the multi-disciplinary involvement of botanists, pharmacognosy, ethnomedicines, pharmacology, toxicology, cheminformatics, biotechnology, biochemistry, drug development and regulatory/economic affairs. During these processes, each step needs to be carefully evaluated so as to safeguard the efficacy, quality, reproducibility and safety of the herbal preparation (Chawla *et al*., 2013).

2.3.2 Herbal dosage form design

Pharmaceutical dosage forms consist of a combination of active ingredients and excipients. The formulation of a dosage form containing a herbal extract, or a "phytomedicinal product", like its standardisation, is quite complicated due to the mixture of myriad phytochemicals contained therein (Ogaji *et al.,* 2012). In contrast to allopathic medicines that usually contain only one chemical entity, these elements each has a different mechanism of action, while also acting on different parts of the body to produce the desired effect (Robotin, 2012). A single therapeutic target approach, as often used by orthodox drug regimens to investigate the efficacy of drugs, would fail to detect the resulting synergistic interactions of phytomedicines on multiple therapeutic targets (Moreira *et al.*, 2014). Proper formulation is required to enable these compounds to reach their biological target areas and exert their pharmacological activities. Meagre solubility and/or permeability limit absorption and bioavailability of active constituents, whereas their susceptibility to degradation reactions accelerated by factors such as pH, temperature, humidity, light and oxygen, necessitate the evaluation of their shelf life with the purpose of ensuring product stability during consumption (Cortés-Rojas *et al.*, 2016). Hence, in order to warrant therapeutic efficacy of a phytomedicinal product, it is of utmost importance to ensure a suitable dosage form design is developed and its subsequent pharmaceutical properties evaluated (Palma *et al.*, 2002).

Liquid dosage forms are most common for herbal products and are obtained from infusions, decoctions and macerations. General disadvantages of these dosage forms include large dosage volumes, mediocre physicochemical stability, microbial contamination susceptibility, and problematic packaging (Qusaj *et al*., 2012; Cortés-Rojas *et al.*, 2016). Solid dosage forms, for example tablets, generally have higher stability, are less complicated to standardise and are convenient dosage forms that consistently deliver accurate doses. Formulation of a suitable tablet dosage form for herbal medicines, however, is still very demanding due to the intrinsic poor rheological and compactability properties of dry herbal extracts, or powdered plant materials. Restricted information regarding the influence of extracts on the physical-mechanical

characteristics of frequently used excipients complicates considerations even more (Palma *et al.*, 2002; Qusaj *et al*., 2012). Pharmaceutical excipients therefore need to be chosen carefully.

2.3.3 The importance of interactions between excipients and active pharmaceutical ingredients

Excipients (e.g. fillers, disintegrants, solvents, binders) are crucial to assist the efficacy; safety; manufacturability; and stability of active compounds within a dosage form (Ogaji *et al.,* 2012; Panakanti & Narang, 2012). Conversely, the core function of any excipient remains to warrant the efficacy and safety of active pharmaceutical ingredients (APIs) during formulation, storage and administration (Abrantes *et al.*, 2016). They are generally considered to be pharmacologically inert, but can still undergo physical and/or chemical interactions with active pharmaceutical compounds, as they are in direct contact (Crowley & Martini, 2001; Bharate *et al.,* 2010; Fathima *et al*., 2011; Hotha *et al*., 2016). Excipients can potentially affect a series of processes, including: disintegration of the dosage form, drug dissolution and stability, as well as interaction of the drug with biological factors by modifying drug substance characteristics; all of which could result in the altered absorption, distribution, metabolism and elimination (ADME) of the API, ultimately resulting in reduced bioavailability (Panakanti & Narang, 2012; Abrantes *et al*., 2016).

Possible sources of interactions include functional groups of excipients and/or impurities, as well as residues contained within the excipients. These interactions can cause degradation of the active compound or produce harmful reaction products, resulting in decreased stability, bioavailability, therapeutic efficacy and safety of the product (Crowley & Martini, 2001; Bharate *et al.,* 2010; Fathima *et al*., 2011; Panakanti & Narang, 2012). Incompatibilities between the active ingredient and excipients must be assessed during pre-formulation studies to ensure optimal dosage form attributes are obtained (Bharate *et al.,* 2010). Many stability problems faced during formulation can be accredited to a lack of vigilance regarding the complexities of physical and chemical drug-excipient interactions, often resulting in the formation of low levels of novel entities. Awareness of a drug's tendency to undergo degradation reactions together with familiarity of excipients' reactivities and the residues they may contain, would assist in anticipating and avoiding the occurrence of undesirable interactions (Fathima *et al*., 2011).

Adsorption of active ingredients to the surfaces of some excipients is an example of a physical interaction. Although it commonly occurs, it is difficult to detect since no chemical changes are involved. Such interactions can either be detrimental or beneficial to drug delivery, as it frequently affects the drug dissolution rate. Adsorption of active compounds to excipient surfaces may either increase the surface area of drug particles and result in increased drug release rates, or decrease the amount of free drug available for dissolution and diffusion, causing low bioavailability (Fathima *et al.,* 2011; Panakanti & Narang, 2012). For example the adsorption of a novel κ-opioid agonist

to the surface of microcrystalline cellulose (MCC) led to incomplete drug release. It may also initiate chemical degradation (Fathima *et al.,* 2011).

Chemical interactions occur when active ingredients and excipients form new and often unstable compounds through a chemical reaction. These interactions are almost always damaging, as the nature of degradation products are often toxic. Examples of possible chemical interactions include hydrolysis, oxidation, Hofmann elimination, and photodegradation, among others (Fathima, 2011; Barnes, 2013; Hotha *et al*., 2016).

2.3.4 The function of fillers in pharmaceutical dosage forms and possible incompatibilities

Fillers are generally included in tablet formulations to increase the bulk powder volume and thus the size of tablets. They should be biocompatible; possess good biopharmaceutical (e.g. hydrophilic and water soluble) as well as technical properties (e.g. dilution capacity and compactability); be non-hygroscopic; and affordable (Alderborn, 2013).

2.3.4.1 Microcrystalline celluloses

Celluloses (Figure 2.6) are one of the most frequently used fillers. They are biocompatible and possess good disintegration properties. Microcrystalline cellulose (MCC) is the most frequently used cellulose powder in tablet formulation. It is an odourless, tasteless, white, porous powder that consists of purified, partially depolymerised cellulose (Alderborn, 2013; Thoorens *et al*., 2014). Depending on the relative position of the cellulose chains within the particle, MCC particles have crystalline and amorphous regions. Physical and technical properties, e.g. compactability and hygroscopicity of particles are affected by the degree of crystallinity. MCC can be utilised in wet and dry granulation, as well as direct compression tableting processes. Recently, MCC formulated nanoparticles and nanogels have been designed for modified drug release. MCC is hygroscopic, but insoluble in water, most organic solvents, and diluted acids. However, it is partially soluble in a 5% w/w sodium hydroxide solution. Celluloses may be incompatible with drugs prone to chemical degradation through hydrolysis, due to their hygroscopic properties. Listed in Table 2.3 are a handful of frequently used drugs that are incompatible with MCC. Commercially available MCCs (e.g. Pharmacel® 101 and Avicel® PH-101) come in numerous grades that differ with regards to particle size, moisture content, manufacturing method, and other physical properties. It is not systemically absorbed following oral administration and demonstrates little toxicity. It is considered a non-irritant and non-toxic material. Though unlikely when used as a pharmaceutical excipient, MCC may have a laxative effect when consumed in large amounts (Alderborn, 2013; Thoorens *et al*., 2014; Quinn & Sun, 2016).

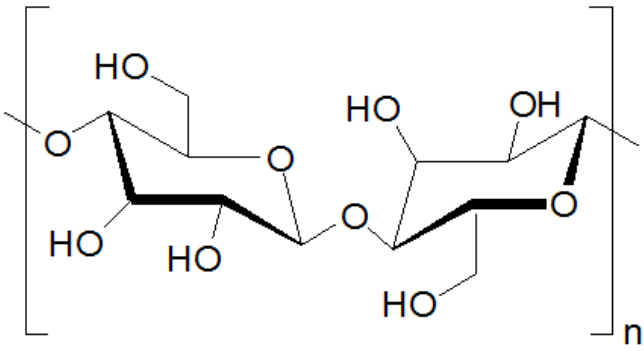


Figure 2.6: *Chemical structure of microcrystalline cellulose – adapted from Quinn & Sun (2016)*

2.3.4.2 Chitosan

Chitosan, a polysaccharide that consists of *N*-acetylglucosamine and glucosamine co-polymers, is produced through the partial deacetylation of chitin. The composition of chitosan may vary, depending on the manufacturer, since the nomenclature with regards to the different degrees of *N*-deacetylation between chitosan and chitin has not yet been clearly defined. Commercially available chitosan products come in numerous grades that vary in their degree of deacetylation, molecular weight and viscosity (Ray, 2011; Jones, 2016). Chitosan (as illustrated in Figure 2.7) has been introduced as a filler often used in various pharmaceutical formulations, including oral, nasal, transdermal and parenteral drug delivery systems. It possesses pronounced film forming properties, enhances drug absorption through muco-adhesion and inhibits bacterial growth and infection (Rinaudo, 2006). The cationic characteristics thereof are unique, as it is a pseudo-natural cationic polymer. It has a high charge density and subsequently adheres to negatively charged surfaces. It also has significant complexation ability with metals, as well as forming electrostatic complexes with oppositely charged molecules; and as such alters the physicochemical characteristics thereof (Ray, 2011; Jones, 2016). Table 2.3 summarises generally used drugs that are incompatible with chitosan.

Factors that affect how the diverse polymer is pharmaceutically used include the salt form, molecular weight, pH and degree of deacetylation. It is sparingly water soluble and insoluble in organic solvents (e.g. ethanol), alkali or neutral pH solutions above 6.5 (Rinaudo, 2006; Jones, 2016). The monomeric unit, D-glucosamine, is protonated in an acidic medium, resulting in a soluble, uncoiled polysaccharide chain. Importantly, its solubility is dependent on the degree of deacetylation, along with the pH of the solution (De Kock, 2005).

Generally, chitosan is regarded as a non-irritant and non-toxic excipient, with a lethal dose 50% (LD_{50}) value of above16 g/kg. It is biodegradable and biocompatible with infected and healthy skin (Jones, 2016). However, some studies have found soluble chitosan salts to display

concentration-dependent toxicity towards cell cultures *in vitro*. It was also found to cause erythrocyte lysis. The chitosan salt used was a definite contributing factor to the observed cytotoxicity, since the counterion governed the interaction of the cationic amine group of chitosan with anionic cellular components. Chitosan salts can be ranked as follows with regards to cytotoxicity: hydrochloride > hydroglutamate > glycol chitosan > hydrolactate; with chitosan hydrochloride (CL 210) revealing an IC_{50}-value of 0.21 ± 0.004 mg/ml (De Kock, 2005).

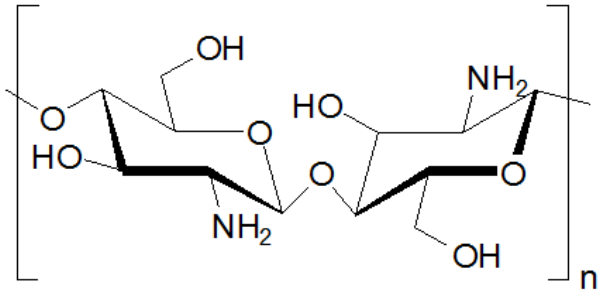


Figure 2.7: *Chitosan or poly(D-glucosamine) - adapted from Rinaudo (2006)*

Table 2.3: *Frequently used fillers and known incompatibilities with pharmaceutical active compounds – adapted from Bharate et al. (2010)*

Excipient	Incompatible active compound
Microcrystalline cellulose	Enalapril, isosorbide mononitrate, clenbuterol,
Chitosan	Diclofenac and piroxicam

2.3.5 Extraction: the importance of polar and nonpolar solvents

Extraction is a process used to remove the active constituents from dried plant material. The plant material is soaked in a solvent during which the constituents are dissolved and diffuse into the liquid. Extract types include liquids, purified and dry extracts,; and tinctures; the choice thereof depends upon the intended application. Liquid extracts are frequently utilised in liquid formulations, whilst dry extracts are most suitable for capsule and tablet formulations. The process to re-dissolve dry extracts to form a liquid dosage form, often results in the presence of turbidity or precipitation of some components. Downstream processing may also modify the composition of the initial extract preventing dissolution thereof. This necessitates the use of co-solvents or pH manipulation to improve solubility and stability (Lockwood, 2013).

Compound extraction from plant material is perplexing and difficult due to the wide-ranging variation in chemical structure and polarities of numerous phytochemical compounds contained therein (Abarca-Vargas *et al.*, 2016). Various extracts need to be prepared with the polarity of target constituents kept in mind, as it will impact the choice of solvent, as well as extract stability, extraction yield and, eventually, biological activity thereof (Lockwood, 2013; Abarca-Vargas *et al.*,

2016). Different solvents will extract varying amounts of active constituents from plant material due to their polarity differences. For example: hexane would extract waxes, fixed oils and fats; methanol would extract amino acids, sugars and glycosides; whereas acetone would extract aglycones, glycosides and alkaloids (Bagla, 2012). Solubility of constituents is another important consideration, as only compounds in solution are able to cross cell membranes for absorption (Ashford, 2013). Although the solubility of different compounds will determine the most suitable solvent for extraction and re-suspension processes, formulation constraints often result in partial or complete insolubility as extracts contain various compounds with ranges of differing solubilities (Lockwood, 2013). Coopoosamy *et al.* (2010) stated that solvent selection can serve as a form of separation by utilising the polarity of constituents - less polar compounds are typically extracted with less polar solvents and *vice versa*. Consequently, the selection of a suitable extraction solvent and technique are important parameters to consider, as they will contribute towards the standardisation of a given herbal preparation and enable the extraction of desired, soluble and active compounds, while excluding unwanted compounds (Abarca-Vargas *et al.*, 2016; Dhanani *et al*., 2017).

Evidence found by Stafford *et al.* (2005), Steenkamp *et al.* (2005) and Fouche *et al.* (2011), among others, indicated that numerous extracts exhibited various levels of activity (and cytotoxicity); a possible indication of different compounds present in each extract. Stafford *et al.* (2005), for example, stated that the COX-inhibitory activity of ethanol extracts increased with storage time, whereas water extracts exhibited a loss in activity. Traditional healers should be made aware of these findings through a consultation process in order to improve prescribing practices, as well as to prevent the preparation of potentially toxic extracts. For instance, while traditionally, rhizomes are commonly prepared as hot or cold infusions or steamed for inhaling (Fouche *et al.*, 2013), Light *et al.* (2002) found aqueous rhizome extracts to be potentially cytotoxic, whereas extracts prepared from leaves had little to no cytotoxicity. Such findings are not only possible indicators that different compounds are present within the extracts, but also of the varying levels of stability of these compounds within a selected solvent.

2.4 BUDDING USE OF ETHNOMEDICINES: OBSTACLES IN MONITORING SAFETY

2.4.1 Contributing factors to the recent surge in popularity of herbal medicine use

As mentioned previously, the usage of herbal medicines has of late experienced a resurgence of general public interest in developing and developed countries alike. Consumers consider herbal

remedies to be a more sensible and balanced technique of healing and believe it to promote healthier living (Kunle *et al.*, 2012; Ekor, 2014).

The recent spike in popularity has been ascribed to several elements, including but not limited to (i) numerous claims of efficacy of herbal remedies, (ii) consumers showing greater interest in alternative and complementary medicines, (iii) misperceptions that herbal remedies are safe and superior to orthodox pharmaceuticals, (iv) frustration with regards to deficient results from modern medicines, (v) high financial implications and adverse effects of allopathic drugs, and (vi) the increased campaign toward self-medication. Patients furthermore select herbal drugs through an inferential methodology based upon subjective information, that is, "it worked for a colleague, a relative, or a friend". Mass media marketing has also considerably increased patients' awareness of herbal remedies and has contributed towards their perceived credibility, with manufacturers and sales executives employing a multitude of marketing strategies, including radio and television programs, to cast their products into the public eye (Ekor, 2014).

The renewed popularity of herbal preparations has additionally projected much needed attention on issues relating to their safety and their impact on public health. Though many herbal medicines have auspicious potential, numerous products remain untested and unregulated, resulting in limited awareness of their rational use, adverse effects and contra-indications (WHO, 2002; Ekor, 2014).

2.4.2 Importance of toxicity screening of herbal medicines

As stated, traditional medicine, or more specifically, herbal preparations, play a profound role in the primary health care of not only humans, but animals as well. Nonetheless, our present standpoint on the acute and chronic cytotoxic effects of the majority of medicinal plants is at an undeveloped stage. This is alarming, as a large proportion of poverty stricken communities rely on long term use of these plants to satisfy their health care needs (Bagla, 2012). One might expect bioactive compounds acquired from plants to have a low toxicity, but the reality is that many endemic cultures simply do not have a reporting system to accurately document experienced adverse effects. Acute toxic effects, for example dermatological and gastro-intestinal disturbances, would obviously be noticed resulting in the plant being used cautiously, if at all. Contrary, chronic toxic effects, such as liver or kidney damage and cancer, would less likely be observed, resulting in their continuous use (Fabricant & Farnsworth, 2001; Moreira *et al.*, 2014).

Prior to being launched into the market, herbal medicinal products are not required to undergo any obligatory safety or toxicological evaluation in most countries. Lacking infrastructure to regulate manufacturing practices and implement quality control procedures further complicate trade in these products, not to mention the unrestricted availability of these products to consumers

without a prescription (Kunle *et al.*, 2012; Ekor, 2014). According to Taylor *et al.* (2003), the determination of efficacy and long-term safety of herbal preparations is a vital step towards making them acceptable from a first-world perspective. Therefore, toxicity testing is necessary to identify possible risks that may be detrimental to human health. Attempts have been made to implement global standards for toxicological characterisation, tests to determine acute high-dose and chronic low-dose toxicities, as well as cellular, organ and system based toxicity assays specifically for herbal medicines administered alone or in combination with other herbs or drugs. Through early detection of potential toxic plant extracts or compounds contained within, possible toxicants can be discarded or modified to deliver safer alternatives (Ifeoma & Oluwakanyinsola, 2013). There are several indications that many traditional herbal preparations and/or their active constituents may particularly cause liver injury, or be carcinogenic. Even more so, co-administration of herbal preparations and pharmaceutical drugs may cause pharmacokinetic interactions (Chen *et al.*, 2010; Gouws *et al.*, 2012; Hermann & Von Richter, 2012; Hoenerhoff *et al.*, 2013; Li *et al.*, 2013; Moreira *et al*., 2014; Calitz *et al*., 2015). Table 2.4 lists only a few medicinal plants and their potential toxic effects.

Table 2.4: *Potential toxic effects observed with the consumption of common herbal medicines for different indications – adapted from Ifeoma and Oluwakanyinsola (2013)*

Common name	Plant source	Indications	Potential toxicity
Ginseng	*Panax ginseng* roots	To relieve stress and promote mental and physical performance	Hypertension, central nervous system stimulation, skin eruptions
St. John's Wort	*Hypericum perforatum* aerial parts	Antidepressant and mood stabiliser	Potent cytochrome P450 (CYP450) enzyme inducer, hepatotoxic and nephrotoxic in pregnancy
Aloe	*Aloe vera* leaves	Laxative and wound healing	Cytogenetic toxicity

2.4.3 Scientific evidence of possible cytotoxic and genotoxic effects of *Siphonochilus aethiopicus* plant extracts

Some studies have indicated that certain extracts of *S. aethiopicus* possess genotoxic and cytotoxic effects. Lategan *et al.* (2009) determined crude ethyl acetate extracts to possess an IC_{50}-value of 73.9 µg/ml (±12.8), by employing a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or tetrazolium reduction (MTT) assay. Taylor *et al.* (2003) and Steenkamp *et al*. (2005) both utilised comet assays and observed DNA damage with both methanol:water (9:1) and methanol extracts. Steenkamp *et al.* (2005) furthermore postulated that the DNA damage induction caused by aqueous and methanol *S. aethiopicus* extracts could be a

possible mechanism of antibacterial activity. They similarly observed that aqueous extracts potentiated lipid peroxidation in cell membranes, showing pro-oxidant capacity, but on the contrary, also had substantial hydroxyl radical scavenging activity. It is however not unusual for an extract to exhibit contradictory properties (pro-oxidative *versus* anti-oxidant), as these tests measure different endpoints in oxidative stress.

An analysis of aqueous leaf and rhizome extracts (3.9–1000 µg/ml) was conducted by Light *et al.* (2002) to investigate the cytotoxic effects thereof on vervet monkey kidney (VK) cells over a period of 7 days. Minimal morphological changes were observed through light microscopy with regards to the leaf extracts at concentrations higher than 250 µg/ml; which was possibly indicative of cytotoxicity. The MTT assay confirmed this low level of cytotoxicity with a cell survival of 75% for leaf extracts, whereas it indicated that rhizome extracts were potentially cytotoxic, with a cell survival of less than 50% at a concentration of 125 µg/ml (Light *et al*, 2002). This was an interesting discovery as traditionally the rhizomes are often administered as a hot infusion; or steamed and the vapour inhaled (Fouche *et al.,* 2013).

Due to the multi-compound nature of *S. aethiopicus*, a new interest into investigating the medicinal and toxic properties of specific individual compounds has flared up. Igoli *et al.* (2012) found that the diterpenes (8(17),12E-labdadiene-15,16-dial and 15-hydroxy-8(17),12E-labdadiene-16-al (section 2.2.2) are selectively cytotoxic to peripheral human T lymphocytes (Jurkat), epithelial human bone marrow (SH-Sy5Y) and fibroblast mouse sub-cutaneous connective tissue (L929) cell lines at 100 µg/ml, indicative of their anti-cancer potential, while only displaying a moderate effect on normal cell line, fibroblast human foreskin (Hs 27). However, the sesquiterpenoids (*epi*-curzerenone and furanodienone) showed no *in vitro* cytotoxicity at the same concentration.

Continuous research into novel, herbal therapies is necessary, as it often contains numerous active compounds, acting on multiple biological receptors, which may result in therapeutic success (Fouche *et al.*, 2013). As indicated before, *S. aethiopicus* is traditionally used to treat asthma, a chronic airway disease that requires continuous treatment as no cure exists to date. The necessity of prolonged consumption of *S. aethiopicus* raises concerns for patient safety. To completely comprehend the effects of this plant on human health, further cytotoxicity and selectivity tests are thus necessary (Fennell *et al.*, 2004).

2.5 DRUG DEVELOPMENT AND *IN VITRO* TOXICOLOGY: THE RELEVANCE OF CYTOTOXICITY ASSAYS

2.5.1 Drug development process

As illustrated in Figure 2.8, the drug development process consists of four phases, namely: early drug discovery, late drug discovery, pre-clinical and clinical phases. The two major contributing factors to drug attrition include toxicity and efficacy (Hughes *et al*., 2011). Late stage failures, particularly in pre-clinical and clinical stages, are enormous sources of economic and time inefficiencies; and accordingly represent some of the paramount difficulties faced by pharmaceutical companies today (McKim, 2010).

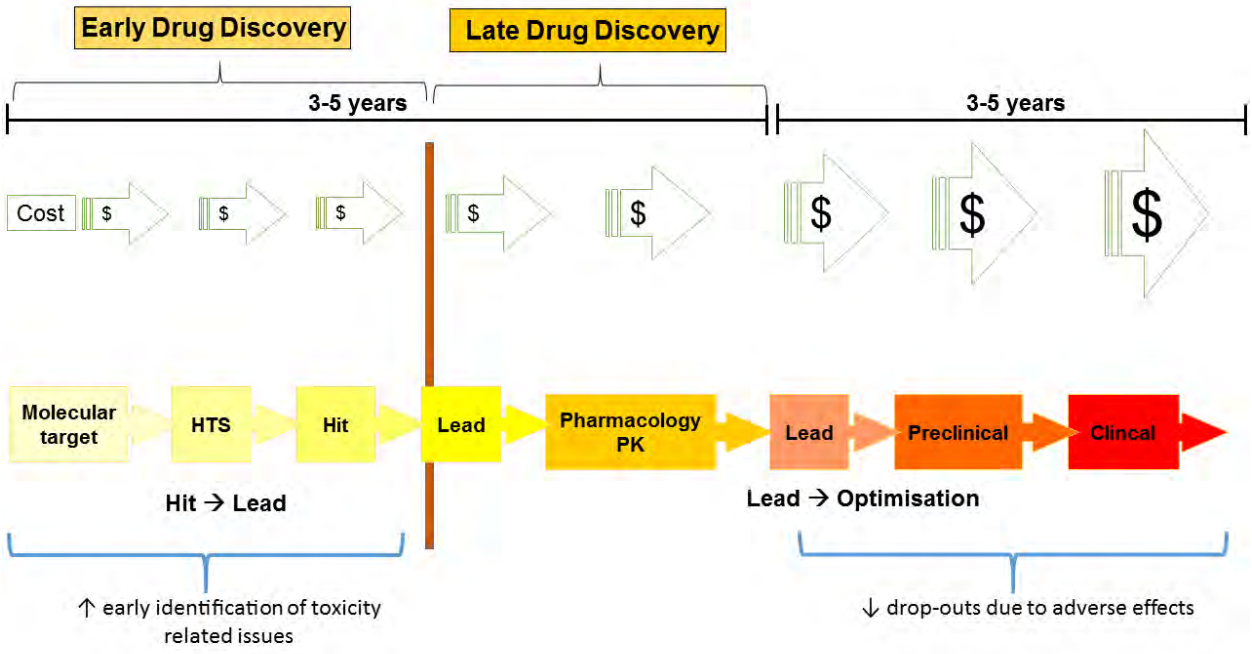


Figure 2.8: *The typical pathway of drug discovery – adapted from McKim (2010) and Hughes et al.* (2011)

Typically, a chemical library of compounds is utilised to screen molecular entities and identify those compounds that may influence or display activity towards the intended target. "Hits" or positive molecules progress to the process of lead identification and optimisation. Successful drug candidates will thereafter advance into pre-clinical and if lucrative, clinical phases. The capability to identify and reduce risk factors early on, would improve the probability of success and accelerate the drug development process (McKim, 2010; Hughes *et al.*, 2011).

2.5.2 *In vitro* toxicity and cell based *in vitro* cytotoxicity assays: more acceptable alternatives

2.5.2.1 Essence of *in vitro* toxicity models

Traditionally, toxicity testing has relied upon studying adverse effects in experimental animal models at later phases of pre-clinical drug development. These methods are economically and ethically time and cost consuming (Li *et al.,* 2003; Gerets *et al.,* 2009). Due to animal welfare concerns, immense pressure has arisen towards developing alternative methods, based upon the three R's (Goh *et al*., 2015): reduction of the sum of animals utilised, refinement of animal test models and replacement of animal use in research (Ifeoma & Oluwakanyinsola, 2013). Presently, nine out of ten potential APIs that enter phase I clinical trials, inevitably fail. Consequently, animal testing performed in the pre-clinical phases, associated with such unsuccessful molecule candidates, could have possibly been avoided. By utilising *in vitro* assays with more relevant predictive value, not only will overall animal use be reduced, but the quality of potential compounds increased and pre-clinical or toxicology related attrition will also be reduced (Chapman *et al.*, 2013).

Many *in vitro* assays have been designed and validated for early stage screening of new active compounds in order to reduce or even replace the use of certain *in vivo* tests and sieve out compounds with a higher potential for toxicity (Goh *et al*., 2015). Scientific and technological developments, together with recent regulatory and judicial changes have provided opportunities to support the implementation of *in silico* and *in vitro* alternatives to *in vivo* tests. For example, the validation of the Cytosensor microphysiometer test and the bovine corneal opacity and permeability (BCOP) test has led to their incorporation in international regulatory guidelines (Chapman *et al.*, 2013; Goh *et al*., 2015). Not only has the introduction of *in vitro* cytotoxicity assays streamlined the process of identifying the threshold safety values for new compounds, but it is deliberated to be almost obligatory during target validation and medicinal modification (Niles *et al.,* 2009). Utilising various biochemical endpoint *in vitro* assays can also potentially provide imperative indications of undesirable effects, as they provide valuable information pertaining to mechanisms of toxicity and potential subcellular targets (McKim, 2010).

2.5.2.2 Limitations of *in vitro* toxicity assays

It would, nonetheless, be foolish to assume that any single *in vitro* toxicity assay is an adequate indication of toxicity. For *in vitro* models to be valuable, they have to be technically characterised and extrapolative of *in vivo* effects. Data collected should elucidate toxicity mechanisms and provide information on sub-cellular targets (McKim, 2010). Typically, data produced by mechanism centred assays have a higher potential of inter-species translation, whereas bioassay centred models often provide data that can only be translated within a limited context (Chapman

et al., 2013). Nonetheless, it remains important to integrate cell based *in vitro* toxicity models that possess the ability to predict toxicity in rodents, earlier into the drug discovery process. Thereafter, potential drug compounds could be screened using *in vitro* models aimed at detecting human specific toxicity. Another important factor to consider is inter-species variation - even though a compound may be non-toxic in one species, it may still very well be toxic in another (McKim, 2010).

The truth remains that *in vitro* systems may be unable to replicate the complex physiological and biochemical interactions within *in vivo* animal models, but they do provide valuable extrapolative information that enables researchers to identify and reduce risks early on, significantly improve the efficacy of drug development, permit a reduction in animal testing and increase the likelihood of success (Li *et al.*, 2003; McKim, 2010).

2.5.2.3 Cell based cytotoxicity assays

Cell based assays utilised during high throughput screening (HTS) consists of three categories: reporter gene assays, second messenger assays and cell proliferation assays, of which the last is of particular importance to this study. They are particularly employed during early drug discovery and have delivered lead compounds of high quality (Zang *et al*., 2012). As stated by Zang *et al.* (2012), cell based assays have been used "from target identification and validation to primary screening, lead identification, optimisation, safety, and toxicology screening".

Cell based cytotoxicity assays are utilised to determine how cellular functions of cultured cells are affected by exposure to test substances. These tests are performed prior to animal safety studies and generally provide insight towards the ability of compounds, in this case plant extracts, to affect cell viability and either inhibit cellular growth, or have genotoxic and carcinogenic effects. Several parameters are used to determine cytotoxicity, including cell viability markers, for example membrane and metabolic markers, inhibition of proliferation, as well as morphologic and intracellular differentiation markers. These assays are valuable tools for medium and high throughput screens of diverse phytochemicals concurrently over wide concentration ranges. A selection of numerous cell lines is encouraged, as compounds (or herbal extracts) may display cytotoxic effects only against a specific cell type (Ifeoma & Oluwakanyinsola, 2013).

Advantages of cell based *in vitro* tests include: the provision of data to estimate the efficacy to safety window for compounds; requires a minimal amount of compound; results are easily reproducible; and they shorten the drug development process. Contrary, these models do have limitations, including a risk of false negative and/or positive results, a lack in solubility of compounds, low cell permeability, and short exposure times that may cause toxic compounds to slip through undetected (Li *et al.*, 2003). The incidence of false negative and/or positive results can be significantly reduced if multiple parameters of cell death are assessed. Moreover, *in vitro*

exposure concentrations often have diminutive significance to *in vivo* reference values, such as toxic plasma concentration, since only a few exposure concentrations are used (McKim, 2010). Even so, these cell based systems bare the characteristics of a more relevant biological micro-environment and consequently epitomises a respectable concession between *in vitro* biochemical assays and whole organisms. They can provide information concerning the test compound's cell permeability and intracellular stability, as well as its acute cytotoxicity and tissue specific responses (Zang *et al.*, 2012).

2.5.3 Cultured human hepatocellular liver carcinoma (HepG2) and human epithelial colorectal adenocarcinoma (Caco-2) cells

Two of the most widely studied cell lines, HepG2 and Caco-2, were utilised during this study and consequently their particular relevance is be discussed. These cell lines were chosen due to their respective predictive nature to assess liver and gastro-intestinal tract relevant organ cytotoxicity (Sahu *et al*., 2014). Both cell lines are utilised in key *in vitro* assays during drug discovery; Caco-2 cells are used to investigate permeability and drug absorption, whereas HepG2 cells are used to scrutinise possible hepatotoxicity (Hughes *et al.*, 2011). Hepatotoxicity is one of the most prominent factors that contribute towards the failure of potential drug candidates (Noor *et al.*, 2009). Various plant species have also been associated with hepatotoxicity or liver injury, including comfrey (*Symphytum officinale*), kava kava (*Piper methysticum*) and senna (*Cassia acutifolia*) to name only a few (Calitz *et al*., 2015).

HepG2 cells have been well characterised and are generally used to determine cytotoxicity as they possess higher predictability for humans compared to other animal cell lines. The HepG2 cell line presents similar phenotypic and genotypic properties to normal liver cells and are highly differentiated (Dehn *et al.*, 2004; Gerets *et al.*, 2009; Gerets *et al.,* 2012; Sahu *et al.*, 2014). Cells retain the ability to produce plasma- and lipoproteins, as well as cell surface receptors. In addition they have proven to preserve cytochrome (CYP) P450 reliant mono-oxygenase enzymes, as well as sulphate- and glucuronic conjugation enzymes. These properties render the HepG2 cell line useful for toxicological studies, particularly for detecting possible hepatotoxic compounds (Dehn *et al.*, 2004). Low metabolism capacity compared to primary liver cells, especially low phase I CYP enzymes, could be responsible for high false negative results and represents the main limitation of this cell line (Gerets *et al.*, 2009). Various parameters can be used to determine possible toxicity of compounds on hepatocytes, including: membrane integrity, metabolic functions and other non-specific viability indicators. These parameters detect functional or structural perturbations that impair cellular functions (Dehn *et al.*, 2004). Regardless of their metabolic limitations, screening of multiple cytotoxicity parameters on HepG2 cells can still yield a hepatotoxicity prediction of 90% specificity and 80% sensitivity (Noor *et al*., 2009).

The Caco-2 cell line has been comprehensively employed as a model to investigate the physiology of the intestinal barrier and is one of the foremost cell lines for investigating *in vitro* toxicology (Sambuy *et al.*, 2005; Sahu *et al.*, 2014). These cells differentiate spontaneously in culture and form a cellular mono-layer. Caco-2 cells display many biochemical and morphological attributes of mature small intestine enterocytes. In their differentiated state, they also bear a close resemblance to intestinal epithelium (Sambuy *et al.*, 2005; Natoli *et al.*, 2012; Sahu *et al.*, 2014). Caco-2 cells, furthermore, display a cylindrical morphology of a polarised nature. Microvilli can be located on the apical membrane, while duodenum hydrolase enzyme activities are similarly expressed here. Tight junctions are furthermore present between neighbouring cells (Sambuy *et al.*, 2005). Several studies have indicated the correlation between a compound's permeability through Caco-2 cells and its oral absorption (Fernandes *et al.,* 2012). However, reproducibility problems, due to intrinsic variability of cells utilised in various laboratories, limit the comparability of results (Natoli *et al.*, 2012).

2.5.4 Cytotoxicity, apoptosis and necrosis: the underlying relationship

Cell death is a normal part of the cell cycle and is a resulting response to many endogenous modulations, including inflammation, while also occurring post exogenous xenobiotic exposure (Kanduc *et al*., 2002). Cytotoxicity can broadly be described as the cell killing property of a chemical compound, independent of the mechanisms of cell death (Rode, 2008). A cytotoxic compound can be defined in terms of *in vitro* cell culture systems as any given compound that alters cellular attachment, has any undesirable effects on cell growth, considerably alters cellular morphology, or causes cell death (Niles *et al.*, 2008). The MTT, lactate dehydrogenase (LDH) and neutral red assays (Figure 2.9) are of the most regularly used assays in the detection of cytotoxicity or cell viability; and are frequently utilised when searching for an anti-cancer drug, or unwanted cytotoxic effects of drugs, before investing in further development. Through the utilisation of several *in vitro* cytotoxicity assays, instead of only one, the reliability of obtained results are increased (Fotakis & Timbrell, 2006; Igoli *et al*., 2012). However, the mechanism by which cells die remains an important consideration in cytotoxicity testing (Du Plessis & Hamman, 2014).

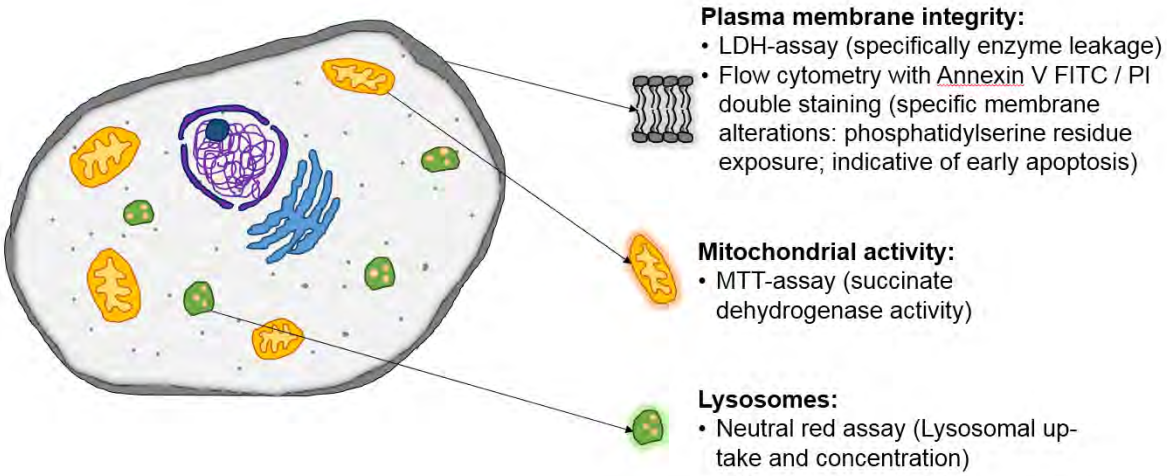


Figure 2.9: *A schematic representation and summary of common cytotoxicity and cell viability assays along with the specific cellular area they each affect, including plasma membrane integrity, mitochondrial activity, and lysosomal activity*

2.5.4.1 The morphologic appearance of cell death: apoptosis versus necrosis

Cell death can be classified by its morphological appearance as apoptotic, necrotic, and autophagic (Kroemer *et al.*, 2009). Only apoptosis and necrosis are discussed in this section.

Apoptosis is characterised by pyknosis (reduction of cellular volume); nuclear morphology changes, including chromatin condensation and fragmentation (karyorrhexis); plasma membrane blebbing; however, plasma membrane integrity is maintained until the final stages; followed by *in vivo* engulfment of shed apoptotic bodies by resident phagocytes. The occurrence of chromatin fragmentation is caused by activation of Ca^{2+}/Mg^{2+} endonuclease, once condensation has occurred (Kepp *et al.*, 2011; Stanley, 2014). It should be noted that "apoptosis" and "programmed cell death" (PCD) are not synonyms, although some mechanisms involved in both processes are similar (Darzynkiewicz *et al.*, 1992; Kroemer *et al.*, 2009). Necrosis, on the other hand, is characterised by overall oncosis (increase in cell volume), increasingly translucent cytoplasm, swelling of organelles, early rupture of the plasma membrane, and loss of intracellular contents. Necrotic cell death can be described as cell death lacking the characteristics of apoptosis or autophagy in negative terms, since cells undergo early plasma membrane rupture and do not display cytoplasmic vacuolisation (Kroemer *et al.*, 2009; Kepp *et al.*, 2011). In contrast to apoptosis, the chromatin structure of cells during necrosis is not grossly altered (Stanley, 2014). Table 2.5 lists the primary differences between apoptosis and necrosis.

Table 2.5: *A summary of the fundamental differences between apoptosis and necrosis (Kanduc et al.,* 2002)

	Apoptosis	Necrosis
Inflammation	• Generally not associated with inflammation.	• Traditionally associated with inflammation.
Type of cellular injury	• Occurs in response to minor injury.	• Occurs in response to more serious injury.
Pathway	• Occurs via a predictable, coordinated and pre-determined pathway.	• Occurs as a result of depletion of cellular energy stores that activate several independent biochemical events.
Possibility of prevention	• Can potentially be modified to sustain cell viability.	• Very difficult to prevent.

Apoptosis is utilised by the body to remove undesirable and harmful cells in a 'silent' manner, without initiating an inflammatory response. Many cell growth regulatory genes are also involved during apoptosis; consequently cell proliferation and death are closely co-regulated to ensure tissue homeostasis. A large number of anti-cancer therapies rely on the activation of apoptotic pathways, as evasion of apoptosis is a characteristic of cancer as well as other degenerative diseases (Schulze-Osthoff, 2008; Stanley, 2014; Badmus *et al.*, 2015).

2.5.4.2 Activation pathways of apoptotic cell death

Activation of cysteine proteases, particularly caspases, is considered a key process in apoptosis. Caspases is a common effector molecule in several cell death pathways. Particularly caspases 3, 8 and 9, which each plays a vital role in apoptosis (Sawai & Domae, 2011; Stanley 2014). Once caspases is activated, either through oligomerisation or proteolytic cleavage of procaspases, other intracellular proteins are cleaved resulting in the precise killing of the resident cell. Intrinsic and extrinsic pathways exist by which apoptosis can be initiated within a cell (Demchenko. 2013; Stanley 2014; Aydemir *et al.,* 2015).

2.5.4.3 Role of apoptosis in the eukaryotic cell cycle

Both intrinsic and extrinsic pathways result in the activation of executioner caspases (caspases 3 and 7). Apoptosis is considered the main mechanism of cell death elicited by DNA damage, as it eliminates genetically damaged cells and counteracts carcinogenesis in order to maintain homeostasis (Stanley, 2014). The eukaryotic cell cycle can be divided into four stages (Figure 2.10), during which the DNA content will vary, due to replication and division. These stages include the G_1-phase (cell growth), S-phase (DNA replication), G_2-phase (cell growth and error control) and M-phase (cell division). Some cells, on the other hand, also possess the ability to go into quiescence, or the G_0-phase. The cell cycle is closely regulated to ensure correct

protein synthesis, DNA replication, and inspection for DNA damage. Prior to proceeding to the next stage of the cell cycle, feedback signals are required at checkpoints within the cycle. Key checkpoints are present in the G_1-phase, before the start of the S-phase; prior to entry into the M-phase at the end of the G_2-phase; and after the M-phase is completed. These checkpoints are rigorously controlled by proteins, such as cyclins and cyclin-dependent kinases (CDKs). During the G_1-phase, cyclins bind to CDK proteins to enable entry into the S-phase, mitotic cyclins again bind to CDK proteins during the G_2-phase to allow initiation of mitosis (Stanley 2014).

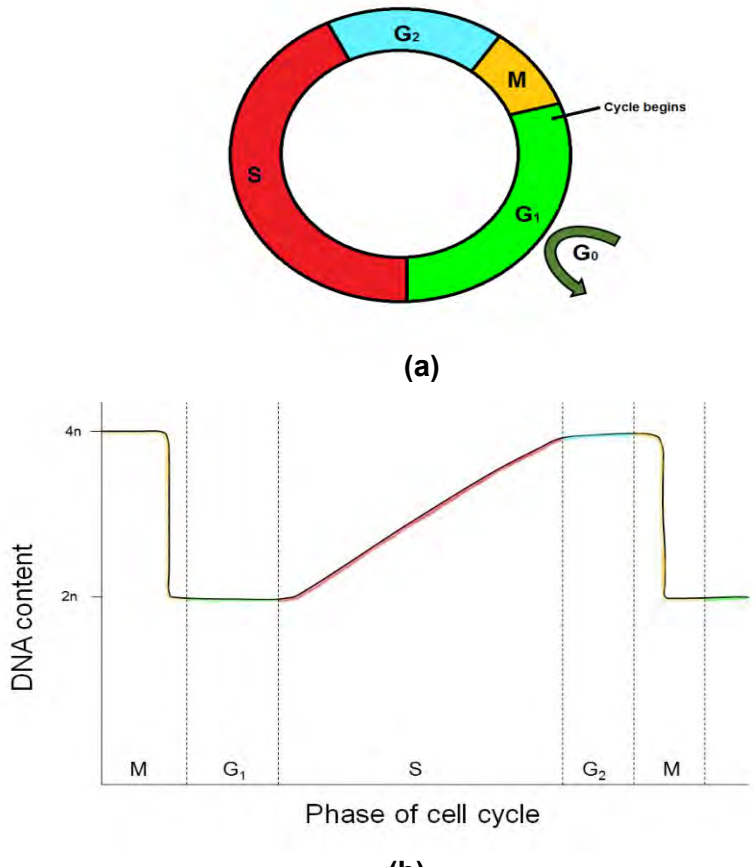


Figure 2.10: *The eukaryotic cell cycle.* ***(a)*** *Phases of the cell cycle including G_1, S, G_2, M and G_0.* ***(b)*** *DNA content of cells vary during each phase of the cell cycle* – adapted from Stanley (2014)

2.5.4.4 A better clarification of necrosis

Necrotic cell death was previously considered as an uncontrolled pathway of cell death, but evidence increasingly suggests that it is finely regulated by several transduction signalling pathways and catabolic mechanisms. The term “necroptosis” has furthermore been advocated to describe regulated necrosis, in order to distinguish it from accidental necrosis. Due to the early loss of plasma membrane integrity, necrotic cells spill their intracellular content and initiate pro-inflammatory signalling cascades (Festjens *et al.*, 2006; Kroemer *et al.*, 2009). Necrotic cell death

occurs in both physiological and pathophysiological processes; as well as being capable of killing tumour cells that have developed strategies to evade apoptosis (Festjens *et al*., 2006; Schulze-Osthoff, 2008). In addition, necrosis and apoptosis often occur as mixed features during cell death processes (Kroemer *et al.*, 2009).

Though their inter-dependency is not yet fully clarified, numerous cellular mediators, processes and organelles have been identified as playing a role in necrotic cell death. Some occurrences include mitochondrial, lysosomal and nuclear alterations, lipid degradation and the induction of non-caspase type proteases through elevated cytosolic calcium concentrations. Nonetheless, no absolute consensus exists with regards to the biochemical characteristics that may be used to unambiguously identify necrosis (Kroemer *et al.*, 2009).

2.5.4.5 Differentiation of primary necrosis from post-apoptotic necrosis

From a pharmaceutical perspective, chemical entities that cause apoptosis are preferred to entities that cause primary necrosis in cell cultures, since the cytotoxic liabilities associated with necrosis are unacceptable (Niles *et al.*, 2008). Pathologically, necrosis accompanies gratuitous cell loss and inflammation initiated by pro-inflammatory agents liberated from the dead cells (Golstein & Kroemer, 2007). Figure 2.11 illustrates a comparison of some cytotoxicity markers and the expected influence of apoptosis and necrosis thereon. Secondary or post-apoptotic necrosis should nonetheless be distinguished from primary necrosis. Post-apoptotic necrosis follows apoptotic cell death *in vitro*, due to the absence of phagocytes to eradicate the dying cells, whereas primary or caspase-independent necrosis is mediated by, among others, receptor interacting protein kinase 1 (RIPK1) and RIPK 3 pathways. Necrostatin-1 has been recognised to inhibit the kinase activity of these proteins and can successively be classified as an inhibitor of caspase-independent necrosis (Sawai & Domae, 2011).

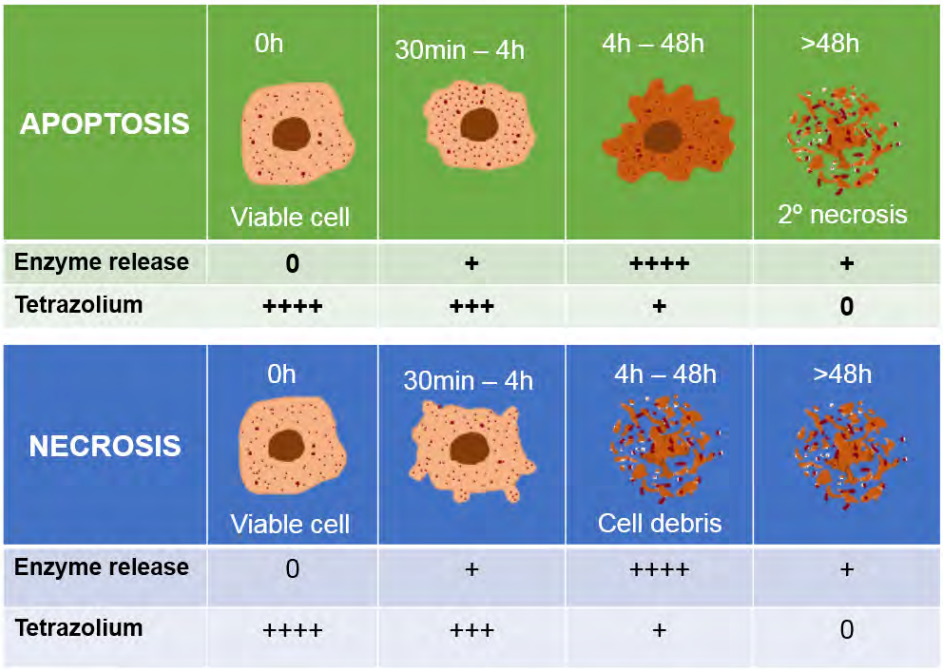


Figure 2.11: *Sequential evaluation of measurable cytotoxicity and cell viability markers to compare apoptosis and primary necrosis. Apoptosis causes an increase in cytotoxicity markers with a subsequent decrease in viability markers. These events are time dependent. Primary necrosis exhibits a similar profile, but in a much shorter time period (30 min to 4 h) – adapted from Niles et al. (2008)*

2.6 CONCLUSION

It is evident that formulation of pharmaceutical dosage forms utilising plant material involve stability and technological difficulties far greater than those for single, isolated compounds. The properties of plant extracts depend upon the interactions within the complete extract, which consist of several phytochemicals and can include synergistic, antagonistic, or additive interactions, which can easily be amplified through interaction with excipients (Taylor *et al.,* 2003; Lockwood, 2013).

Cellular toxicity is often only determined right at the end of a study, resulting in the late discovery of an extract, which may have auspicious potential in certain fields, being too toxic for further use (Bagla, 2012). Though several studies have proven its effectiveness against a wide range of ailments (Light *et al.*, 2002; Lategan *et al.,* 2009; Coopoosamy *et al*., 2010; Fouche *et al.,* 2011; Igoli *et al.*, 2012; Fouche *et al.*, 2013), the general cytotoxic effects and interactions of *S. aethiopicus* extracts, as well as in combination with specific excipients, have yet to be explored. Fillers included in this study comprised of chitosan and Pharmacel® 101 (microcrystalline cellulose), whereas aqueous, diethyl ether and ethanol crude extracts, as well as a traditional infusion and commercial solution were prepared. A wide range of characteristics could thus be explored for the identification of any possible cytotoxic effects, API-excipient interactions, and

mechanisms of induced cell death elucidated. The outcomes of this study, could in future, assist with the choice of fillers and extraction procedures to be used for safe pharmaceutical formulations containing *S. aethiopicus*.

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CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

Considering the well-established botanical classification of *S. aethiopicus,* one must remain aware of the incomplete nature of this plant's biological activity, as well as the lack in scientific evidence regarding its safety and efficacy. Very limited information is available on the *in vitro* cytotoxicity of African ginger extracts, with only a few authors reporting on routine cell viability assays such as MTT (Light *et al.,* 2002; Taylor *et al.,* 2003; Steenkamp *et al*., 2005; Lategan *et al.,* 2009; Igoli *et al.,* 2012). No further efforts have been made to elaborate on cytotoxic effects, IC_{50}-values and mechanisms of induced cell death (apoptotic or necrotic) of various extracts.

This study aimed to investigate the *in vitro* cytotoxicity of ethanol, diethyl ether and aqueous extracts, as well as a commercial solution and traditional infusion of *S. aethiopicus* in cultured HepG2 and Caco-2 cells using cell viability assays (MTT and LDH) and flow cytometry (fluorescein isothiocyanate or FITC conjugated Annexin V/PI). To achieve this aim, several solvent extracts were prepared from dried, powdered *S. aethiopicus* rhizomes, including ethanol, diethyl ether and aqueous extracts. A 1% v/v ethanol in serum free media (SFM) solution was used to re-suspend the extracts. Not only are the solubility and stability of biomarker compounds affected by the polarity of the chosen solvent system, it can also be employed as a separation technique (Coopoosamy *et al.*, 2010; Sasidharan *et al.*, 2011; Lockwood, 2013).

Extract-excipient combinations were compounded in a 20:80 ratio to investigate possible physicochemical extract-excipient interactions and the forthcoming effects thereof on cytotoxicity. Furthermore, in light of the readily available nature of several commercial products claiming to contain African ginger despite lacking proof of its safety and stability, a solution was prepared utilising one such product, namely Phyto Nova African Ginger® tablets. This particular product was chosen due to the accessability thereof in large retail pharmacy groups. It was only fitting to also prepare a traditional extract in accordance with methods used by traditional healers. By doing so, cytotoxic effects relevant to realistic prescription practices could thus correspondingly be explored. In view of the complexity of standardisation and quality control of herbal medicines as a result of the inherent convolution and widespread variation of raw constituents, it is important to note that a single extract was compounded for each solvent from the same batch of dried plant powder (Seasotiya *et al.,* 2014). This methodology was considered to increase accuracy and eliminate batch-to-batch variation.

Since herbal extracts contain a rather complex and heterogeneous concoction of compounds, some with biological activity and others that serve a more dietary function, an effort was made to

chemically analyse and characterise each extract by means of UPLC-Q-TOF/MS in order to comprehensively identify the major chemical constituents within a given sample (Jing *et al.*, 2015). Precise identification of the compounds present in herbal extracts is often very challenging and the lack of reference substances further complicates the process. UPLC-Q-TOF/MS has demonstrated its usefulness as a qualitative instrument to identify the assorted compounds present in herbal products (Yun *et al.*, 2014). The presence or absence of four biomarker molecules in each African ginger extract - (4aR,5S,8aS)-3,5,8a-trimethyl- 4a,5,8a,9-tetrahydro-naphtho[2,3-b]furan-8(4H)-one (Compound 6, section 2.2.2), (4aR,5S,8aS)-2-hydroxy-3,5,8a-trimethyl-4a,5,8a,9-tetrahydro-naphtho[2,3-b]furan-8(4H)-one (Compound 7, section 2.2.2), (4aR,5S,8aS,9aR)-9a-hydroxy-3,5,8a-trimethyl-4a,5,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,8aH)-dione (Compound 9, section 2.2.2) and a fourth unknown compound - as previously highlighted by Bergh (2016) - would accordingly assist in informing us on the compounds responsible for the observed cytotoxic effects. However, one must still bear in mind herbal medicines often yield certain properties by means of synergistic interactions of various major and minor components present in said extracts (Sasidharan *et al.*, 2011).

Several cytotoxicity assays were performed (including MTT, LDH and FITC conjugated Annexin V / propidium iodide double staining for flow cytometry) to determine the cytotoxic properties of the extracts in different concentrations. The justification for utilising various or combined assays lie therein that the predictability thereof is significantly higher than that of individual assays. Measuring a single parameter can easily lead to erroneous conclusions, whilst integration of several parameters should allow a better interpretation of toxicity results. End points measured in each assay provide information on different cytotoxic markers such as cell viability, membrane integrity and mechanisms of cell death (apoptosis and necrosis). Calculation of the IC_{50} was particularly important as it represented the concentration of the extract that killed half of the cell population and provided insight regarding the extent of cell injury (Li *et al.*, 2003; Gerets *et al.*, 2009). Data from the MTT assays were utilised and integrated for this purpose (Gerets *et al.*, 2009).

3.2 MATERIALS

3.2.1 Plant material

Due to the critically endangered status of African ginger in South Africa, ethical approval of this study was obtained from the Health Research Ethics Committee (HREC) of the NWU (approval number: NWU-00335-16-S1), foregoing the procurement of any *S. aethiopicus* material. Plant material (rhizomes) of *S. aethiopicus* was purchased from the muthi market in Potchefstroom, and Phyto Nova African Ginger® Tablets were purchased from a local pharmacy group in Potchefstroom.

3.2.2 Mammalian cell cultures and cell culture reagents

Mammalian cell cultures, including HepG2 (catalogue number HB-8065) and Caco-2 (catalogue number HB-8065) cell lines, were previously purchased from the American Type Culture Collection (ATCC) and, subsequently, were available and registered at the NWU PharmaCen (Centre of Excellence for Pharmaceutical Sciences). HyClone Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (catalogue number SH30243.01) was obtained from Separations (Johannesburg, South Africa), and Gibco foetal bovine serum (FBS; catalogue number 10499044) was purchased from LTC Tech (Pty) Ltd. (Johannesburg, South Africa). Pen-Strep combination antibiotics (catalogue number DE17-602E), non-essential amino acids 100 X (NEAA, catalogue number BE13-114E) and trypsin-EDTA 10 X with Versene (catalogue number BE02-007E) were all purchased from Whitehead Scientific (Pty) Ltd. (Lonza) (Cape Town, South Africa). Phosphate buffered saline (PBS; catalogue number P4417-100TAB) was obtained from Sigma-Aldrich (Johannesburg, South Africa).

3.2.3 Materials utilised during cytotoxicity assays

MTT (catalogue number M5655-1G) and staurosporine (catalogue number S6942-200UL) were obtained from Sigma-Aldrich (Johannesburg, South Africa). The Pierce™ LDH cytotoxicity assay kit (cat. no. 88954) was purchased from Thermo Fisher Scientific (Thermo Scientific™; Johannesburg, South Africa), whereas the BD Annexin V FITC apoptosis detection kit I (catalogue number BD/556547) was procured from The Scientific Group (Pty) Ltd. (Johannesburg, South Africa).

3.2.4 Selected fillers and excipients to investigate possible cytotoxic drug-excipient interactions

Chitosan (batch number 030912) and Pharmacel® 101 (batch number 894123, lot. Number 100043) were obtained from Warren Chem Specialities (Pty) Ltd. (Johannesburg, South Africa). Other chemicals were of analytical grade and used without further purification.

3.3 PREPARATION OF PLANT MATERIAL AND CRUDE EXTRACTS

3.3.1 Preparation of dry plant powder

Rhizomes of *S. aethiopicus* were grated and dried in an oven at 55–60ºC for 24 h. By using a stainless steel grinder (Kenwood®, South Africa) the resulting dried material was pulverised to a course powder. The powder material (which will be referred to as dry plant powder) was stored in an airtight glass container at room temperature (± 25ºC) prior to the compounding of crude extracts (Light *et al.*, 2002; Fouche *et al.*, 2011).

3.3.2 Preparation of an aqueous extract

An aqueous extract was prepared through the addition of 50 g dry plant powder to 500 ml hot water and stirred for 1 h at 250 rpm with a Heidolph MR 3001K magnetic stirrer (Sigma-Aldrich, Johannesburg, South Africa). Thereafter the pulp was centrifuged for 10 min at 5 500 rpm utilising a Sigma 3-16KL benchtop centrifuge (Sigma-Aldrich, Johannesburg, South Africa) and vacuum filtered through Whatman no. 1 filter paper (Sigma-Aldrich, Johannesburg, South Africa). A second overnight and third 1 h extraction was carried out with the collected pulp and the resulting extracts combined and lyophilised for 1 week using a Virtis SP Scientific Sentry 2.0 freeze-dryer (United Scientific (Pty) Ltd., Johannesburg, South Africa) (Light, 2002; Fouche *et al*., 2011).

3.3.3 Preparation of diethyl ether and ethanol extracts

Solvent (200 ml), either ethanol 95% (EtOH) or diethyl ether (Et_2O or DiEt), was added to 50 g dry plant powder and left to stand for 1 h with occasional stirring. The formed pulp was vacuum filtered using Whatman no. 1 filter paper and subjected to an additional overnight extraction. After a third and final 1 h extraction, the subsequent filtrates of each solvent were individually combined and the pulp discarded. These extracts were concentrated and dried at room temperature (± 25ºC) utilising nitrogen gas (Fouche *et al*., 2011; Fouche *et al*., 2013).

3.3.4 Preparation of a traditional infusion

In accordance with traditional practices, dry plant powder (16 mg) was infused in boiling hot PBS (5 ml) for several minutes to prepare an infusion. The resulting infusion was allowed to cool to room temperature (± 25ºC) prior to being centrifuged at 3 000 x g for 3 min. The supernatant was recovered by decanting and the pulp discarded. A stock solution was finally compounded through 1:1 dilution with 1% v/v ethanol in SFM, resulting in a 1.6 mg/ml concentration. This concentration translates to a traditional 200 mg dose taken with 125 ml of fluids (Manzini, 2005). It will be referred to as the traditional infusion.

The rationale for using a 1:1, PBS to SFM, preparation was due to the lack of magnesium and calcium in PBS. The normal function of many cellular adhesion molecules depend on these cations (Aplin *et al.*, 1998; Lee *et al.*, 2014; Ohnuma *et al*., 2014). On the other hand, SFM cannot be boiled due to the various constituents contained within. PBS was furthermore used instead of distilled water as it is a water-based buffer solution of which the osmolality as well as ion concentrations are isotonic and accordingly non-toxic to cells during short term exposure.

3.3.5 Preparation of commercial solution: Phyto Nova African Ginger® tablets

Phyto Nova African Ginger® tablets, a commercially available product, was prepared as a solution by crushing a tablet, said to contain 100 mg freeze-dried extract, into a fine powder utilising a mortar and pestle. A powder mass (40.61 mg), equivalent to 8 mg active ingredient, was re-suspended in 1% v/v ethanol in SFM (5 ml) to a final concentration of 1.6 mg/ml; and will be referred to as the commercial solution. This concentration is equivalent to the manufacturer's recommended oral dosage of 200 mg active ingredient (2 tablets), taken with fluids (125 ml).

3.3.6 Dilution of dry compounded extracts

Dry aqueous, ethanol and diethyl ether extracts (5 mg) were re-suspended in 1% v/v ethanol in SFM (5 ml) by firstly dissolving the concentrate in ethanol (50 µl). Once dissolved, 450 µl SFM was added, mixed well and the remaining 4.5 ml SFM was subsequently added to form a stock solution (1000 µg/ml). It should be noted that aqueous extracts were centrifuged at 5 000 rpm and filtered through an Acrodisc® syringe filter (Pall Corporation; Sigma-Aldrich, Johannesburg, South Africa) prior to being applied to cells.

3.4 COMPOUNDING OF BINARY EXTRACT FILLER MIXTURES

Binary mixtures consisting of a particular extract combined with a filler were compounded through mixing and suspension. Fillers normally constitute 65–85% of a formulation's composition (Railkar, 2013); therefore, a ratio of 20:80, extract to filler, was prepared to investigate possible cytotoxic, physicochemical extract-excipient interactions. All reconstituted extracts, with the exception of traditional infusions and commercial solutions, were mixed with selected fillers, as listed in Table 3.1. Annexin V/PI double staining was only performed on those extracts which demonstrated toxicity during MTT and LDH assays, as the reagents used were expensive and limited.

Table 3.1: *Binary mixtures consisting of African ginger extracts and selected fillers intended for MTT assay, LDH assay and Annexin V/PI double staining, where: E (extract only), C (extract and chitosan), P (extract and Pharmacel®101). The symbol (♦) indicates assays performed on each extract and binary mixture*

	Aqueous			**EtOH**			**DiEt**			**Traditional**			**Commercial**		
	E	C	P	E	C	P	E	C	P	E	C	P	E	C	P
MTT	♦	♦	♦	♦	♦	♦	♦	♦	♦	♦			♦		
LDH	♦	♦	♦	♦	♦	♦	♦	♦	♦	♦			♦		
Annexin V/PI				♦			♦								

3.5 CHEMICAL PROFILING OF EXTRACTS

3.5.1 Ultra performance liquid chromatographic analysis (UPLC)

Characterisation of the numerous constituents of *S. aethiopicus* was performed by Dr Marietjie Stander and Mr Malcolm Taylor (Mass Spectrometry Unit, Central Analytical Facilities: Stellenbosch). An Ultra Performance Liquid Chromatographic (UPLC) analysis was performed using a Waters Acquity UPLC system equipped with a sample manager, binary solvent manager, column heating compartment and photodiode-array (PDA) detector (Waters, Milford, USA). Chromatographic separation of compounds were achieved on a Waters Acquity UPLC ethylene bridged hybrid (BEH) C_{18}-column (100 mm x 2.1 mm, 1.7 µm particle size; Waters, Milford, USA) with the thermostat at 45ºC. A mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.35 ml/min was used. The gradient elution was optimised as follows: an initial ratio of 98:2 (A relative to B) where it was maintained for 7 min, changed to 40:60 (A relative to B) for 1 min, after which it was changed to 1:99 (A relative to B) for 50 sec. Finally it was changed back to the initial ratio for 1 min and 10 sec. The total running time was 10 min and the injection volume was set at 2.0 µl (partial loop injection). The method was based on that used by Bergh (2016) with minor alterations. Table 3.2 provides a short summary of the analytical instrumentation.

Table 3.2: *Summary of the analytical instrumentation and chromatographic conditions utilised to perform UPLC analysis of S. aethiopicus extracts*

Analytical instrumentation and conditions	Description
Analytical instrument	• Acquity Ultra Performance Liquid Chromatographic system with a photodiode array (PDA) detector. • The system is interfaced with a Synapt G2 quadruple time of flight (Q-TOF) mass spectrometer equipped with an electrospray ionization source.
Column	• Waters Acquity BEH C_{18}-column • (100 mm × 2.1 mm, 1.7 µm particle size)
Thermostatted temperature	• 45ºC
Mobile phase	• 0.1% formic acid (solvent A) and acetonitrile (solvent B)
Flow rate	• 0.35 ml/min
Sample volume injection	• 2.0 µl (partial loop injection with needle overfill)
Gradient method (A:B)	• 98:2, changed to 40:60 after 7 min and maintained for 1 min; changed to 1:99 for 50 sec; and changed back to initial ratio for 1 min and 10 sec.

3.5.2 Ultra performance liquid chromatographic quadruple time of flight mass spectrometry (UPLC-Q-TOF/MS) analysis

The UPLC system was coupled to a Synapt G2 Q-TOF system equipped with an electrospray ionisation source (Waters, Milford, USA). The instrument was operated in positive ionisation mode. Nitrogen (N_2) was selected as the desolvation gas. In short, the following parameters were set: capillary voltages 3500 V; sampling cone voltage 15 V; source temperature 120ºC; desolvation temperature and gas flow rate of 275ºC and 650 L/Hr, respectively. The instrument was operated in MS to the E (MS^E) mode whereby a low collision energy scan was followed by a high collision energy scan (Energy ramp from 20 to 60 V) to obtain fragmentation data as well as molecular ion information. The scan range was 200 to 1500 *m/z*. All the raw chromatographic data was recorded and processed using chromatographic software (Masslynx 4.1, Waters, Milford, USA) (Bergh, 2016). Leucine encephalin was used as lock mass for accurate mass determinations and sodium formate was used for mass calibration.

3.6 DETERMINATION OF AVERAGE PARTICLE SIZE AND SIZE DISTRIBUTION

3.6.1 Particle size determination of excipients

Particle size and particle size distribution (PSD) analysis of excipient powders (chitosan and Pharmacel® 101) were determined by means of laser diffraction using a Malvern Mastersizer 2000 instrument fitted with a Hydro 2000 SM small volume sample dispersion unit and a Hydro 2000 MU dispersion unit (Malvern Instruments, Malvern, UK), correspondingly. This apparatus possesses a measurement range of 0.2–2000 μm. During particle size analysis of the fillers, the Hydro 2000 SM dispersion unit was engaged at a stirring rate of 1 200 rpm. Absolute ethanol was chosen as the dispersion medium in order to prevent dissolution of particles of both fillers. In order to compensate for possible interference from the dispersion medium, a background measurement was taken prior to particle size analysis. The small volume dispersion unit was filled with 100 ml absolute ethanol. Preceding addition to the small volume dispersion unit, each filler was separately dispersed in 10 ml absolute ethanol. Consequently, a sufficient quantity of the dispersed sample was added to the small volume dispersion unit in order to obtain an obscuration of 10–15%. Each measurement was conducted for 12 s, recording 12 000 diffraction images or events (Esterhuizen-Rudolph, 2015). The particle size and size distribution of each sample were measured in duplicate; followed by calculation of the average size and size distribution compiled with Malvern Software (Malvern Instruments, Malvern, UK).

3.6.2 Particle size determination of organic extract suspensions

In order to determine the particle size and PSD of ethanol and diethyl ether *S. aethiopicus* extracts, a method similar to that utilised for the excipients (section 3.6.1) was followed, with the exception of a different dispersion medium. The small volume dispersion unit was filled with 100 ml distilled water and a background measurement captured prior to particle size analysis as to counteract dispersion medium interference. Foregoing their addition to the dispersion unit, extracts were initially dissolved in a small volume of absolute ethanol and subsequently dispersed with distilled water (40 ml) due to the insolubility of both extracts in an aqueous medium. An appropriate volume of each dispersed sample was added to the small volume dispersion unit to obtain an obscuration of 10-15%. Duplicate measurements were taken of the respective samples; with each measurement conducted for 12 s, recording 12 000 diffraction images or events. Average size and size distribution were compiled with Malvern Software (Malvern Instruments, Malvern, UK).

3.7 CULTIVATION OF MAMMALIAN CELL CULTURES

3.7.1 Mammalian cell cultures

Both the HepG2 and Caco-2 cell cultures were maintained in 75 cm^2 flasks at 37ºC and 5% CO_2 atmosphere in a Forma™ Steri-Cycle™ CO_2 incubator (Thermo Scientific™, Labotec (Pty) Ltd., Johannesburg, South Africa). These cell lines were maintained in complete growth medium (CGM) consisting of DMEM supplemented with FBS to a final concentration of 10%, 1% penicillin-streptomycin antibiotics and 1% non-essential amino acids. CGM was replaced twice a week, or as frequently as necessary. Once ± 80% confluency was reached, the cells were sub-cultured to new cell culture flasks. The cell layer was rinsed with 1 X PBS (10 ml) to remove all traces of serum containing trypsin inhibitor. Next, 3.0 ml Trypsin-EDTA solution (1 X) was added to the flask for 3–5 min (for HepG2) and 10–15 min (for Caco-2) in order to detach the cell layer. CGM (7 ml) was added to the flask and the resulting suspension triturated to ensure a single cell suspension. Appropriate aliquots of the cell suspension were added to new culture vessels by pipetting, followed by incubation under previously mentioned conditions. The same procedure was applied during experimental analysis, with the exception of adding cells to 96-well plates.

3.7.2 Cell proliferation studies

Due to the difference in each cell line's response to external factors, it was imperative to determine the optimal cell number for seeding in multi-well plates as this formed the basis of all *in vitro* assays performed. A simple MTT cell proliferation assay was performed for this purpose. The assay is based on the principle that the absorbance measured is directly proportional to the number of viable cells (Riss *et al.,* 2016).

Following harvesting of the cells by means of trypsination, cells were re-suspended and the cell concentration determined employing the Invitrogen™ Countess™ automated cell counter. Using a 96-well plate, serial dilutions of the cells ranging from 1 x 10^4– 1 x 10^5 cells/ml were prepared by plating appropriate aliquots of the cell suspension to wells and adding CGM to a volume of 100 µl per well. Dilutions were plated in triplicate; and three wells containing SFM were also included as blanks for absorbance readings. Multi-well plates were incubated for 8 h and 24 h, respectively, for HepG2 and Caco-2 cells. MTT stock solution (5 mg/ml) was prepared by diluting 5 mg MTT powder in 1 ml PBS. Volumes of 10 µl MTT stock solution and 90 µl SFM were added to each well, after removal of CGM and incubated for 3 h at 37ºC. Formation of purple formazan precipitate was periodically observed under a microscope. Following the incubation period, all the media was carefully removed by pipetting, where after DMSO (100 µl) was added to all wells. The wells were left at ambient temperature (± 25^{o}C) for 1 h in order to solubilise the purple crystals. Absorbance of each well was measured at 560 nm and background absorbance of multi-well plates at 630 nm, which was subsequently subtracted from the 560 nm measurement using

a SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer (Molecular Devices, Sunnyvale, California, USA) with SoftMax® Pro Microplate Data Acquisition & Analysis software. Average values were determined for triplicate readings and the average value for the blank subtracted. Final absorbance was plotted against the number of cells/ml. The optimum number of cells used in multi-well assays was chosen within the linear portion of the plot and yield an absorbance between 0.75 and 1.25.

3.8 MORPHOLOGICAL OBSERVATION OF CELLS UNDER LIGHT MICROSCOPE

HepG2 and Caco-2 cells were equally seeded in 96-well plates at densities of 2 x 10^4 cells/well and 4 x 10^4 cells/well (100 µl), respectively. Once cells formed a monolayer (an overnight incubation period for HepG2 cells and 24 h for Caco-2 cells, at 37°C and 5% CO_2), the growth media was aspirated and cells briefly rinsed with PBS (100 µl). Following the removal of PBS, cells were exposed to varying concentrations of extracts for 4 h. A concentration range consisting of 50, 100, 150, 200, 300, 500 and 1000 µg/ml was used for organic and aqueous extracts, whereas 0.8 and 1.6 mg/ml concentrations were used for commercial and traditional solutions. Controls included consisted of positive controls (cells treated with 0.4% Triton X-100; TX) and vehicle controls (cells treated with 1% v/v ethanol in SFM; VC). Following the incubation period, the morphology of the cells were observed under a light microscope (Nikon Eclipse TS100 microscope equipped with a Nikon TV Lens C-0.35X camera) and images captured at 40x magnification using IC Capture Ver.2.3 capturing software (The Imaging Source Europe GmbH).

3.9 CYTOTOXICITY ASSAYS

3.9.1 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay

3.9.1.1 Introduction

Utilisation of the MTT assay offers a homogeneous, colorimetric assay suitable for high throughput screening (HTS) that does not require radioisotopes; has simplified processing; and is sensitive enough to be performed using 96-well plate setups. The assay is considered a golden standard to compare new cytotoxicity and viability assay methods with; and to date is exceptionally well characterised and referenced in many published articles (Niles *et al.,* 2008; Fernandes *et al.*, 2012; Sigidi *et al.*, 2016; Kuete *et al*., 2017).

It is presumed that the cellular reduction of water soluble MTT to insoluble formazan involves the transfer of electrons from reduced nicotinamide adenine dinucleatide (NADH) to MTT by mitochondrial reductase enzymes (as indicated in Figure 3.1), although the precise mechanism is not yet clear (Riss *et al.,* 2016; Kuete *et al*., 2017). The cell membrane impermeable formazan crystals accumulate within viable cells and consequently the quantity of formazan (measured utilising a spectrophotometer) is directly proportional to the number of viable cells (Mossman, 1983; Fotakis & Timbrell, 2006). Cells lose their ability to convert MTT to formazan once they die and accordingly, the colour formation functions as a marker for viable cells only (Riss *et al.*, 2016). Similarly, the MTT assay can be used during cell proliferation studies (e.g. determining optimal cell number) and cell viability studies (e.g. determining cytotoxic activity of exogenous compounds (Kuete *et al.*, 2017). Initially, Mossman (1983) who originally described the MTT assay, assumed the tetrazolium ring of MTT was cleaved by mitochondrial succinate dehydrogenase enzymes in living, metabolic active cells to form purple formazan – thus measuring mitochondrial activity (Riss *et al.,* 2016) - but more recently it has been described that these reduction enzymes may also be located in other cellular organelles, for example the endoplasmic reticulum (Kuete *et al*., 2017).

During excitotic insults, oxygen radicals can potentially accumulate within cells, affecting the reduction of MTT (Uliasz & Hewett, 2000). Furthermore, non-enzymatic reduction of MTT can moreover be triggered by chemical interference often caused by plant extracts and polyphenolic compounds. These factors may all cause discrepancies, resulting in false positive outcomes and subsequently necessitate the need for alternative cell viability measurements (Riss, 2014).

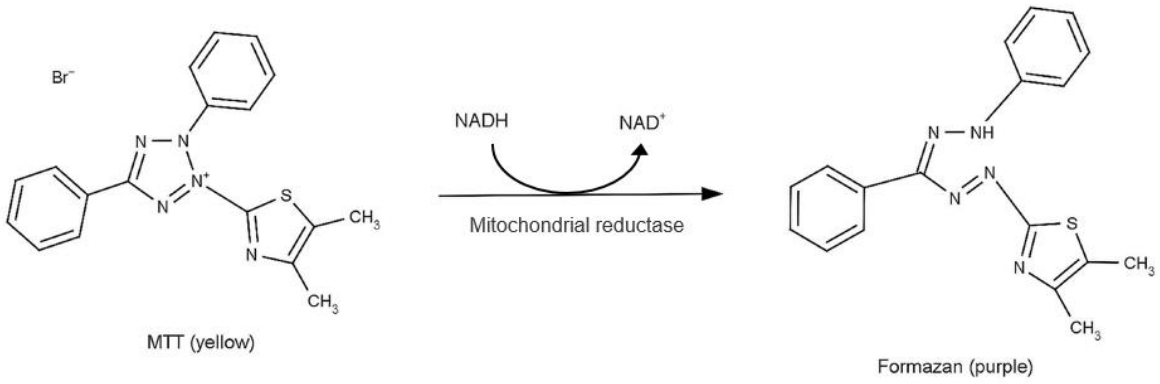
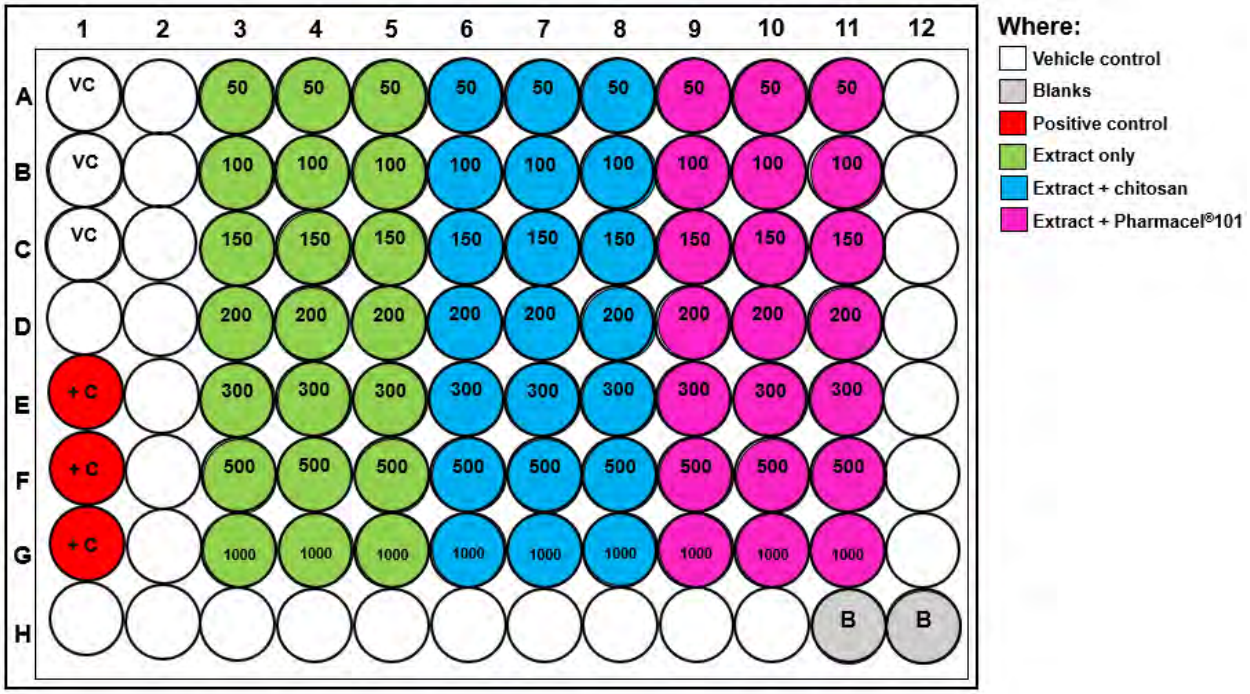


Figure 3.1: *Conversion of yellow, water soluble MTT to purple, insoluble formazan crystals by mitochondrial reductase enzymes – adapted from Riss et al. (2016)*

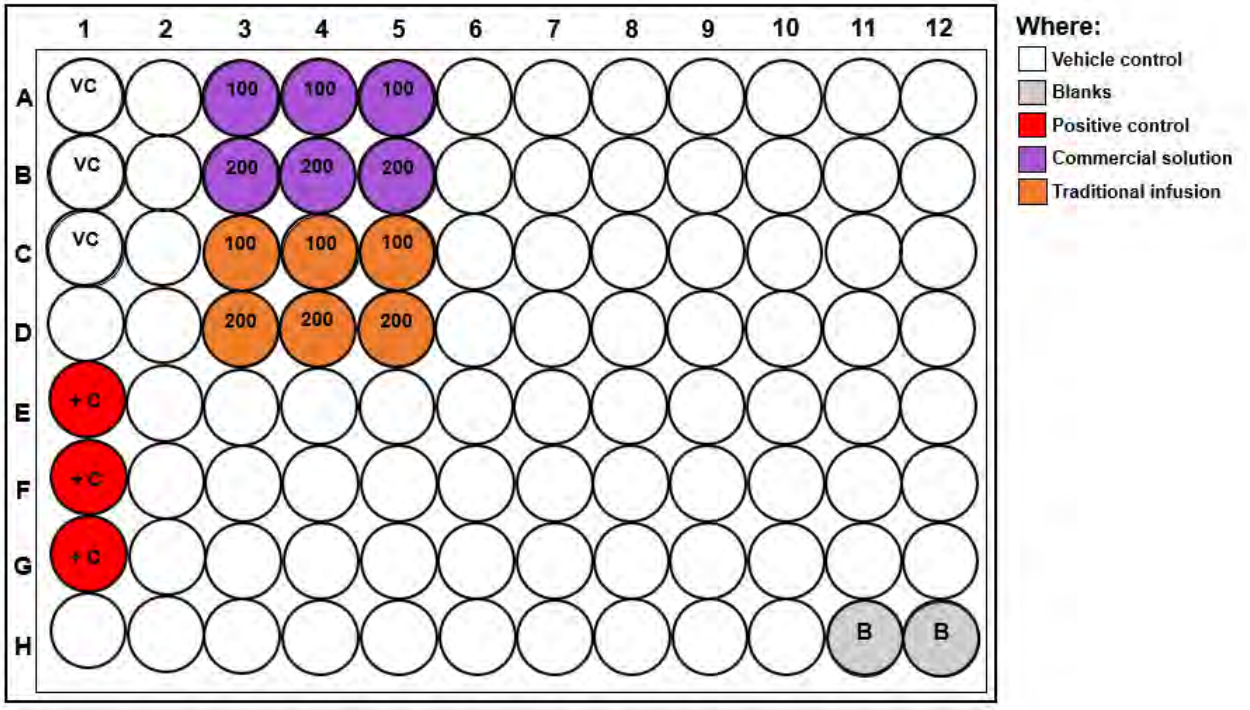
3.9.1.2 Plate layout of 96-well plates for MTT assay

Cells seeded in 96-well plates were exposed to various concentrations of ethanol-, diethyl ether- and aqueous extracts following resuspension (50, 100, 150, 200, 300, 500, 1000 µg/ml), as well as commercial solutions and traditional infusions (0.8 and 1.6 mg/ml) for 4 h prior to MTT assays.

Figure 3.2 provides an illustration of the layout of (a) organic and aqueous compounded extracts, as well as (b) commercial and traditional solutions.



(a)



(b)

Figure 3.2: *Experimental plate layout of* ***(a)*** *ethanol, diethyl ether and aqueous extracts, as well as* ***(b)*** *commercial solutions and traditional infusions for MTT assays. Each experimental concentration (µg/ml* or mg/ml*) was plated in triplicate and assays performed in duplicate*

3.9.1.3 Method

Viable cells were seeded at densities of 2 x 10^4 cells/well and 4 x 10^4 cells/well (100 µl per well) for HepG2 and Caco-2 cells, respectively. In order to form a cell monolayer, cells were incubated at 37°C and 5% CO_2 overnight (HepG2) or for 24 h (Caco-2). Thereafter, media was aspirated and monolayers washed with PBS (100 µl). Once PBS was removed, cells were exposed to varying concentrations of extracts, as indicated in Figure 3.2 and incubated for 4 h. Controls included in this assay consisted of positive controls (cells treated with 0.4% Triton X-100) and vehicle controls (cells treated with 1% v/v ethanol in SFM). After removal of the extracts, 10 µl MTT stock solution (5 mg/ml) and SFM to a final volume of 100 µl were added to each well and incubated for 3 h. Again, media was removed from each well and supplemented with DMSO (100 µl), after which the wells were incubated for 1 h to solubilise the resulting formazan crystals. Experiments were performed in triplicate and individually repeated. Absorbance of the solution was measured at 570 nm, whilst background absorbance was measured at 690 nm and subtracted from the 570 nm measurement. The SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer (Molecular Devices, Sunnyvale, California, USA) with SoftMax® Pro 6.2.1 Microplate Data Acquisition & Analysis software was utilised. As with the cell proliferation application of MTT, the principle of the viability assay application was based on the fact that the absorbance value is directly proportional to the amount of viable cells. However, it differs in the sense that during the cell viability assay, a relative constant number of cells are used and exposed to the plant extracts. Cell viability was calculated as a percentage relative to the untreated or vehicle control cells using Equation 3.1.

$$\%\text{viability} = \frac{(\Delta\text{Absorbance}_{\text{test sample}} - \Delta\text{Blank})}{(\Delta\text{Control} - \Delta\text{Blank})} \times 100 \qquad \textbf{[3.1]}$$

Where:

- Δ Absorbance $_{\text{test sample}}$ = absorbance at 560 nm – absorbance at 630 nm
- Δ Blank = mean blank at 560 nm – mean blank at 630 nm
- Δ Control = control absorbance at 560 nm – control absorbance at 630 nm

3.9.2 Lactate dehydrogenase assay

3.9.2.1 Introduction

Lactate dehydrogenase (LDH) is a water soluble enzyme present in the cytoplasm of nearly all cells and is released into the extracellular fluid following plasma membrane damage (Chan *et al.*, 2013). It can subsequently be utilised as a marker to quantify the number of non-viable cells in multi-well plates, either through fluorescence or through absorbance measurements. It is a simple, reliable and fast assay (Fotakis & Timbrell, 2006). The principal of the Pierce™ LDH cytotoxicity assay kit (Thermo Scientific™) is based on the catalysis of lactate oxidation to

pyruvate by LDH and concurrent production of NADH through a reversible reaction as demonstrated in Figure 3.3 (Legrand *et al.*, 1992; Chan *et al.*, 2013). During the assay, a tetrazolium salt (2-piodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride or INT) is converted to a coloured formazan compound in solution. The amount of formazan produced can be spectrophotometrically quantified as an indication of cytotoxicity (Chan *et al.*, 2013).

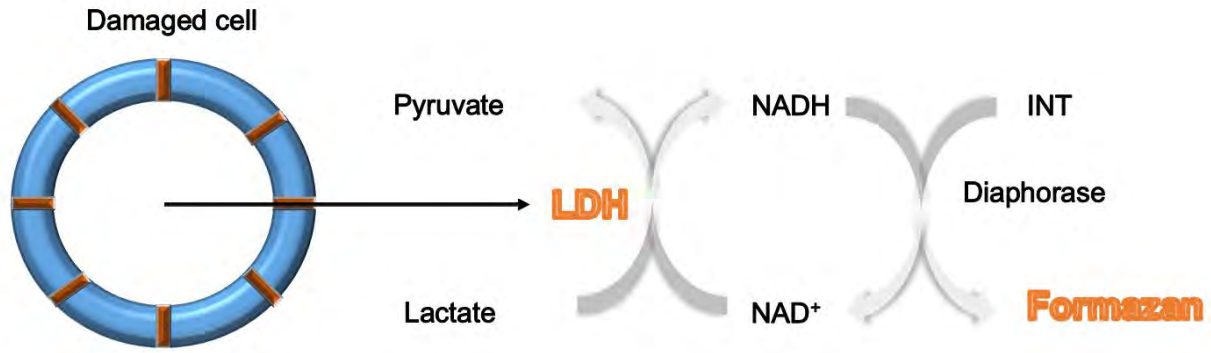
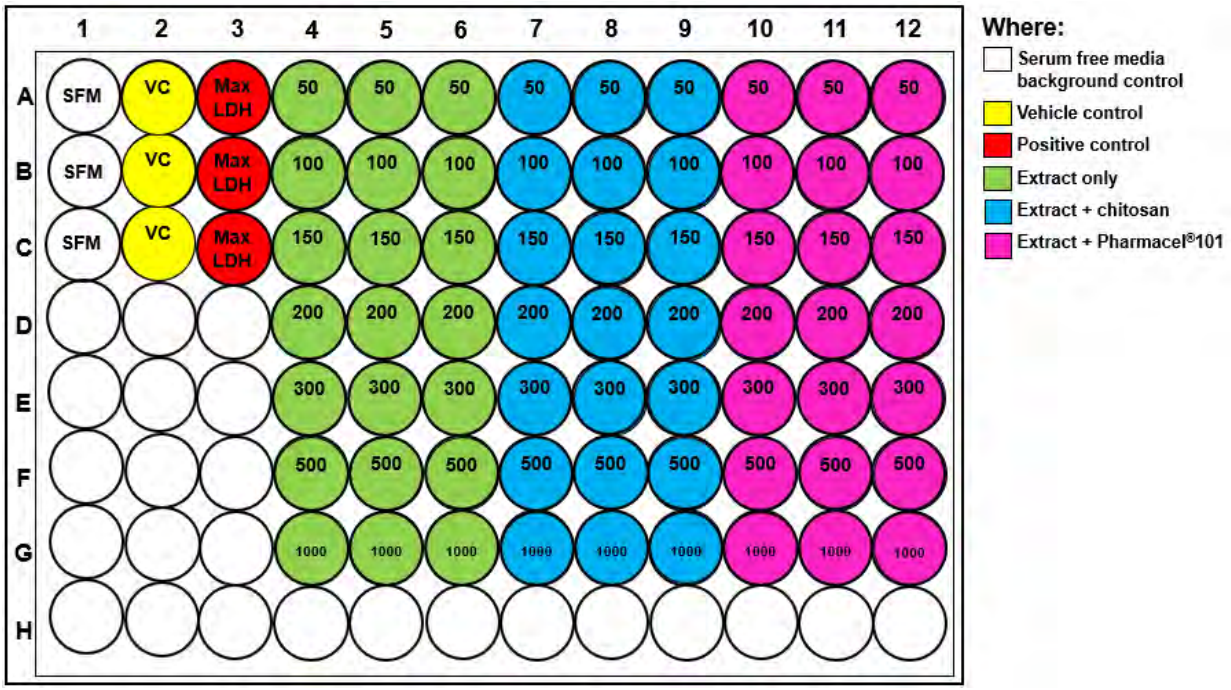


Figure 3.3: *A simple diagram explaining the biochemistry of the LDH assay: firstly lactate is oxidised to pyruvate by LDH and produces NADH. In the second step, diaphorase catalyses the reduction of INT, a tetrazolium salt, to a formazan compound with the concurrent oxidation of NADH to NAD^+ - adapted from Thermo Fisher Scientific (2014)*

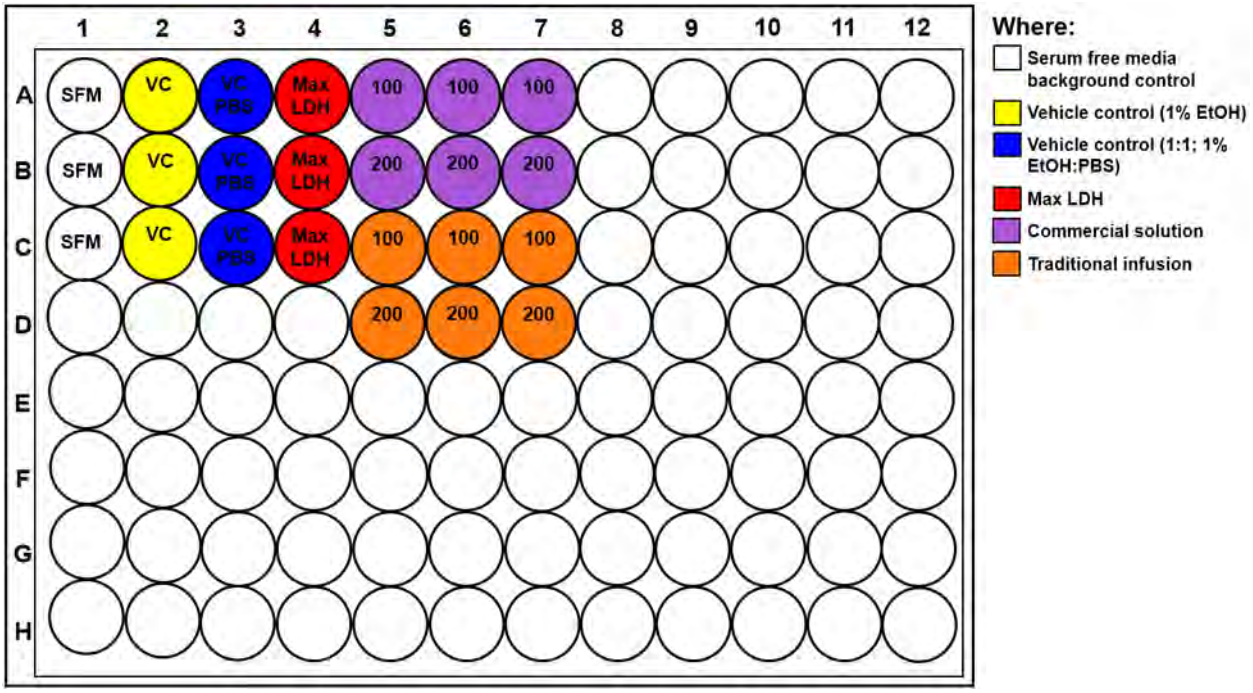
When cell viability is close to 100%, almost no LDH will be present in the extracellular fluid, but as the damage to the plasma membrane increases, effluence of the enzyme and subsequent increase in extracellular concentration increases (Legrand *et al.*, 1992; Arechabala *et al.,* 1999). The LDH assay is normally performed in high-density plate HTS, however, its main criticism is that serum-containing medium can negatively affect the assay's sensitivity and hence, medium-only controls that are cell-free must be incorporated (Niles *et al.,* 2008).

3.9.2.2 Plate layout of 96-well plates for LDH assay

As with the MTT assay, HepG2 and Caco-2 cells were seeded in 96-well plates and exposed to various *S. aethiopicus* extracts for 4 h prior to performing LDH assays. Figure 3.4 (a) indicates the plate layout of ethanol-, diethyl ether- and aqueous extracts following re-suspension (50, 100, 150, 200, 300, 500, 1000 µg/ml), whereas Figure 3.4 (b) indicates commercial solutions and traditional infusions (0.8 and 1.6 mg/ml).



(a)



(b)

Figure 3.4: *Experimental plate layout of* ***(a)*** *ethanol, diethyl ether and aqueous extracts, as well as* ***(b)*** *commercial solutions and traditional infusions for LDH assays. Each experimental concentration (µg/ml* or mg/ml*) was plated in triplicate and assays performed in duplicate*

3.9.2.3 Method

The Pierce™ LDH cytotoxicity assay kit (Thermo Scientific™) was utilised to quantify cell viability. HepG2 (2 x 10^4 cells/well) and Caco-2 (4 x 10^4 cells/well) cells were exposed to extracts and concentrations, as specified in Figure 3.4 (a) and (b), for 4 h at 37°C and 5% CO_2. All test conditions were repeated thrice and the experiment performed in duplicate. Controls included SFM only (background absorbance), vehicle controls (spontaneous LDH activity of cells) and maximum LDH activity (positive control; 10X Lysis buffer solution; Thermo Scientific™) control wells. Following exposure, plates were centrifuged at 300 x g for 3 min. The supernatant (50 µl) of each well was transferred to a clear flat-bottom 96-well plate and reconstituted Reaction Mixture (50 µl; Thermo Scientific™) was added using a multipipettor. After an incubation period (30 min) while protected from light and at room temperature (± 25 C), Stop Solution (50 µl; Thermo Scientific™) was added to each well and gently mixed. By using the SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer with SoftMax® Pro 6.2.1 Microplate Data Acquisition & Analysis software, absorbance of the solution was measured at 490 nm, whereas background absorbance was measured at 680 nm. LDH activity was calculated by subtracting the background (680 nm) value from the sample (490 nm) value and further utilised to determine percentage cytotoxicity (expressed relative to the positive control) with equation 3.2.

$$\%\,\text{LDH release} = \frac{(\Delta\,\text{Extract treated LDH}_{\text{activity}} - \Delta\,\text{Spon LDH}_{\text{activity}})}{(\Delta\,\text{Max LDH}_{\text{activity}} - \Delta\,\text{Spon LDH}_{\text{activity}})} \times 100 \qquad \textbf{[3.2]}$$

Where:

- Δ Extract treated LDH $_{activity}$ = absorbance of extract treated wells at 490 nm – background control absorbance at 680 nm
- Δ Spon LDH $_{activity}$ = absorbance of spontaneous LDH release wells 490 nm – background control absorbance at 690 nm
- Δ Max LDH $_{activity}$ = absorbance of maximum LDH release wells - background control absorbance at 690 nm.

3.9.3 Flow cytometry: fluorescein isothiocyanate conjugated Annexin V and propidium iodide cell staining

3.9.3.1 Introduction

Fluorochrome conjugated Annexin V / propidium iodide (PI) double staining utilises morphological changes in the plasma membrane structure of cells to distinguish between apoptotic and necrotic cells. During apoptosis phosphatidylserine (PS) residues are exposed on the outer leaflet of the plasma membrane (Figure 3.5) while the plasma membrane integrity remains intact (Fadok *et al*., 1992; Engel *et al.*, 2014). PS exposure seems to be a universal phenomenon of apoptosis in all

cell types and lasts from the early execution phases of apoptosis until the cell is broken down into apoptotic bodies (Vermes *et al.*, 1995; Van Engeland *et al.,* 1998; Demchenko, 2013). Annexin V is a Ca^{2+}-dependant phospholipid-binding protein with a high affinity for negatively charged PS. It is used to distinguish between vital and apoptotic cells, but cannot alone distinguish between apoptotic and necrotic cells, since necrotic cells lose their plasma membrane integrity early on and the inner membrane is accessible to extrinsically applied Annexin V (Andree *et al*., 1990; Vermes *et al.*, 1995; Van Engeland *et al.,* 1998; Demchenko, 2013; Nair *et al.*, 2014). However, simultaneous double labelling of fluorescently conjugated Annexin V with propidium iodide (PI), a membrane impermeable DNA stain, can be employed to discriminate between apoptotic and necrotic cells by exploiting the differences in their cell membrane integrity (Darzynkiewicz *et al*., 1992; Vermes *et al*., 1995; Van Engeland *et al.,* 1998; Demchenko, 2013). Due to the intactness of their cell membranes, PI does not stain apoptotic cells; however, necrotic cells are easily stained as a result of early cell membrane permeation. Vital cells were Annexin V and PI negative; apoptotic cells were Annexin V positive, but PI negative; and necrotic cells were Annexin V and PI positive, as illustrated in Figure 3.6 (Van Engeland *et al*., 1996). Due to the morphological changes in cells during apoptosis, their flow cytometric light-scattering properties also vary and can consequently be utilised to detect apoptosis. During apoptotic cell death, the cytogram shifts from high forward/low side scatter to low forward/high side scatter due to increased granularity and a reduction in cell size (Van Engeland *et al*., 1996), whereas necrosis will result in an immediate decrease in forward and side scatter (Darzynkiewicz *et al.*, 1992).

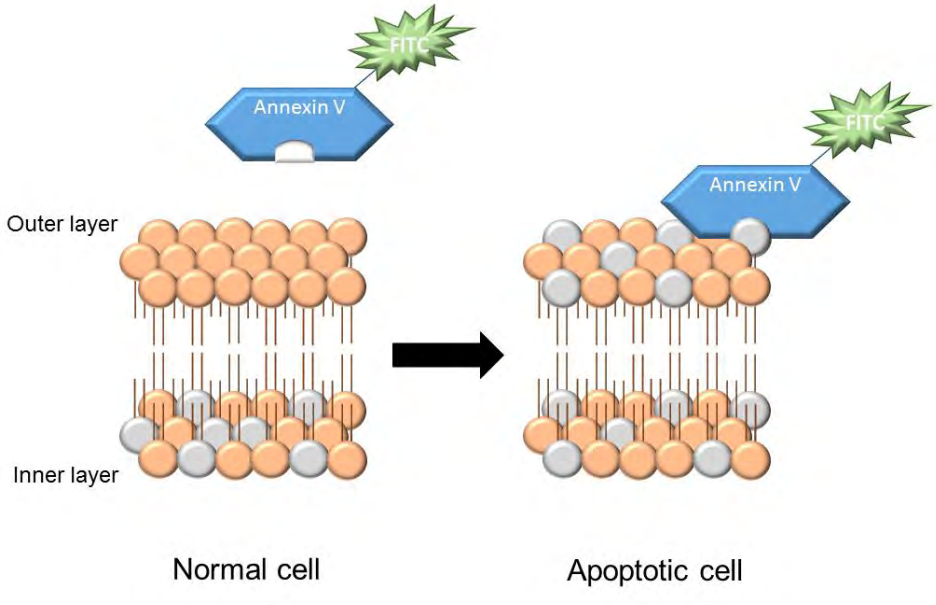


Figure 3.5: *Schematic illustration of the loss of plasma membrane asymmetry and subsequent exposure of PS-residues (orange circles) during apoptosis at the outer membrane and specific binding of labelled Annexin V to these residues – adapted from Van Engeland et al. (1998)*

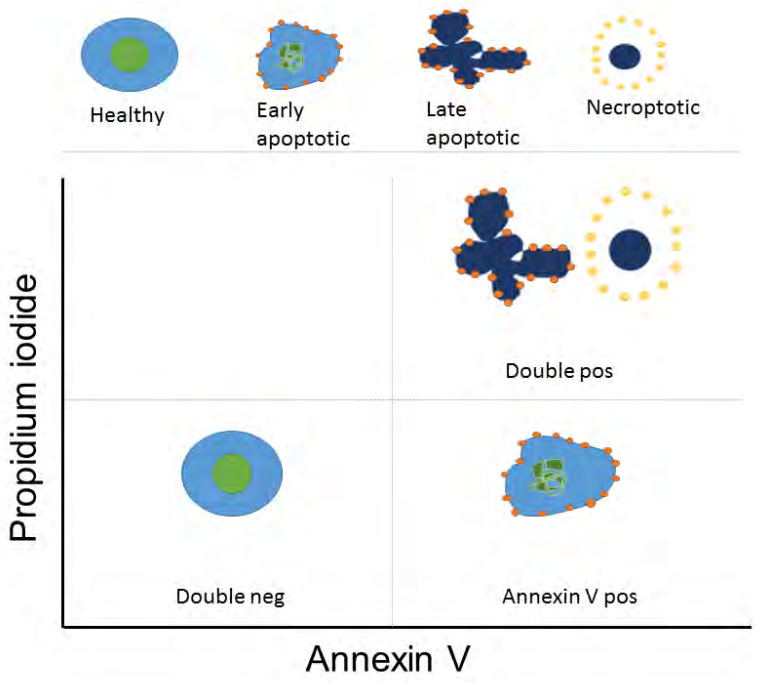


Figure 3.6: *An illustration of vital, early apoptotic, late apoptotic and necroptic cells and the possible distribution thereof on a cytogram - adapted from Pietkiewicz et al. (2015)*

It should be noted that assays were conducted immediately after exposure, since the assay cannot distinguish between primary and post-apoptotic necrosis, which will both have a double positive result. Post-apoptotic necrosis occurs in cell culture conditions due to the lack of phagocytosis, resulting in membrane rupture during late apoptotic phases (Pietkiewicz *et al*., 2015).

3.9.3.2 Method

The commercially available FITC Annexin V Apoptosis detection kit I (BD Pharmingen™) for flow cytometry was obtained to detect apoptotic cell death within a cell population. Assays were performed according to the manufacturer's specifications with minor adjustments for optimal results (Wentzel *et al*., 2017).

Briefly, cells were seeded in 96-well plates and incubated for 24 h. The cells were exposed to ethanol and diethyl ether extracts (50, 100, 150 µg/ml) for 4 h while incubated at 37ºC and 5% CO_2 atmosphere. Following exposure, cells were washed twice with PBS (200 µl) and suspended in 1 X binding buffer at a concentration of approximately 1 x 10^6 cells/ml. The resulting cell suspension (100 µl) was transferred to 12 x 75 mm flow tubes (BD Biosciences) and the reagents, FITC Annexin V (5 µl) and PI (5 µl), added to each tube. Samples were fleetingly vortexed and subsequently incubated for 20 min at room temperature (± 25 C) while protected from light. Succeeding incubation, 1 X binding buffer (100 µl) was added to each sample and analysed by means of the BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, California, USA) with BD FACSuite™ software, measuring the fluorescence emission at 530 nm and 575 nm using

488 nm excitation. As a positive apoptosis control, cells were treated with 1 mM staurosporine (Sigma-Aldrich) for 4 h. A total of 10 000 events were recorded per sample and all experiments were performed in triplicate as well as independently repeated (Wentzel *et al*., 2017).

3.9.4 Flow cytometry instrumentation and data analysis

Fluorescence of single cells was measured by a FACSVerse™ (BD Biosciences) bench top flow cytometer equipped with blue (488 nm) and red (640 nm) lasers. Events were acquired on BD FACSuite™ software, version 1 (Becton & Dickson, Mountain view, CA, USA). The FACSVerse was calibrated using FACSuite™ CS&T research beads (Becton & Dickson, Mountain view, CA, USA). Amplification of signals was carried out at logarithmic scale and measurement of events plotted on forward scatter (FSC), side scatter (SSC), green fluorescence (FL1) and red fluorescence (FL2). For each sample at least 10 000 cells were analysed. Forward and side scatter events (FSC/SSC) were acquired on a log scale and gates were set to the FSC/SSC dot-plot to exclude debris. Positive as well as stained and unstained negative controls were included in each analysis. The following controls (summarised in Table 3.3) were used to establish instrument compensation and setup: unlabelled cells, negative control cells labelled with Annexin V-FITC only (no PI), positive control cells labelled with Annexin V-FITC only (no PI), negative control cells labelled with PI only (no Annexin V-FITC) and positive control cells labelled with PI only (no Annexin V-FITC). Where possible, a total of 10 000 events were counted per sample. A summary of results with basic statistics for untreated and treated samples was calculated and included the absolute number of events acquired, percentage cells, percentage Annexin V–/PI–, percentage Annexin V+/PI–, percentage Annexin V+PI+ and percentage Annexin V–PI+. Data was processed with FlowJo X (10.0.7r2) single cell analysis software (Wentzel *et al*., 2017).

Table 3.3: *Positive, negative and unlabelled controls included in the Annexin V / PI flow cytometry assay*

Positive controls		**Reason for inclusion**
• **Apoptosis (1 mM staurosporine)**	a) Annexin V	Determine boundaries of Q4
	b) PI	Determine boundaries of Q1
	c) Annexin V / PI	Record double labelling of samples
	d) Unstained cells	Control how the cell population changed in FSC/FSS plot
Negative controls		
• **Cells and medium, untreated**	c) Annexin V /PI	
	d) Unstained cells	To adjust the cloud of cell population in FSC/FSS plot and define boundary of Q3
Unlabelled cells • Cells and solvent in medium		

3.10 STATISTICAL EVALUATION

All data were analysed by GraphPad Prism software (version 5; GraphPad Software Inc., San Diego, CA). Data are expressed as the mean (± SEM or standard error of mean) of experiments done in triplicate and independently repeated. Briefly, for MTT assays, data were normalised to positive (presumed to represent 0% viability) and negative controls (presumed to represent 100% viability), whereas LDH data were normalised to positive controls (presumed to represent 100% viability). MTT data were further utilised to determine IC_{50}-values of extracts and their respective filler-combinations through the log-transformation of the concentration values (x-axis). By using the nonlinear least squares regression, log(inhibitor) versus normalised response-variable slope function, data was simultaneously analysed, the curve fitted and IC_{50}-values calculated.

Furthermore, Statistica software (ver.12; TIBCO Software Inc., Palo Alto, CA) was used to perform statistical analysis of MTT and LDH data. Concisely, the one-way analysis of variance (ANOVA) test was performed in order to detect any statistical significant differences between extracts and their filler combinations at a particular concentration. P-plots were used as a graphical indication of normality, followed by Levene's *F*-test to test the assumption of homogeneity of variance. Concluding, the non-parametric Kruskal-Wallis test was used in order to do multiple comparisons between extracts and their filler combinations at a particular concentration. A p value ≤ 0.05 was considered statistical significant throughout all tests. Furthermore, GraphPad Prism was also used to statistically analyse FITC annexin V/PI data by means of the non-parametric Kruskal-Wallis test followed by the Dunn's multiple comparison test in order to determine whether extracts reduced cell viability in a significant manner. Again significance was indicated by a $p \leq 0.05$.

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CHAPTER 4: RESULTS AND DISCUSSION

4.1 INTRODUCTION

Increasing awareness of possible risks concomitant with the use of traditional medicine or botanicals have made it clear that 'natural' does not imply 'safe'. Accordingly, attempts to warrant the safety of these products and prevent their misuse are also increasing (Prinsloo *et al.*, 2017). During preclinical testing of potential compounds, the use of cell culture practices has become customary since the type of cultured cells can be chosen to signify the biochemical anomalies of the illness under investigation. Unfailingly, data obtained from *in vitro* cytotoxicity assays selected during preclinical screening must be reliable and accurate (Van Tonder *et al*., 2015). Despite the critically endangered status of *S. aethiopicus,* several commercially available products said to contain African ginger are currently freely available to consumers and extracts containing the active compound siphonochilone, have been patented for the application thereof in the treatment of allergic conditions such as asthma (Horak *et al*., 2009; Berg, 2016). This raises major trepidations, especially when considering the lack of quality control protocols and the complete lack of cytotoxicity data available for African ginger. It simply cannot be assumed that an extensive history of use or absence of toxicity evidence provides confirmation of safety or absence of toxicity (Moreira *et al.*, 2014).

In order to establish a cytotoxic profile for *S. aethiopicus*, numerous extracts were prepared during this study including ethanol, diethyl and aqueous extracts. A commercial solution and traditional infusion was included in the study for comparative purposes. Since plant extracts are very intricate mixtures of phytochemicals, the cellular response will rely on the action of the complete extract, which might include synergistic, additive or antagonistic interactions. Consequently, diverse response patterns might be observed for different extracts, resulting in a lack of anecdotal toxicity data (Taylor *et al.*, 2003). The extracts where chemically characterised based on methods described by Bergh (2016). Of these extracts ethanol, diethyl ether and aqueous extracts were furthermore compounded with chitosan and Pharmacel® 101 excipients due to the increasing commercial availability of products claiming to contain African ginger extracts in several oral pharmaceutical dosage forms. Pharmacel® 101 consists of MCC and is one of the most frequently used pharmaceutical excipients, with particular relevance as a filler during tablet formulation (Alderborn, 2013; Thoorens *et al.*, 2014). On the other hand, the pharmaceutical applications of chitosan exceeds conventional tablet formulation. It can be utilised in various drug delivery systems (i.e. microspheres, hydrogels, micelles and nanoparticles) for oral, ocular, pulmonary and nasal administration, as well as injectable and implantable dosage forms. Chitosan is furthermore suggested to be an effective absorption enhancer due to its potential to open tight

junctions and its muco-adhesive properties. Nevertheless, various dosage forms formulated with chitosan indicated desirable effects on drug delivery with prolonged drug release, enhanced solubility, and reduced physiological or enzymatic degradation (Sonaje *et al.*, 2012; Cheung *et al.*, 2015).

MTT and LDH assays (routine cell viability assays) as well as apoptosis analysis (by means of FITC conjugated annexin V/PI) were performed on HepG2 and Caco-2 cells. These cells were chosen as a representation of the possible cytotoxicity extracts may have on liver and small intestine cells. Following oral administration (the preferred route of administration of African ginger) small intestine cells are directly exposed to extracts, whereas hepatocytes encounter active compounds during detoxification (Vihola *et al.*, 2005; Wentzel *et al.*, 2017). We set out to answer three basic questions: first, what extracts cause cytotoxic effects? Secondly, are these effects concentration-dependent? And finally, do these extract-excipient combinations have a statistically significant effect on cell viability when compared to the effects of the extracts alone?

To the best of our knowledge, this is the first study that truly investigated the *in vitro* cellular effects of *S. aethiopicus* extracts on cell lines, including the mechanism of cell death evoked by the extracts and whether combination with certain excipients altered the effects of each extract. As mentioned in previous chapters, only superficial cytotoxicity assays have been conducted with some African ginger extracts. Through our findings we hope that further investigation might follow in order to clarify the precise molecular mechanism of cell death, as well as identification of what compound(s) might be responsible for the observed cytotoxic effects, and whether these compounds might possess some form of selectivity towards cancerous cells.

4.2 PLANT EXTRACT PREPARATION

It is a well-known fact that the phytochemical composition and quantities of phytochemicals are influenced by several factors including the geographical source of collection, season, time, altitude, *et cetera*. As a result, it was of utmost importance to reduce the influence of these variable factors as much as possible (Dash & Nivsarkar, 2016). Furthermore, ethical approval (see Annexure F) was obtained prior to the initiation of this study, due to the endangered status of *S. aethiopicus.* Therefore a sufficient amount of plant material (rhizomes) was bought to suffice the need of the entire study and avoid variability.

While grating the *S. aethiopicus* rhizomes it was observed that some of the rhizomes were a deeper yellow colour compared to others that were paler. This observation was also visible after drying of the rhizomes as some of the grated rhizome pieces were a light brown colour, whereas others were more ivory coloured (Annexure A, Figure A1). A very sharp and repulsive ginger-like smell was furthermore noted during grating.

Yields of ethanol and diethyl ether extracts were as follow: 5.10% w/w (2.55g) and 4.9% w/w (2.50 g) for ethanol and diethyl ether extracts, respectively, were produced. The aqueous extract yielded a freeze-dried mass of 3.19 g and delivered a yield of 6.38% w/w (Annexure A, Table A1 for all calculations of percentage yield). Considering the yields produced by Fouche *et al*. (2011) and Fouche *et al*. (2013), the yields obtained in this study were almost double for ethanol and diethyl ether extracts; in both studies ethanol extracts produced yields of 2.60% w/w, whereas diethyl ether extracts from their studies yielded 2.10% w/w. On the contrary, the aqueous yield of Fouche *et al*. (2011) was 15.20% w/w; more than twice the yield we produced. However, our aqueous yield was congruent with that of Bergh (2016), who produced a yield of 6.64% w/w. These results again stress the difficulties and clear variability associated with herbal preparations, as pointed out previously.

Dry ethanol and diethyl ether extracts had to be dissolved with a small amount of ethanol prior to being diluted to the appropriate stock solution concentration (1000 µg/ml) with SFM since they were completely insoluble in regular aqueous media. Consequently, the formed dilution was more of a milky, resuspended extract, rather than a clear solution. This was the first indication of possible solubility-related issues. However, preparations intended for oral consumption, as is the case with African ginger, may be formulated as suspensions (Dash & Nivsarkar, 2016). It should be noted that all extracts (including the commercial solution, but excluding the traditional infusion) were resuspended in exactly the same manner, in other words, with 1% v/v ethanol in SFM, as described in section 3.3 for comparison purposes.

4.3 CHEMICAL PROFILING OF EXTRACTS

4.3.1 Ultra performance liquid chromatographic analysis (UPLC)

UPLC analysis was performed to separate the individual phytochemical compounds present in each selected extract from one another, followed by mass determination and structure elucidation thereof by means of Q-TOF/MS. The characteristic UPLC chromatogram of the diethyl ether African ginger extract is presented in Figure 4.1 as respective ultraviolet-diode array detection (UV-DAD) and base peak intensity (BPI) chromatograms.

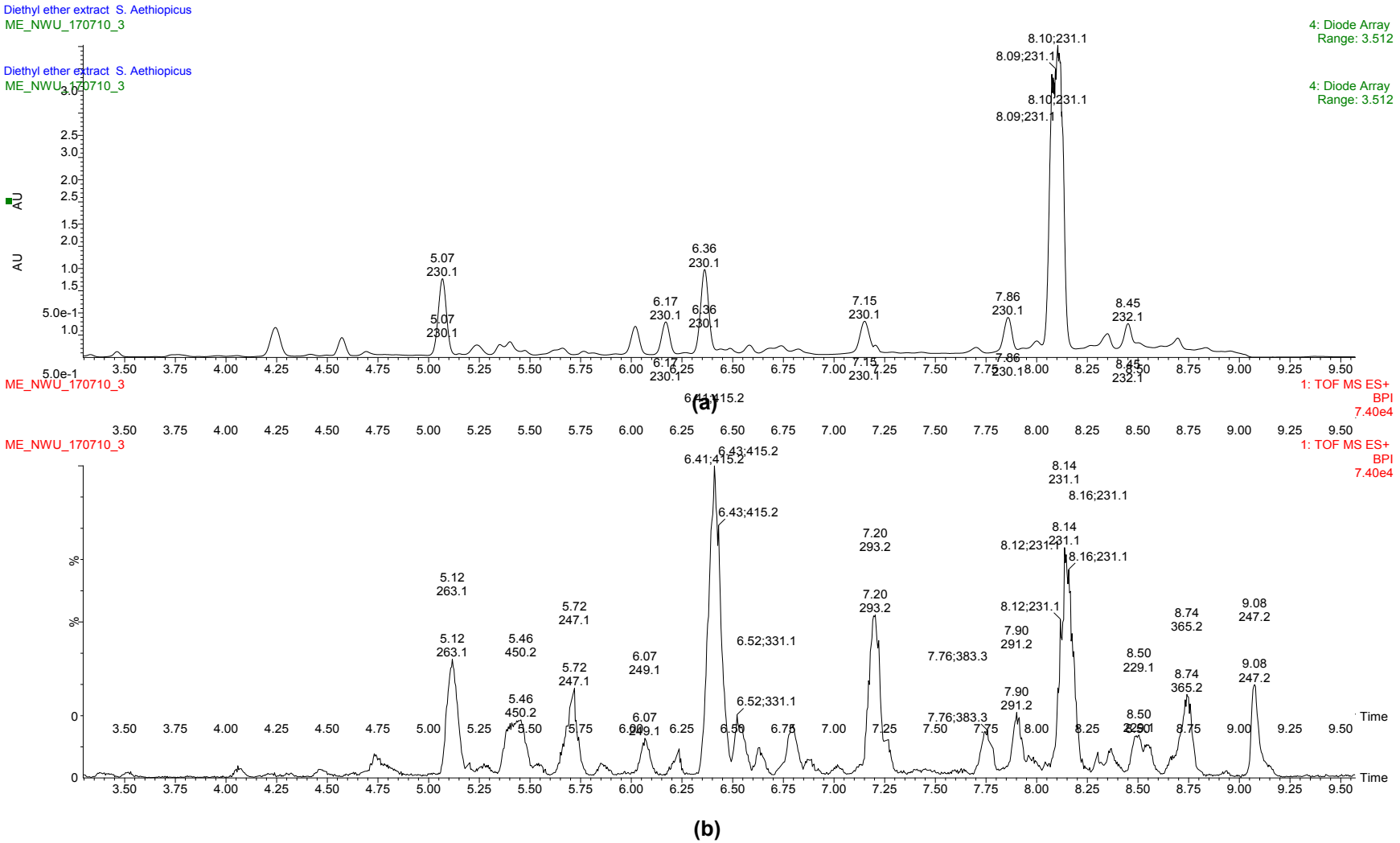
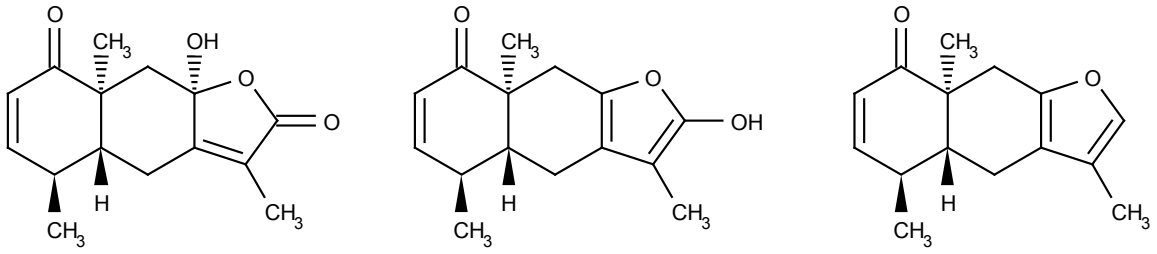


Figure 4.1: *UPLC chromatogram of the diethyl ether extract of S. aethiopicus; **(a)** UV-DAD chromatogram and **(b)** BPI chromatogram*

The following compounds as identified by Bergh (2016) were chosen as marker molecules for the chemical characterisation of the extracts studied: compound 6, compound 7, compound 8 and compound 9 (as listed in section 2.2.2 and illustrated in Figure 4.2). These compounds were chosen due to their strong peak intensities over the chromatographic spectrum (Bergh, 2016) and will be named AG 1 (compound 6), AG 2 (compound 7), AG 3 (compound 8) and AG 4 (compound 9) from here forth for practicality purposes.

AG 1 AG 2 AG 4



(a) Compound 6 **(b) Compound 7** **(c) Compound 9**

Figure 4.2: *Chemical structure of AG 1, AG 2 and AG 4 as previously identified by Bergh (2016). The chemical structure of AG 3 has not been elucidated, however its peak intensity is noteworthy*

In line with the findings of Bergh (2016), the base peaks (BP) of AG 2, 3 and 4 were found at 247, 415 and 231 m/z, respectively, in this study (Figure 4.3). Contradictory to Bergh (2016), the BP of AG 1 was observed at 263 m/z. The major peak in the chromatograms was identified as AG 3. This is very interesting, since AG 4 (compound 9) has been identified as the major constituent of African ginger extracts in numerous studies (Holzapfel *et al.*, 2002; Viljoen *et al.*, 2002; Fouche *et al.*, 2011; Katele Zongwe, 2015; Bergh, 2016). The abundance of AG 3 could not be attributed the autoxidation of AG 4 since it was not identified as one of the oxidation products (Katele Zongwe, 2015).

The ethanolic and diethyl ether extracts depicted similar qualitative chemical profiles containing all four marker molecules, though the peak intensities of each compound varied. Aqueous and commercial extracts, on the other hand, lack AG 3 and AG 4 in their composition; nonetheless they have comparable chemical profiles. The difference in phytochemical composition of organic and aqueous extracts was expected, as it is a known fact that diverse compounds may possess differing solubilities in solvents of varying polarities (Lockwood, 2013). Evidently, different compounds will be present in varying amounts in different extracts, possibly resulting in contrasting effects (Stafford *et al.,* 2005; Fouche *et al.*, 2011; Złotek *et al.*, 2015).

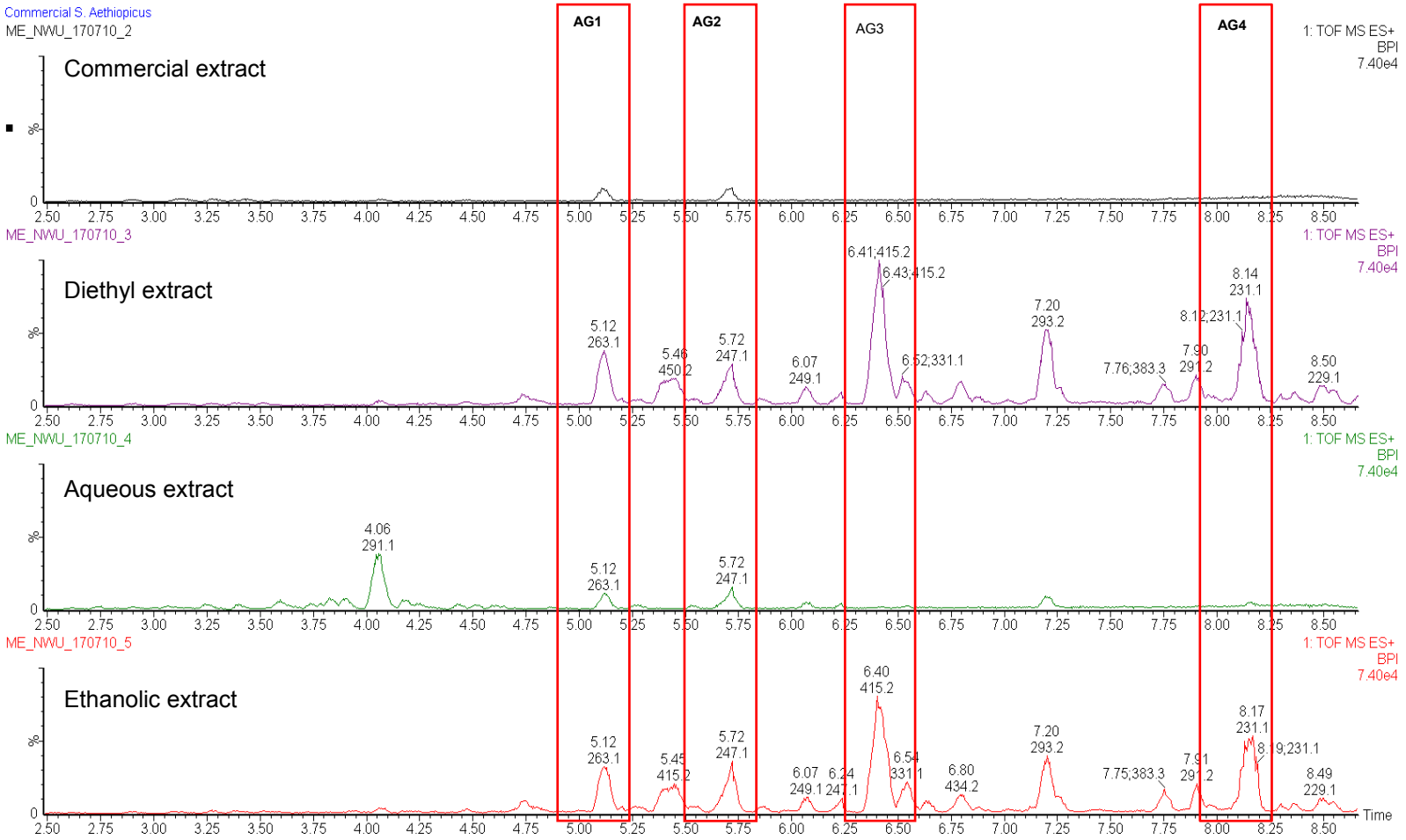


Figure 4.3: *Comparison of AG 1, AG 2, AG 3 and AG 4 peaks in the numerous extracts of S. aethiopicus used during cytotoxicity assays by means of their individual BPI chromatograms*

4.3.2 Ultra performance liquid chromatographic quadruple time of flight mass spectrometry (UPLC-Q-TOF/MS) analysis

The selected biomarker molecules, AG 1, AG 2, AG 3 and AG 4 were fractionated from the diethyl ether *S. aethiopicus* extract by means of the Synapt G2 Q-TOF system coupled to the UPLC system. The time of flight mass spectrometry (TOF/MS) chromatogram for each of these compounds is presented in Figures 4.4–4.7. Though the structure of AG 3 has not been fully elucidated as of yet, its peaks on the chromatograms of Figures 4.1 (b) and 4.4 cannot be ignored as it has the highest intensity. In Annexure B is given the complete UPLC-Q-TOF analysis report.

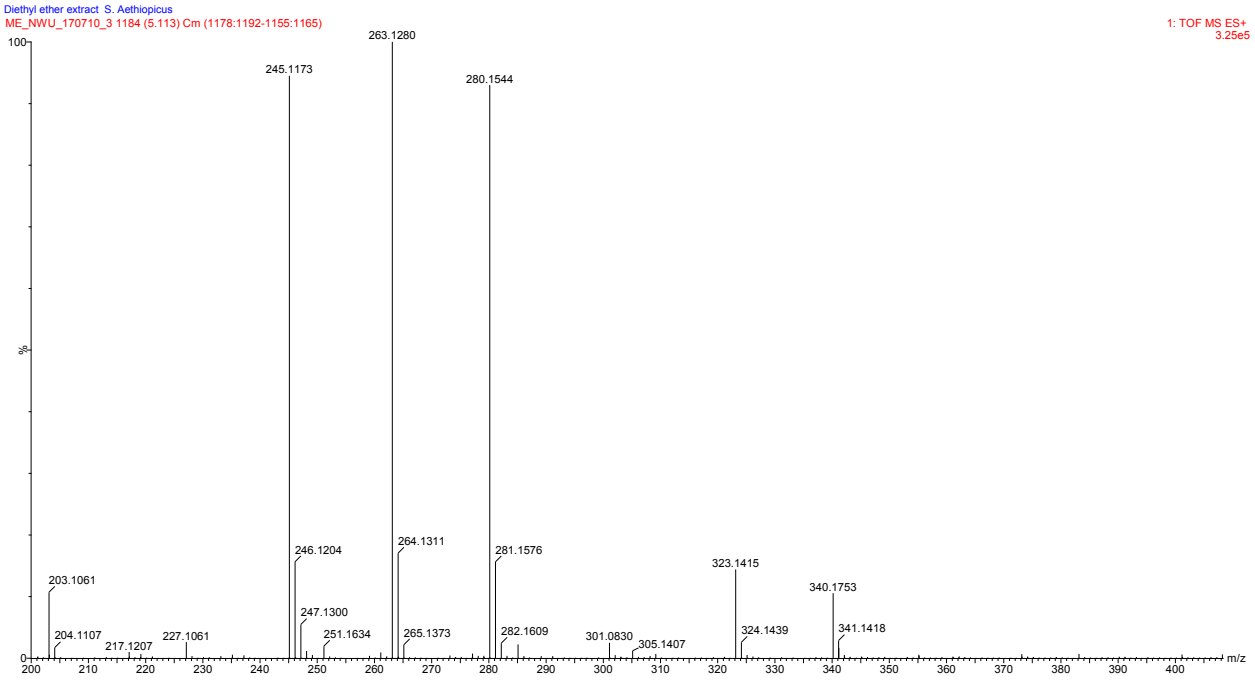


Figure 4.4: *TOF/MS fragmentation spectra of AG 1 identified by UPLC-Q-TOF/MS analysis*

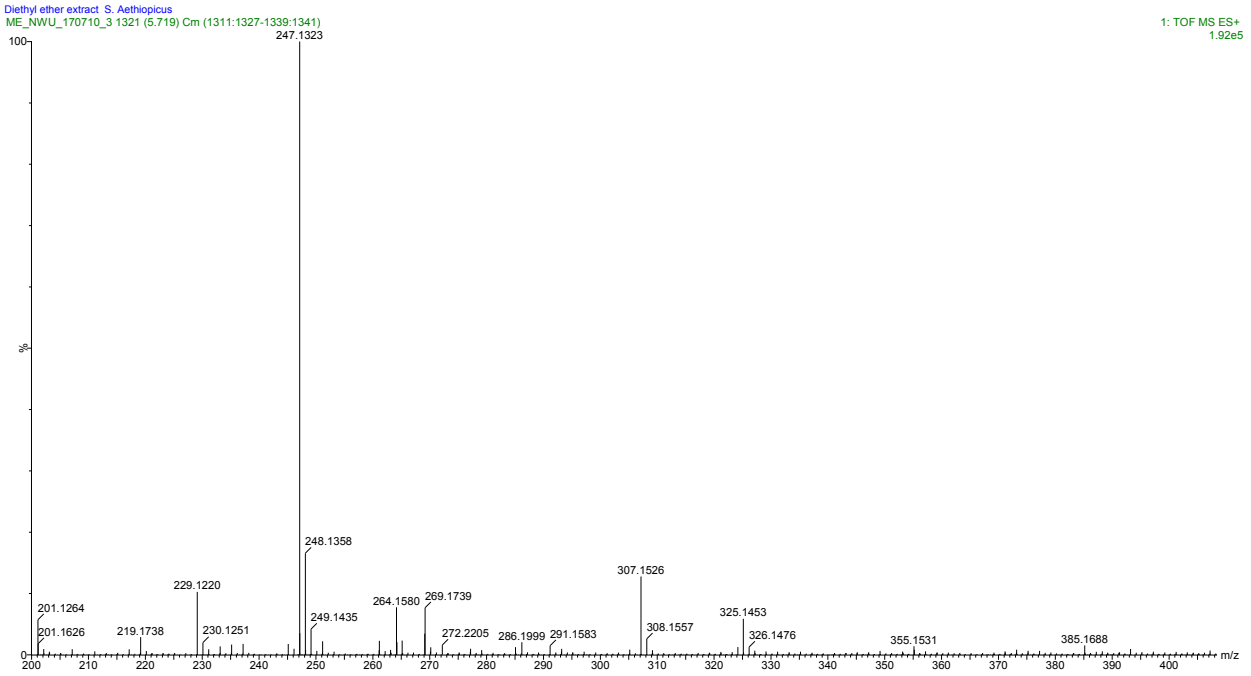


Figure 4.5: *TOF/MS fragmentation spectra of AG 2 identified by UPLC-Q-TOF/MS analysis*

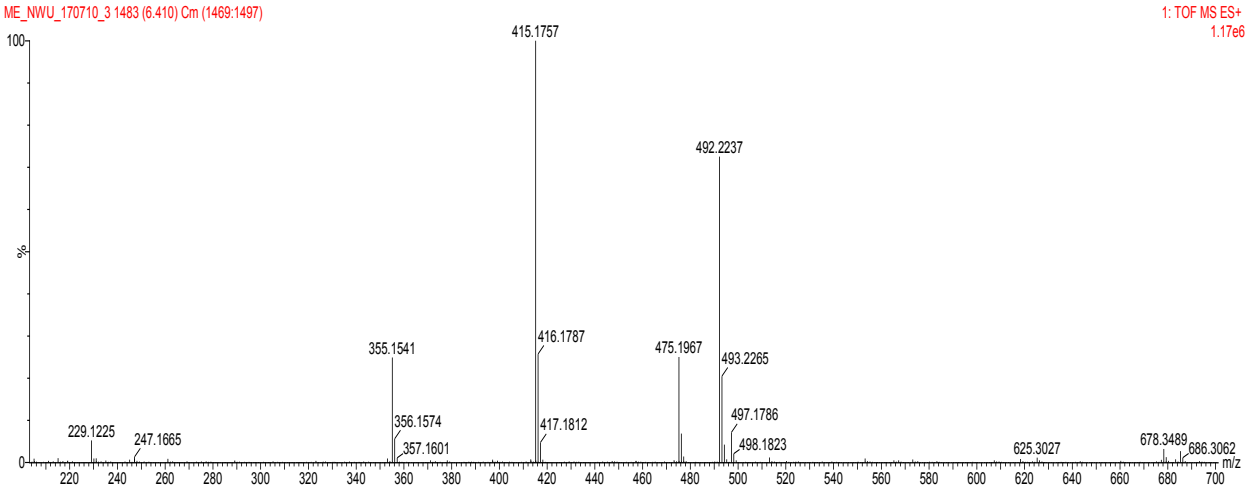


Figure 4.6: *TOF/MS fragmentation spectra of AG 3 identified by UPLC-Q-TOF/MS analysis*

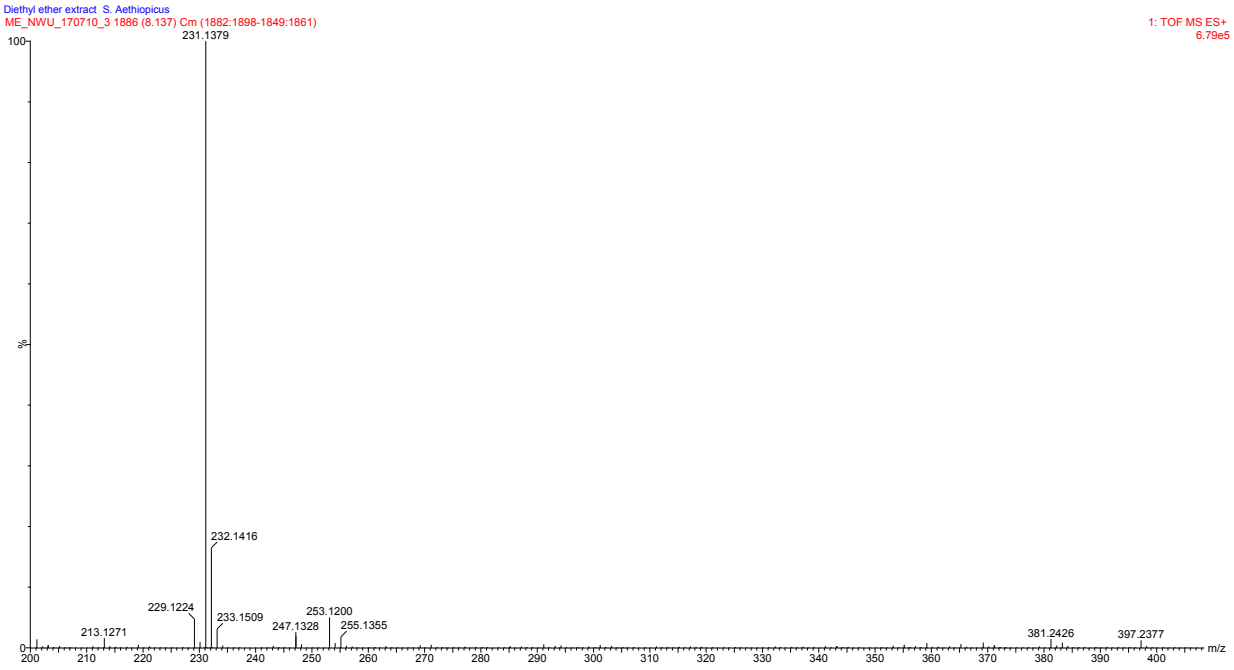


Figure 4.7: *TOF/MS fragmentation spectra of AG 4 identified by UPLC-Q-TOF/MS analysis*

TOF/MS data of all four marker molecules were analysed with MassLynx 4.1 software in order to determine possible molecular formulae and masses for each. A summary of this data is listed in Table 4.1. The iFit Confidence % indicates how well an isotope pattern compares to the theoretical pattern. It should be noted that the iFit Confidence % of AG 3 is extremely low and thus accordingly not reliable.

Table 4.1: *The TOF/MS data of AG 1, 2, 3 and 4*

Compound	Retention time (min)	TOF/MS (m/z)	MassLynx calculated molecular formulae	MassLynx calculated molecular mass	iFit Confidence %
AG 1	5.11	263.13	$C_{15}H_{19}O_4$	263.13	99.95
AG 2	5.72	247.13	$C_{15}H_{19}O_3$	247.13	71.22
AG 3	6.41	415.18	$C_{25}H_{31}O_9$	231.14	0.17
AG 4	8.14	231.14	$C_{15}H_{19}O_2$	475.19	78.70

4.4 DETERMINATION OF AVERAGE PARTICLE SIZE AND DISTRIBUTION

4.4.1 Average particle size and size distribution of excipients used

Due to the insolubility of both Pharmacel® 101 and chitosan in aqueous mediums, it was decided to determine the particle size of both selected excipients since they would be in direct contact with cells during cytotoxicity studies.

Particle size analysis of Pharmacel® 101 and chitosan with a Malvern Mastersizer 2000, both delivered negative skew particle size distributions. As indicated in Table 4.2, a mean volume particle diameter of 74.014 µm was measured for Pharmacel® 101, whereas a measurement of 177.992 µm was acquired for chitosan. Though the average particle size of chitosan was larger than that of Pharmacel® 101, its span (1.503) was less compared to Pharmacel® 101 (1.789), indicating a more narrow particle size distribution.

Table 4.2: *Particle size and distribution analysis of Pharmacel® 101 and chitosan*

	Pharmacel® 101			**Chitosan**		
	1	2	Average	1	2	Average
Mean volume particle diameter (µm)	72.025	76.003	74.014	180.914	175.070	177.992
Span	1.809	1.756	1.783	1.480	1.526	1.503

4.4.2 Average particle size of organic extract suspensions

The observation of resuspended organic extracts becoming milky in colour once diluted with SFM indicates that the crude extract was a dispersion rather than a solution. Microscope images (section 4.6), also revealed ethanol and diethyl ether extracts to possibly form an aggregated extract particle sediment during *in vitro* cellular exposure. This prompted the need for particle size and PSD analysis of the organic extracts.

Once more, a Malvern Mastersizer 2000 was utilised to analyse the particle size of organic extracts. Table 4.3 lists the mean volume particle diameter and span of ethanolic and diethyl ether *S. aethiopicus* extracts. The mean volume particle diameter of ethanol extracts (11.505 µm) was slightly smaller compared to that of diethyl ether extracts (14.054 µm). Strikingly, the span measurement of the ethanol extract (172.649) was considerably larger, compared to the measurement for the diethyl ether extract (2.672), indicating a significantly wider and heterogeneous PSD. This could possibly be attributed to the large number of particles (left-hand

spike, Figure C5–C6; Annexure C) possessing a particle size of ±0.1 µm observed within the ethanol extracts, but absent in diethyl ether extracts.

Table 4.3: *Particle size and distribution analysis of ethanol and diethyl ether S. aethiopicus extracts*

	Ethanol extract			Diethyl ether extract		
	1	2	Average	1	2	Average
Mean volume particle diameter (µm)	12.817	10.192	11.505	15.046	13.062	14.054
Span	192.716	152.583	172.649	2.889	2.455	2.672

4.5 OPTIMISATION OF ASSAY CONDITIONS

4.5.1 Cell proliferation studies: optimal cell number

Well studied cell lines usually have established culture conditions as well as protocols for maintenance thereof. Cell culture conditions that alter the metabolism of cells might also affect the rate of MTT reduction into formazan product. It was therefore crucial to establish optimal cell numbers to use in cytotoxicity assays. MTT cell proliferation assays were performed in order to determine the optimal number of cells per well when using HepG2 and Caco-2 cells, respectively, on a 96-well plate. Results can be viewed in Figure 4.8.

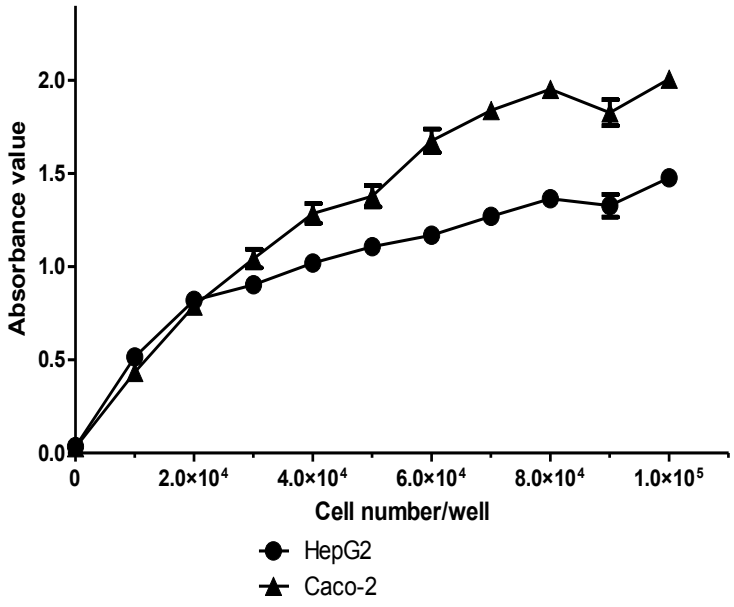


Figure 4.8: *The optimal number of cells per well of HepG2 and Caco-2 cells for cytotoxicity assays performed on 96-well plates. Data is presented as mean (n=3) ± SEM*

According to the MTT cell proliferation assay guide of the ATCC (ATCC, 2011), the average absorbance values of triplicate wells should yield an absorbance of 0.75–1.25 and lie within the linear portion of the graph. For HepG2 cells, this value was determined as 20 000 cells/well, whereas for Caco-2 cells the value was estimated as 40 000 cells/well.

4.5.2 Solvents

Typically *in vitro* cytotoxicity assays are performed in growth media; hence, no solubility problems are signified by water-soluble samples. Since plant extracts contain water-soluble and water-insoluble components, a co-solvent must be used to facilitate solubilisation (Timm *et al.*, 2013). In order to determine which solvent is most appropriate to assist in re-suspending extracts, optimisation studies were performed on HepG2 cells. Figure 4.9 displays the results obtained.

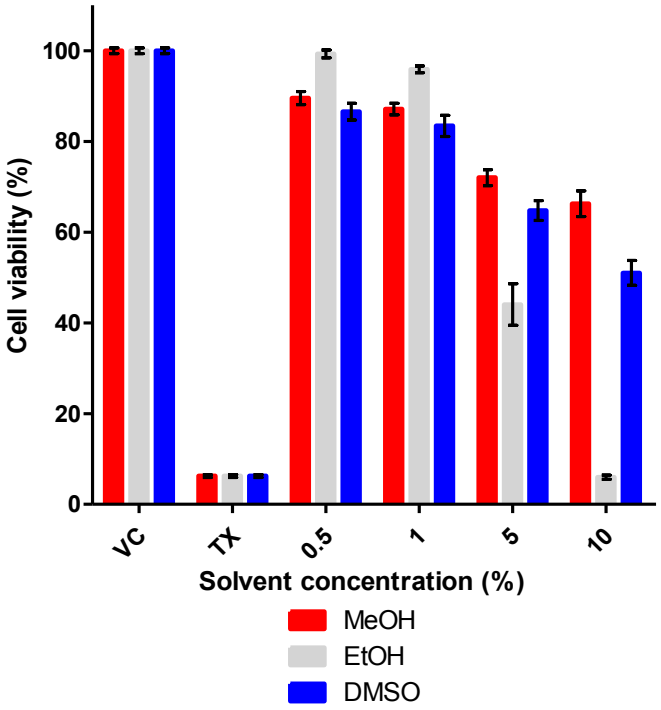


Figure 4.9: *Cell viability (%) of HepG2 cells, as determined with MTT assays, after exposure to numerous concentrations (0.5, 1, 5 and 10% v/v) of methanol (MeOH), ethanol (EtOH) and dimethyl sulfoxide (DMSO) diluted in SFM. Cells in vehicle control wells (VC) were exposed to SFM only, whereas positive control wells (TX) were exposed to 0.4% Triton X-100. Data is presented as mean (n=3) ± SEM*

The cytotoxic effects of co-solvents are frequent concerns when used in cellular systems. The MTT assay is normally used to determine the viability of treated cells (Timm *et al.*, 2013). As seen in Figure 4.9, ethanol only slightly affected the cell viability of cells at low concentrations (0–1% v/v), whereas methanol and DMSO almost reduced cell viability by 10%. However, at concentrations of 5% v/v and more ethanol severely affected cell viability, with a meagre viability

of only 6% at a concentration of 10%; whereas methanol and DMSO revealed cell viabilities higher than 50%, even at the highest concentration.

A 1% v/v ethanol solution was therefore chosen as the co-solvent and concentration to re-suspend the organic plant extracts. Literature furthermore revealed that ethanol is regularly utilised as a solvent for plant extracts. Concentrations of up to 2.8% v/v moreover did not affect the cell viability of mouse Abelson murine leukaemia virus-induced macrophage (RAW 264.7) cells when evaluated with a trypan blue staining method (Wakabayashi & Negoro, 2002), whereas a study by Timm *et al*. (2013) indicated that RAW 264.7 cells tolerated ethanol concentrations of 1% reasonably well.

4.5.3 Concentration range and time of exposure

MTT assays were performed according to ISO recommendations (ISO 10993-5, 2009) during optimisation studies to determine the experimental concentrations and exposure times. It was decided to use the diethyl ether extract during these optimisation studies due to the abundance of all biomarker compounds within the extract. Initial concentrations of 1, 10, 100 and 1000 µg/ml were selected and assayed for 4, 8 and 24 h respectively. Experimental data can be viewed in Figure D1, Annexure D. After a 4 h exposure to a 100 µg/ml extract, cell viability already decreased to below 50%. Consequently, a broad concentration range consisting of 50, 100, 150, 200, 300, 500, 1000 µg/ml was chosen, as well as an exposure time of 4 h.

4.6 MICROSCOPIC EVALUATION OF MORPHOLOGICAL CHANGES

With the aim of examining the effect of ethanol, diethyl ether, and aqueous extracts, commercial solutions, as well as traditional infusions on the morphology of HepG2 and Caco-2 cells, light microscope images were captured according to methods described in section 3.8. Images are presented in Figures 4.10–14.

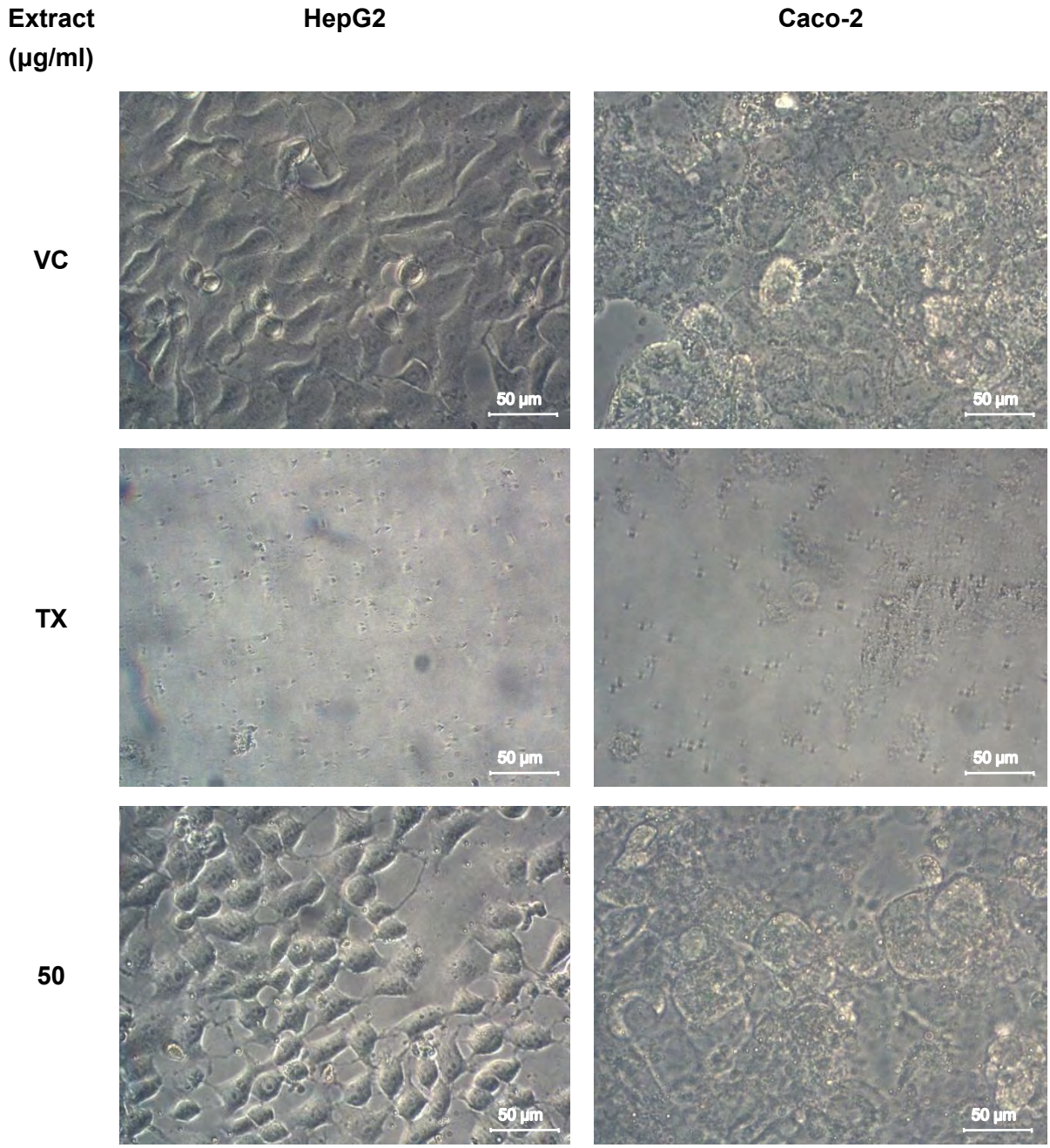


Figure 4.10: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of an ethanol extract of S. aethiopicus as captured by light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100*

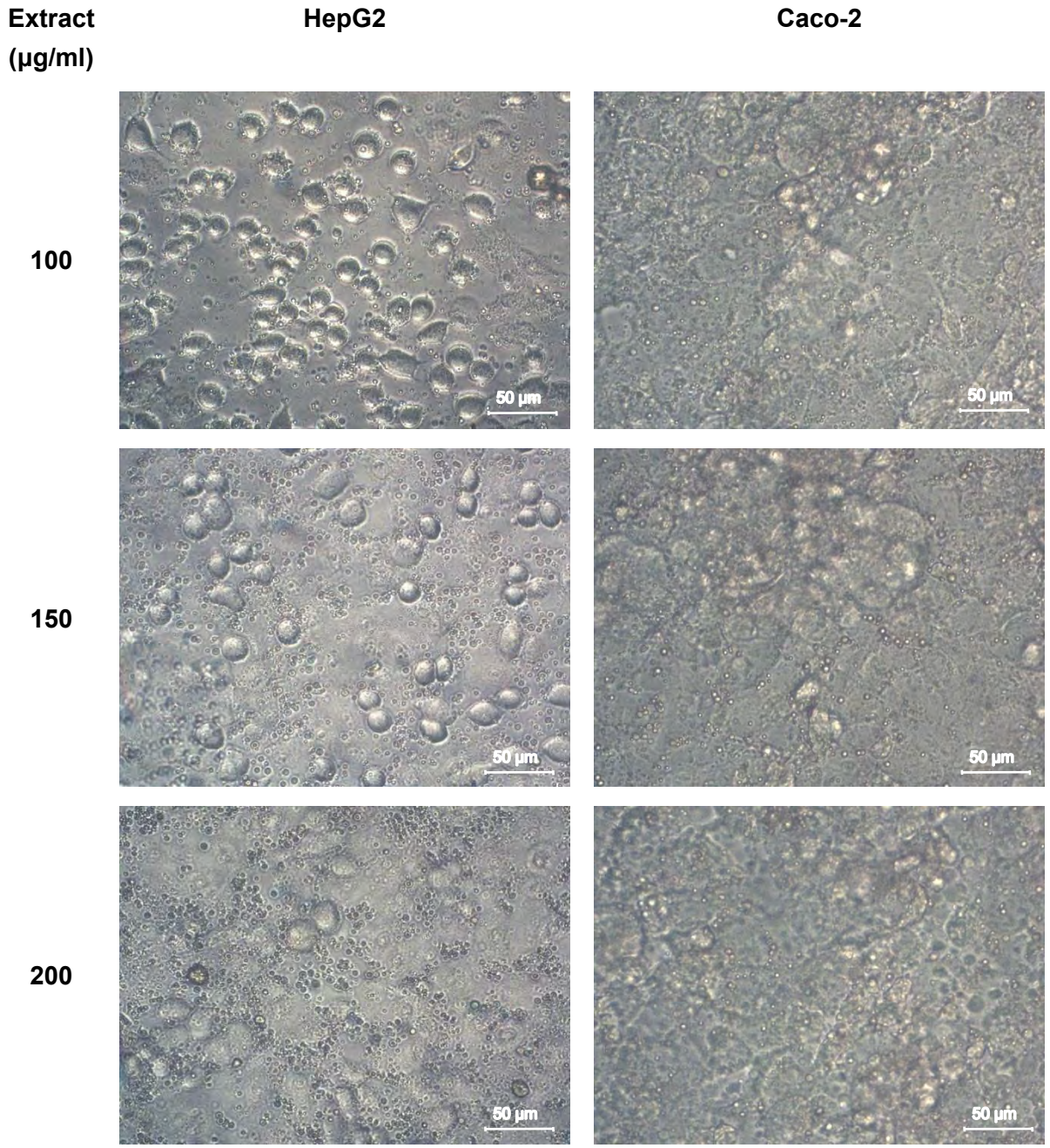


Figure 4.10: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of an ethanol extract of S. aethiopicus as captured by light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

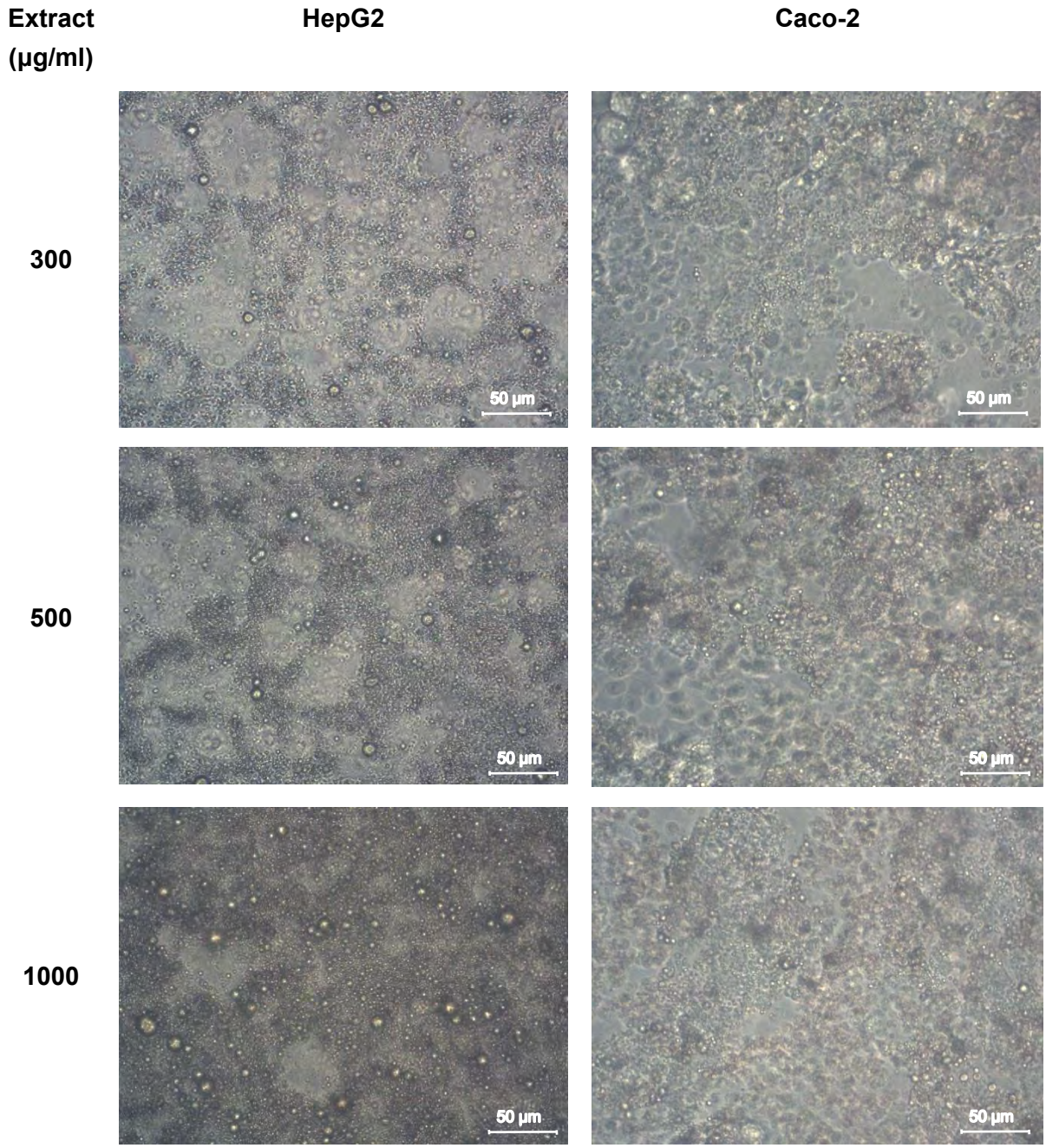


Figure 4.10: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of an ethanol extract of S. aethiopicus as captured by light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

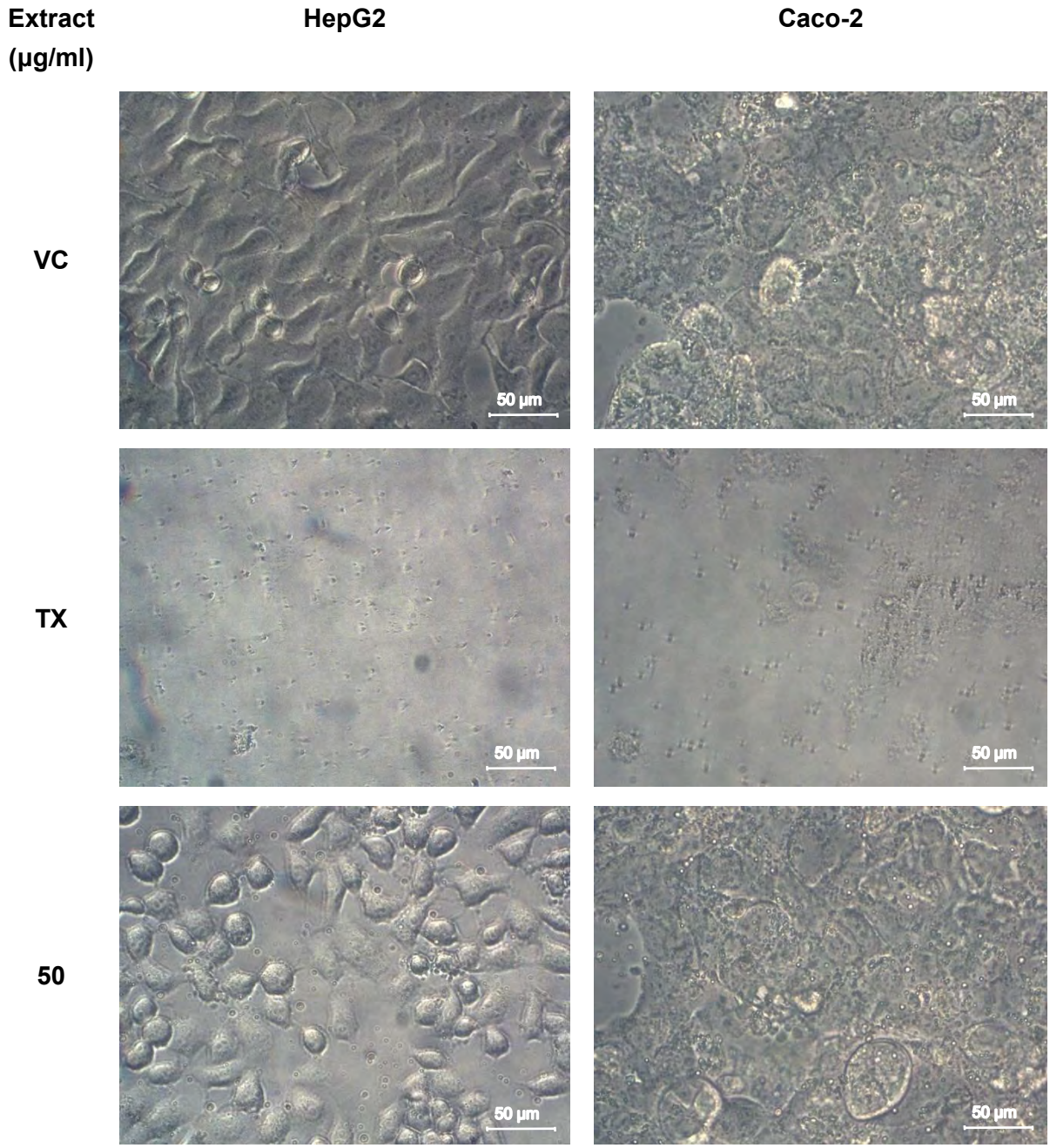


Figure 4.11: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of a diethyl ether extract of S. aethiopicus. Images were captured with captured light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100*

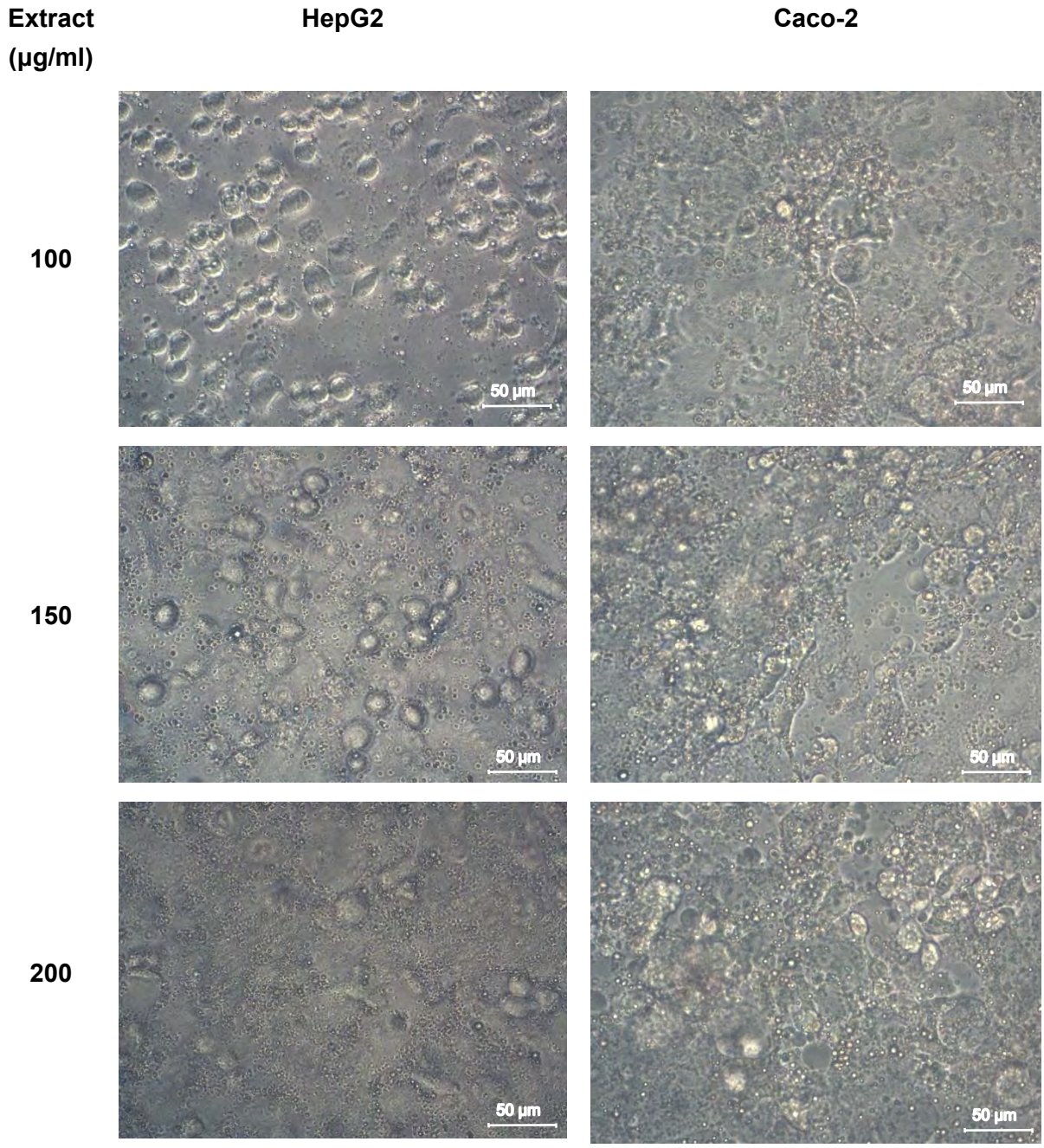


Figure 4.11: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of a diethyl ether extract of S. aethiopicus. Images were captured with captured light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

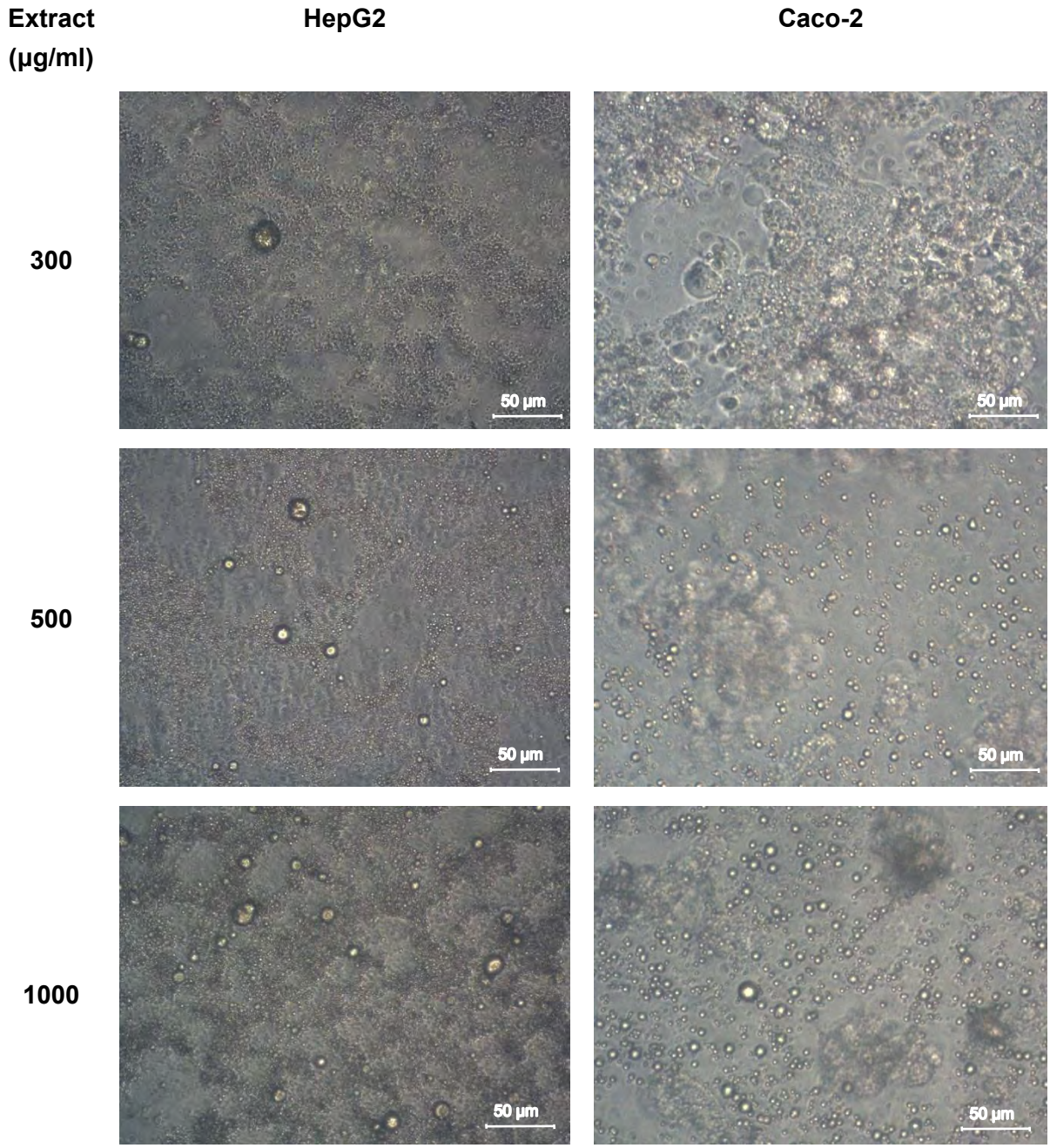


Figure 4.11: *Morphological changes of HepG2 and Caco-2 cells induced by several concentrations (µg/ml) of a diethyl ether extract of S. aethiopicus. Images were captured with captured light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

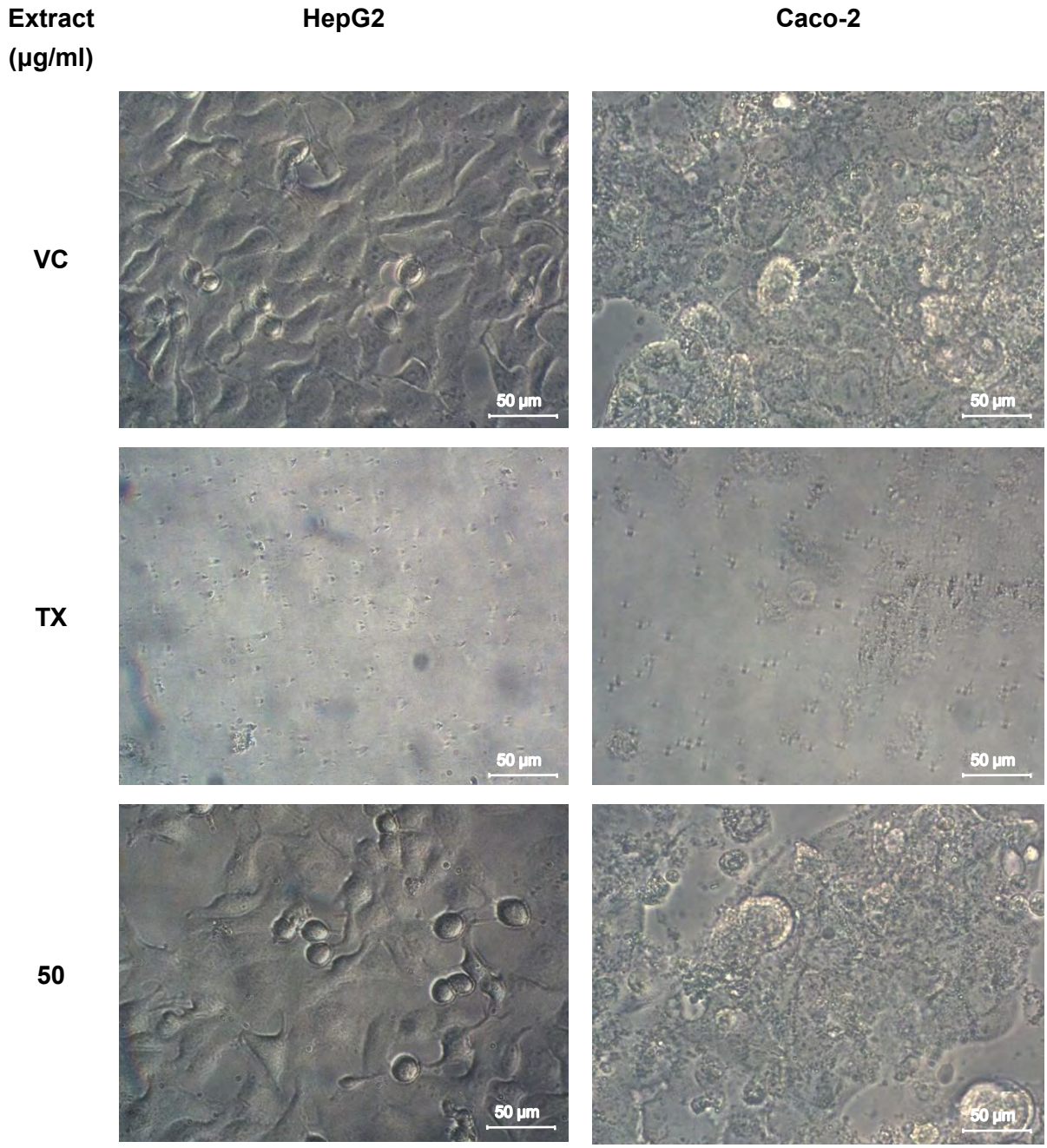


Figure 4.12: *No morphological changes were induced by numerous concentrations (µg/ml) of an aqueous S. aethiopicus extract on HepG2 or Caco-2 cells. Images were captured with light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100*

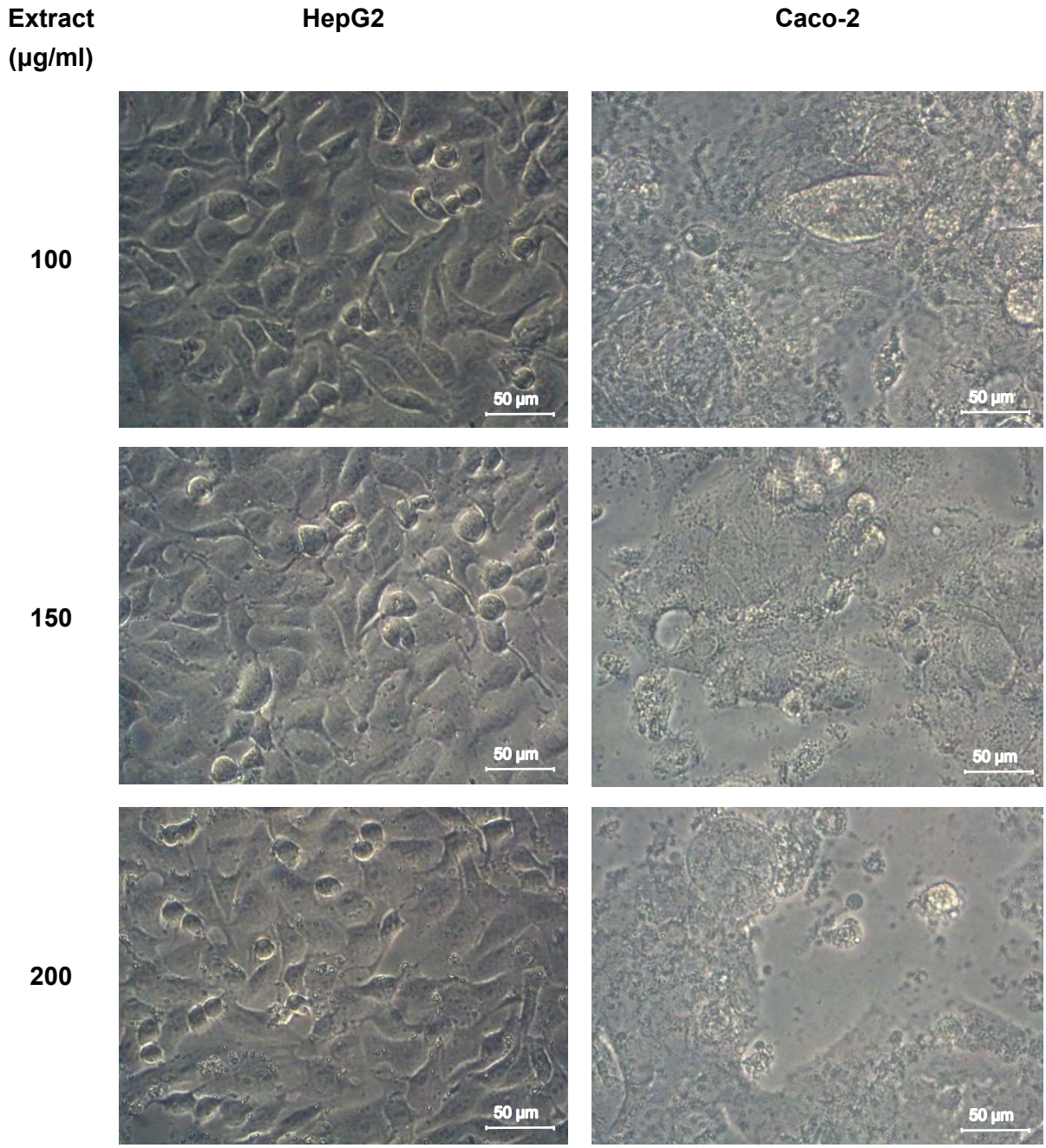


Figure 4.12: *No morphological changes were induced by numerous concentrations (µg/ml) of an aqueous S. aethiopicus extract on HepG2 or Caco-2 cells. Images were captured with light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

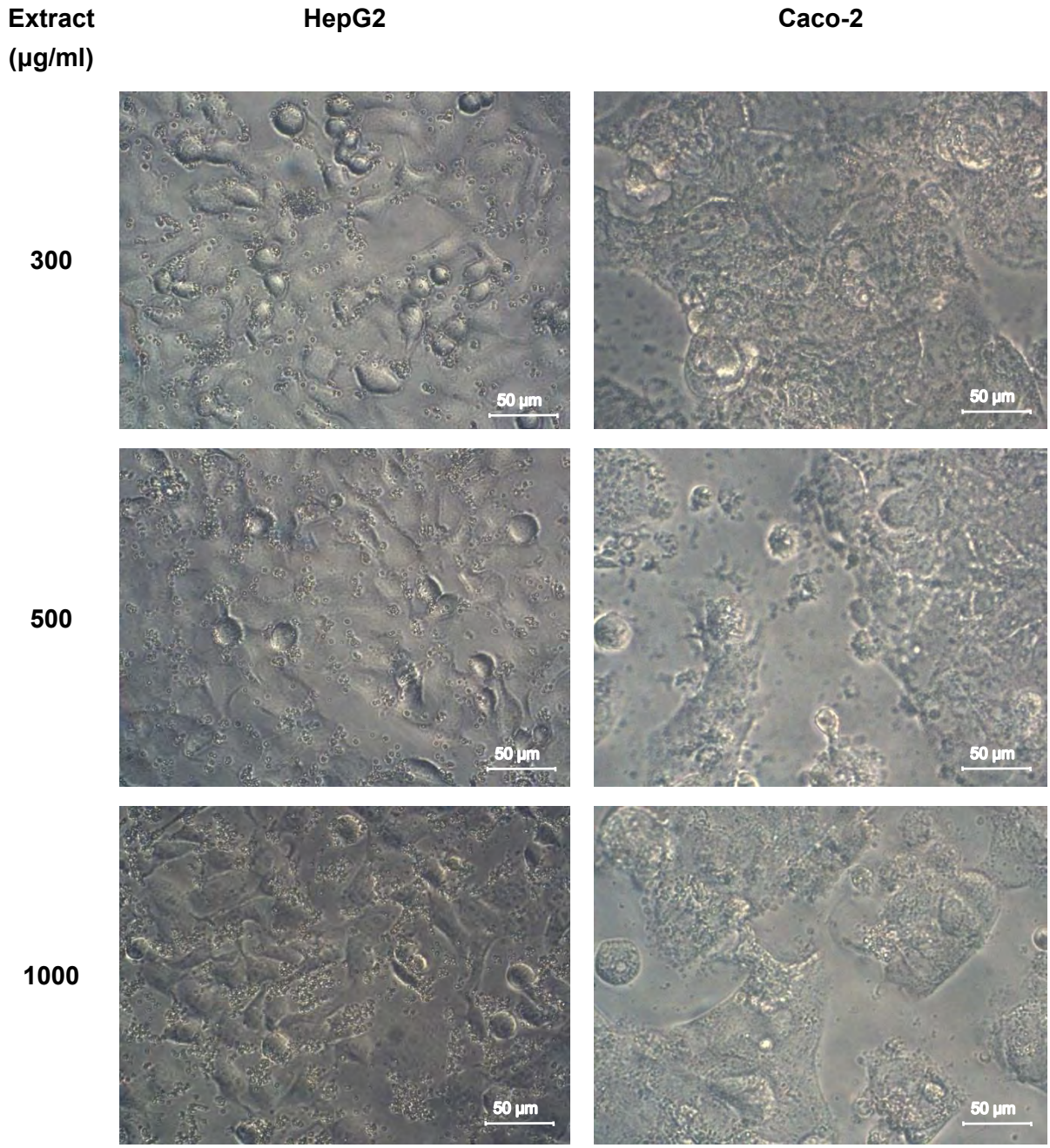


Figure 4.12: *No morphological changes were induced by numerous concentrations (µg/ml) of an aqueous S. aethiopicus extract on HepG2 or Caco-2 cells. Images were captured with light microscopy at a 40x magnification. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

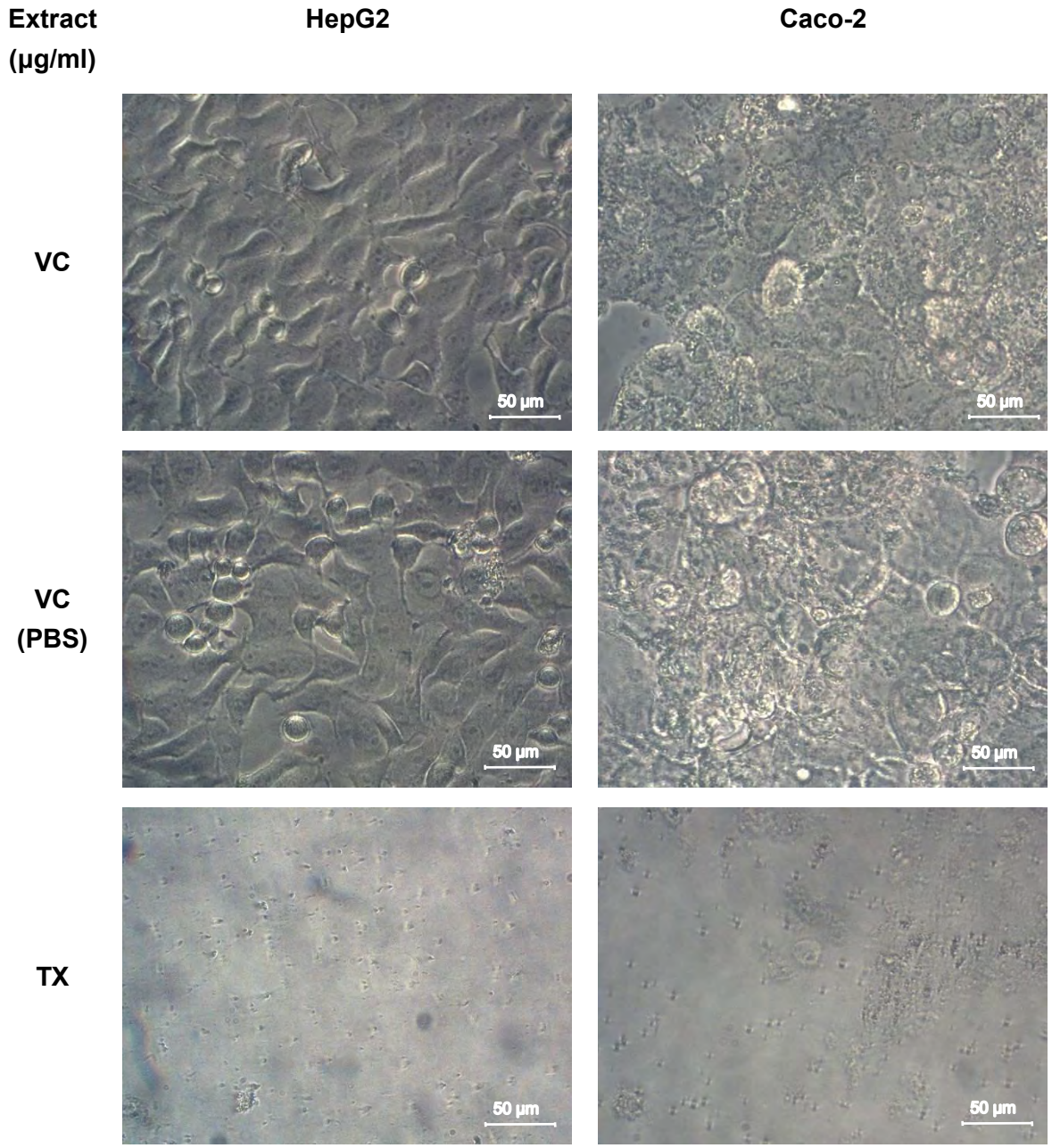


Figure 4.13: *Concentrations (mg/ml) of a commercial solution of S. aethiopicus induced no morphological changes of HepG2 or Caco-2 cells as captured by light microscopy at a 40x magnification. The large, oddly shaped particles are most likely insoluble tablet excipients. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100*

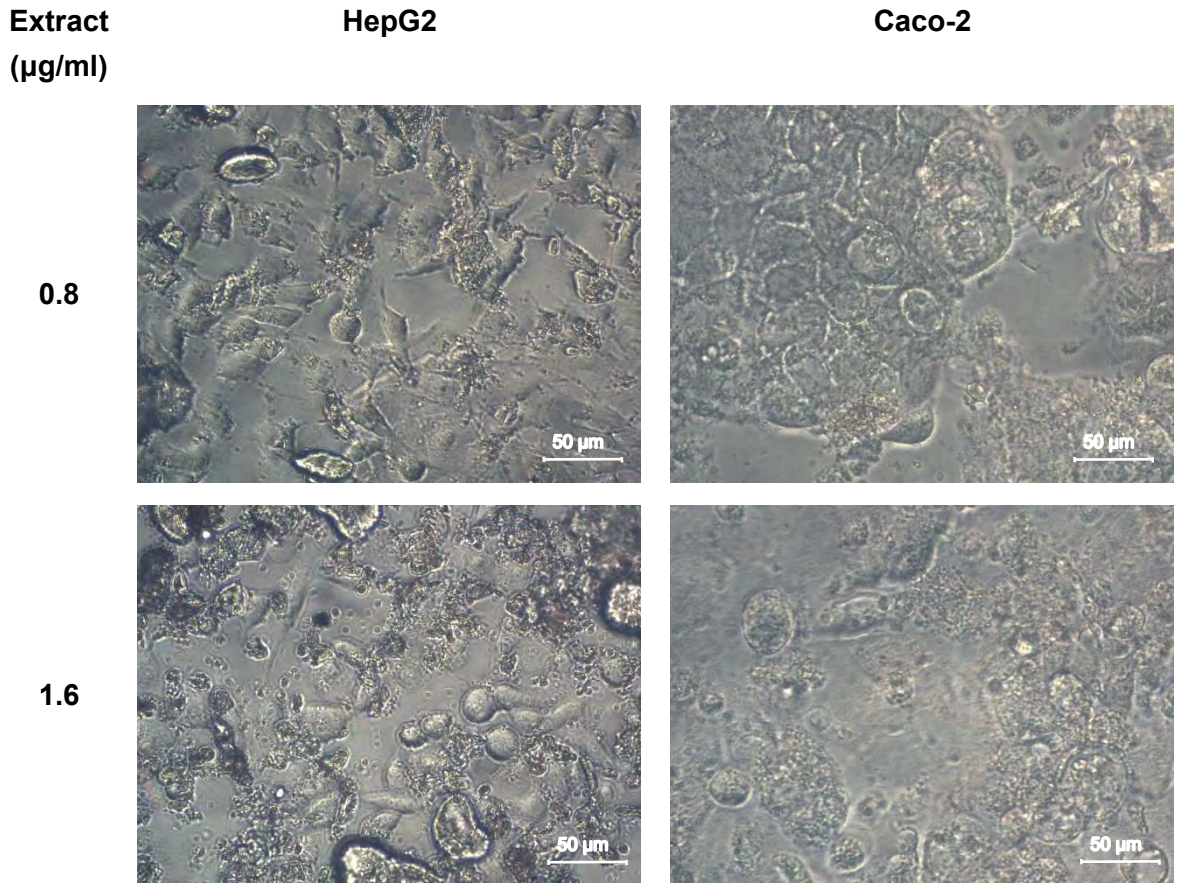


Figure 4.13: *Concentrations (mg/ml) of a commercial solution of S. aethiopicus induced no morphological changes of HepG2 or Caco-2 cells as captured by light microscopy at a 40x magnification. The large, oddly shaped particles are most likely insoluble tablet excipients. VC represents vehicle controls (SFM and cells), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

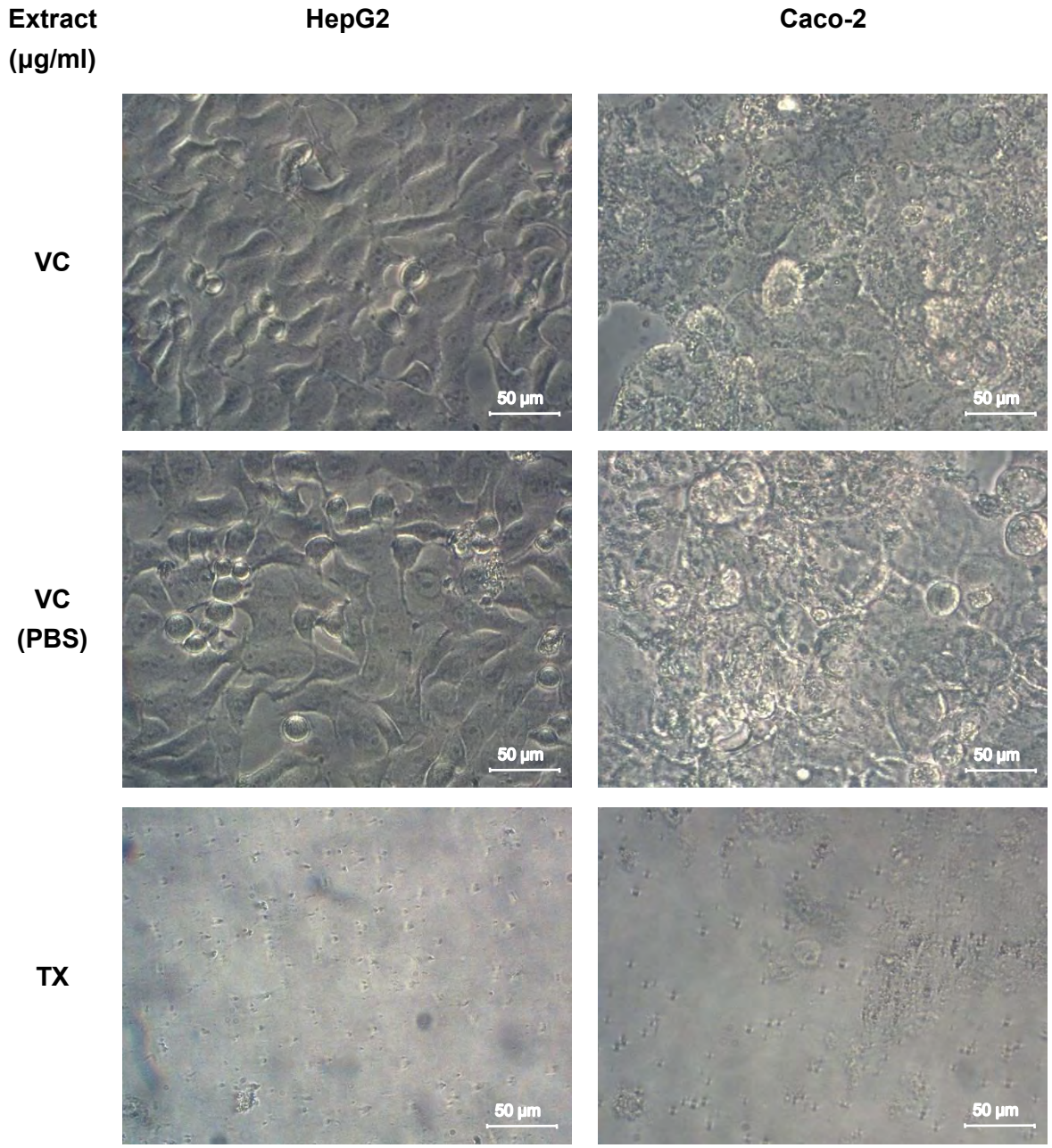


Figure 4.14: *Morphology of HepG2 and Caco-2 cells were not altered by concentrations (mg/ml) of a compounded traditional infusion S. aethiopicus; images were captured by light microscopy at a 40x magnification. Very large dry plant powder particles can be observed, particularly on Caco-2 cells, as the infusion was not filtered. VC represents vehicle controls (SFM and cells), VC PBS represents SFM:PBS vehicle control (1:1 dilution), whereas TX represents cells exposed to 0.4% Triton X-100*

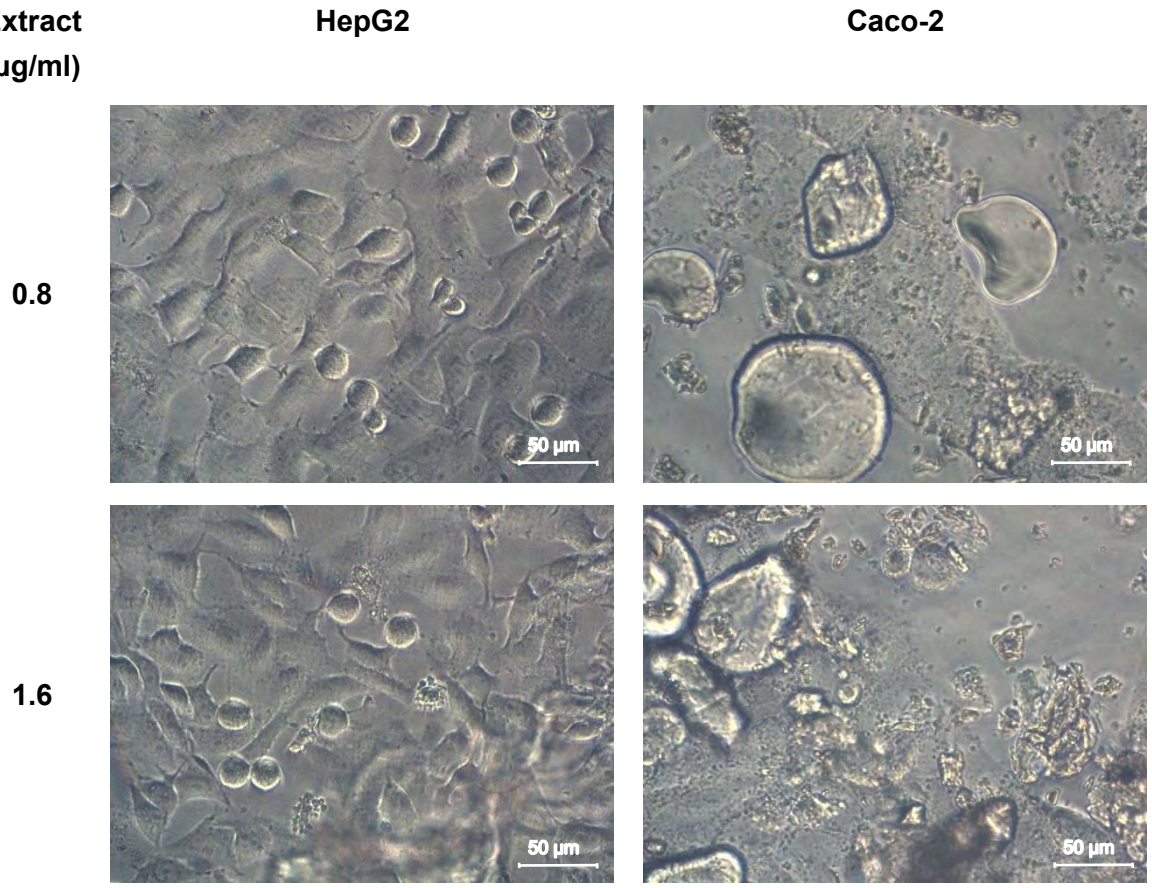


Figure 4.14: *Morphology of HepG2 and Caco-2 cells were not altered by concentrations (mg/ml) of a compounded traditional infusion S. aethiopicus; images were captured by light microscopy at a 40x magnification. Very large dry plant powder particles can be observed, particularly on Caco-2 cells, as the infusion was not filtered. VC represents vehicle controls (SFM and cells), VC PBS represents SFM:PBS vehicle control (1:1 dilution), whereas TX represents cells exposed to 0.4% Triton X-100 (continued)*

The images revealed ethanol and diethyl ether extracts to cause severe morphological changes to HepG2 and Caco-2 cells, which appeared to be possibly concentration-dependent. Caco-2 cells seemed to be less susceptible to the effects of these extracts compared to HepG2 cells; HepG2 cells started to show morphological alterations at concentrations as low as 50 µg/ml, whereas Caco-2 cells only exhibited clear morphological changes at concentrations equal to, and higher than 300 µg/ml. The cell rounding, shrinkage, and decreased cellular adhesion observed were consistent with activation of cell death pathways (Ali *et al.*, 2014; Nair *et al.*, 2014); though it would be inaccurate to make assumptions regarding the mechanism thereof. Due to the heterogeneous cell shape and size of the Caco-2 cells, it was somewhat difficult to observe these alterations. It is important though to note that the small granular particles observed in Figures 4.10 and 4.11 (also mentioned in section 4.4.2) were suspected to be suspended extract particles that formed an aggregated sediment upon standing. These findings affirmed previous

suspicions of solubility issues as mentioned in section 4.2. However, this was also expected as the solubility of phytochemicals present in a herbal extract will vary depending on the selected solvent system used (Dash & Nivsarkar, 2016).

As previously stated, the size of the particulate matter ranges within the micrometre (µm) scale and, as observed from Figures 4.10–4.11, are relatively smaller than HepG2 or Caco-2 cells. It is an established fact that diverse cellular responses may be induced succeeding exposure to particles of various sizes, with smaller particles often inducing higher cytotoxic effects. However, particulate density or concentration might also affect these responses (Santos *et al.*, 2010). A study by Choi *et al.* (2009) found that exposure to microparticles smaller than 3 µm resulted in increased cytotoxicity at concentrations higher than 200 µg/ml. According to Attwood (2013), particles within a dispersed system will unavoidably collide with one another. When these interactions or collisions are permanent, coagulation will occur; this will result in the aggregation and sedimentation of particles, which are challenging to redisperse. Dense aggregate formation at relatively high concentrations (estimated at 300 µg/ml for this study) may therefore result in limited oxygen and nutrient exposure of *in vitro* cells, causing decreased cell viability (Farrell *et al.*, 2015). Nonetheless, as mentioned previously, lower organic extract concentrations still induced morphological alterations in HepG2 cells, even though particles are widely distributed within the cell cultures. Therefore, particle aggregates and subsequent sedimentation might be a contributing factor to the observed cytotoxicity at higher organic extract concentrations; then again the contrary might be true for lower extract concentrations.

On the other hand the aqueous extract, commercial solution and traditional infusion did not cause any cytotoxic morphological alterations on either HepG2 or Caco-2 cells. The aqueous extract was filtered in order to remove any insoluble plant particulate matter; yet still small granular particles could be observed, given it was noticeably less than that of the organic extracts. The commercial solution and traditional infusions were not filtered; the reasoning therefore was to create an intestinal micro-environment as close as possible to the realistic conditions following human consumption. Similarly, the excipient particles (which are larger in size compared to cells) of the commercial solution and the insoluble plant powder particles of the traditional infusion did not seem to exert any deleterious effects on the cells.

It is imperative to keep the difference in extract composition in mind since aqueous extracts, traditional infusions, as well as commercial solutions lack the presence of AG 3 and AG 4; and only contain AG 1 and AG 2 in small amounts. Through these preliminary observations it is evident that compounds AG 3 and AG 4 might be partly responsible for the altered morphological appearance of both HepG2 and Caco-2 cells.

4.7 CYTOTOXICITY ASSAYS

4.7.1 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay

The MTT assay was included in this study as it is considered by many to be the golden standard for determining cell viability and remains a popular cell viability assay frequently utilised in academic labs (Riss, 2014; Van Tonder *et al.*, 2015; Riss *et al.*, 2016; Kuete *et al*., 2017). During the assay, only metabolic active cells possess the ability to convert the MTT reagent into an insoluble, purple formazan product (as mentioned in section 3.9.1.1). Hence, the colour development aids as a marker of viable cells, since dead cells are not able to partake in the MTT reduction. Some authors speculate that specific mitochondrial dehydrogenase enzymes may be involved in the MTT conversion to formazan. However, the precise cellular mechanism is poorly elucidated; it is probable that electrons are transferred to MTT by reducing enzymes from reduced molecules, such as NADH. Reducing enzymes and compounds present in the endoplasmic reticulum, lysosomes and other organelles are also suspected to be involved (Riss, 2014; Van Tonder *et al.*, 2015; Riss *et al.*, 2016).

Figure 4.15 illustrates the cell viability of (a) HepG2 and (b) Caco-2 cells, respectively, when exposed to several concentrations of ethanol, diethyl ether and aqueous extracts of *S. aethiopicus* for 4 h as determined by the quantitative colorimetric MTT assay. Triton-X 100 0.4% was used as a positive control for diminished cell viability.

Initially, aqueous extracts seemed to noticeably increase the cell viability of both HepG2 and Caco-2 cells when compared to vehicle controls. The increase in cell viability was clearly not concentration-dependent as viability plateaued at approximately 120% and 105% for HepG2 and Caco-2 cells, respectively. Figure 4.16 depicts the effects of the commercial solution and traditional infusion to be relatively similar compared to those of aqueous extracts on both cell lines, causing an increase in cell viability. Nevertheless, when considering the effects of ethanol and diethyl extracts, a considerably different profile is observed – almost complete opposite cytotoxic effects are revealed – however, this was not surprising as solvents with varying polarities are often utilised to extract different compounds (Taylor *et al.,* 2003; Abarca-Vargas *et al.*, 2016).

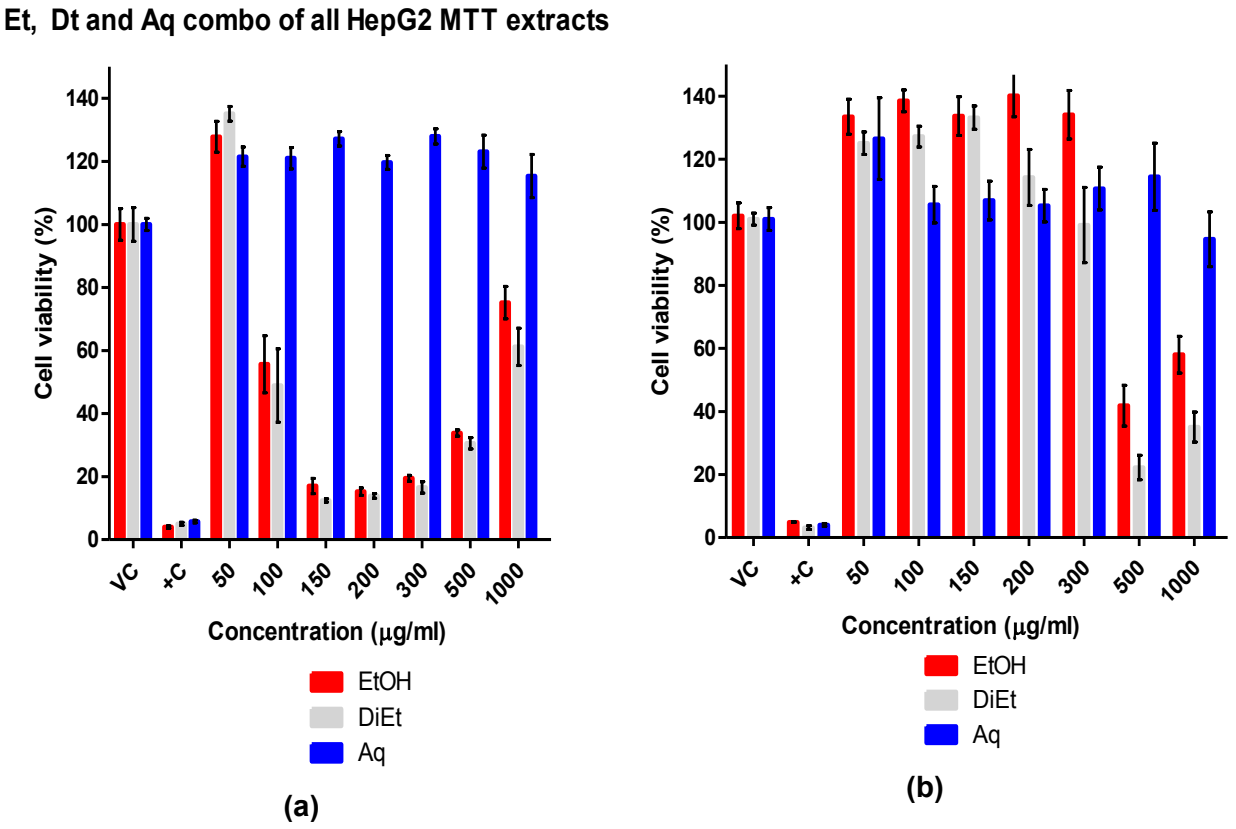


Figure 4.15: *Comparison of effects of various concentrations of ethanolic, diethyl ether and aqueous S. aethiopicus extracts on the cell viability of* ***(a)*** *HepG2 and* ***(b)*** *Caco-2 cells as determined with MTT assay following 4 h of exposure. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data is represented as mean (n=6) ± SEM*

Both cell lines appeared more susceptible to the effects of the diethyl ether extract when compared to those of the ethanolic extract. This finding was interesting when taking the smaller particle size of ethanolic extracts into account. At first, both these extracts increase the cell viability of HepG2 cells at a concentration of 50 µg/ml (127.8% for the ethanol extract and 135.1% for the diethyl ether extract). Thereafter, cell viability of HepG2 cells drastically declined when exposed to a concentration of 100 µg/ml (55.7% for ethanol and 48.9% for diethyl ether extracts). Further reduction in cellular viability is clearly concentration-dependent, however, cell viability seemed to increase again at concentrations of 500 µg/ml and higher. Bearing in mind the microscopic observations of section 4.6, both organic extracts formed a plant extract sediment at these concentrations, up to the point where no clear HepG2 cells could be observed. Attempts to remove the sediment through gently washing the wells with PBS were unsuccessful. This led to the belief that plant extract interference is a possibility, causing false results. Further investigation confirmed these suspicions when extract only wells revealed a clear concentration-dependent conversion of MTT to the purple formazan crystals (Figure 4.16), indicating non-enzymatic reduction of MTT and increased absorbance values. It was, subsequently, decided to disregard the effects of ethanol and diethyl ether extracts at concentrations of 500 µg/ml and higher on HepG2 cells during further data analysis, instead of subtracting the interference

percentages from the corresponding cell viability percentages. The reasoning for this was that the interference was pointedly high and varied noticeably (as indicated by the large error bars at these concentrations), often resulting in negative cell viabilities when included during data analysis. Graphs containing the effects of the complete concentration range (up to 1000 µg/ml) on HepG2 cells can be observed in Annexure D and will be referenced accordingly.

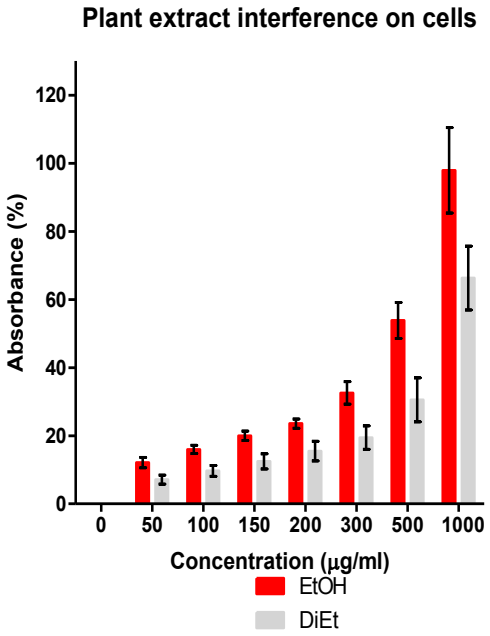


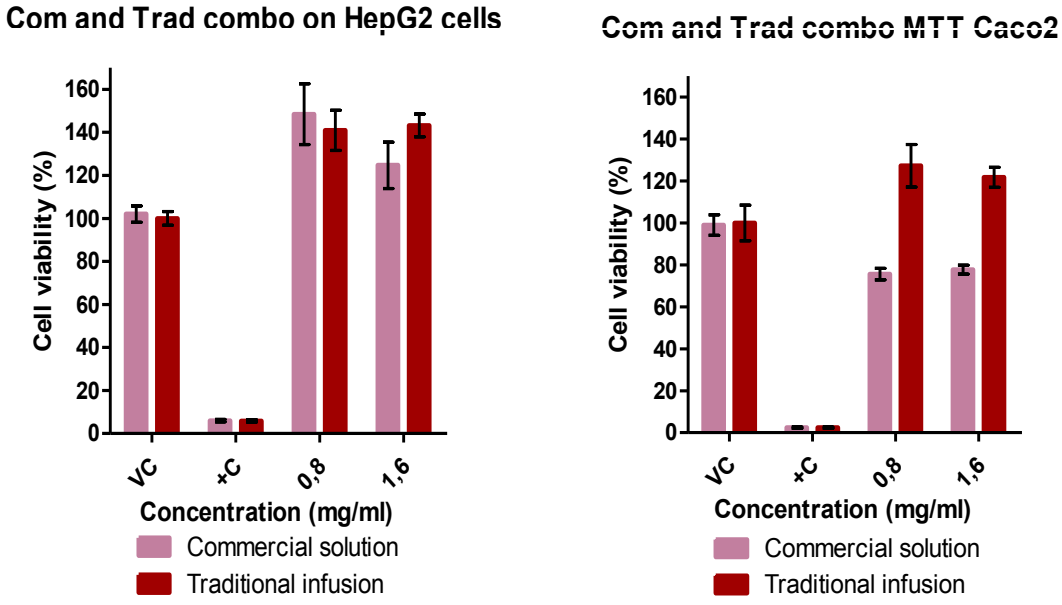
Figure 4.16: *Clear concentration-dependent plant extract interference of ethanolic and diethyl ether S. aethiopicus extracts with the MTT assay in cell-free wells; absorbance is indicated as a percentage when compared to cells exposed to vehicle controls (1% ethanol in SFM). Data is represented as mean (n=6) ± SEM*

In accordance to the findings of Wentzel *et al.* (2017) following exposure to numerous mycotoxins, Caco-2 cells proved to be more resistant to the effects of organic extracts, when compared to HepG2 cells. Cells were viable up to concentrations of 300 µg/ml, after which cell viability vividly decreased (from 134.1% to 41.9% for ethanol extracts and from 99.1% to 22.2% for diethyl ether extracts, at concentrations of 300 and 500 µg/ml, respectively). Though organic extracts again formed a clear sediment at 500 and 1000 µg/ml, microscopic observations this time revealed visible enlarged, cellular clusters at these concentrations. Likewise, one could not disregard the cell viability of Caco-2 cells at said concentrations, as was the case with HepG2 cells, since cell viability clearly only started to diminish at these high concentrations despite extract interference.

On the note of plant extract interference, several authors have more recently emphasised the possibility of chemical interference with the MTT assay. Han *et al.* (2010) found phenolic hydroxyl groups in polyphenolic compounds isolated from green tea to significantly reduce MTT in a non-enzymatic manner. Chemical compounds with reducing potential will convert MTT, non-enzymatically, to formazan, resulting in increased absorbance values and erroneous negative

results. Polyphenolic compounds and other phytochemicals found in plant extracts commonly possess an intrinsic reductive ability. It is suggested that appropriate controls, ergo MTT reagent and test compound or extract in the absence of cells, should be used to detect chemical interference. Furthermore, the MTT assay should be followed with another cell viability or cytotoxicity assay that detects a different viability marker in order to confirm results (Han *et al*., 2010; Riss, 2014; Van Tonder *et al.*, 2015; Wang *et al.*, 2015; Riss *et al.*, 2016). Both these precautionary measures were implemented during this study and so doing justifies the inclusion of the MTT results. This also serves as additional motivation to not rely on one type of viability assay alone. Other assays that utilise different chemical principles and cellular endpoints should be included to confirm results.

It is crucial to bear in mind the differences in chemical composition of *S. aethiopicus* extracts when considering the above mentioned findings. The composition of aqueous extracts differ significantly from that of ethanol and diethyl ether extracts as it does not contain AG 3 and AG 4. When screened, aqueous extracts did not cause any non-enzymatic reduction of MTT, thus suggesting that these two compounds might be somewhat responsible for the interference observed. Subsequently, as the precise functional groups or chemical structures that may result in false MTT data are still unknown (Wang *et al.*, 2015), it is suggested to isolate the major compounds present in *S. aethiopicus* extracts and test their individual ability to interfere with the assay.



(a) **(b)**

Figure 4.17 *Comparison of effects of various concentrations of a commercial solution and traditional infusion of S. aethiopicus on the cell viability of* ***(a)*** *HepG2 and* ***(b)*** *Caco-2 cells as determined with MTT assay following 4 h of exposure. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data is represented as mean (n=6) ± SEM*

As mentioned previously, examination of the effects of the commercial solution and traditional infusions on the viability of HepG2 and Caco-2 cells (Figure 4.17), revealed results similar to that of the aqueous extract. Again, cell viability of both cell lines were increased by the traditional infusion (to 143.2% for HepG2 and 121.7% for Caco-2 cells, respectively, at 1.6 mg/ml), nonetheless the commercial solution slightly decreased the viability of Caco-2 cells (77.7% at 1.6 mg/ml). According to the ISO 10993-5 (2009) guidelines, however, cell viability has to be decreased by at least 30% to be considered cytotoxic. Hence, the effects of the commercial solution can still not be considered cytotoxic in nature, even at the highest tested concentration.

Figures 4.18 and 4.19 depicts the effects of (a) ethanol, (b) diethyl ether and (c) aqueous extracts on the cell viability of HepG2 and Caco-2 cells, respectively, alone and in particular combination with either chitosan or Pharmacel® 101. Statistical analysis revealed that the MTT data is not normally distributed, nor does it comply with the assumption of homogeneity of variance. For this reason, the non-parametric Kruskal-Wallis test (Statistica software, ver.12; TIBCO Software Inc.) was used to compare extracts and their excipient combinations at a particular concentration (refer to Annexure E for full statistical particulars). Clearly, the effects of the excipients on cell viability were seldom significantly different ($p \leq 0.05$) compared to the extract itself, irrespective of the extract or cell line in mention. It should be noted that Figures 4.18 and 4.19 only illustrate pertinent concentrations for each cell line for reasons mentioned earlier. The effects of the full concentration range on the viability of HepG2 and Caco-2 cells are given in Figures D2 and D3 (Annexure D).

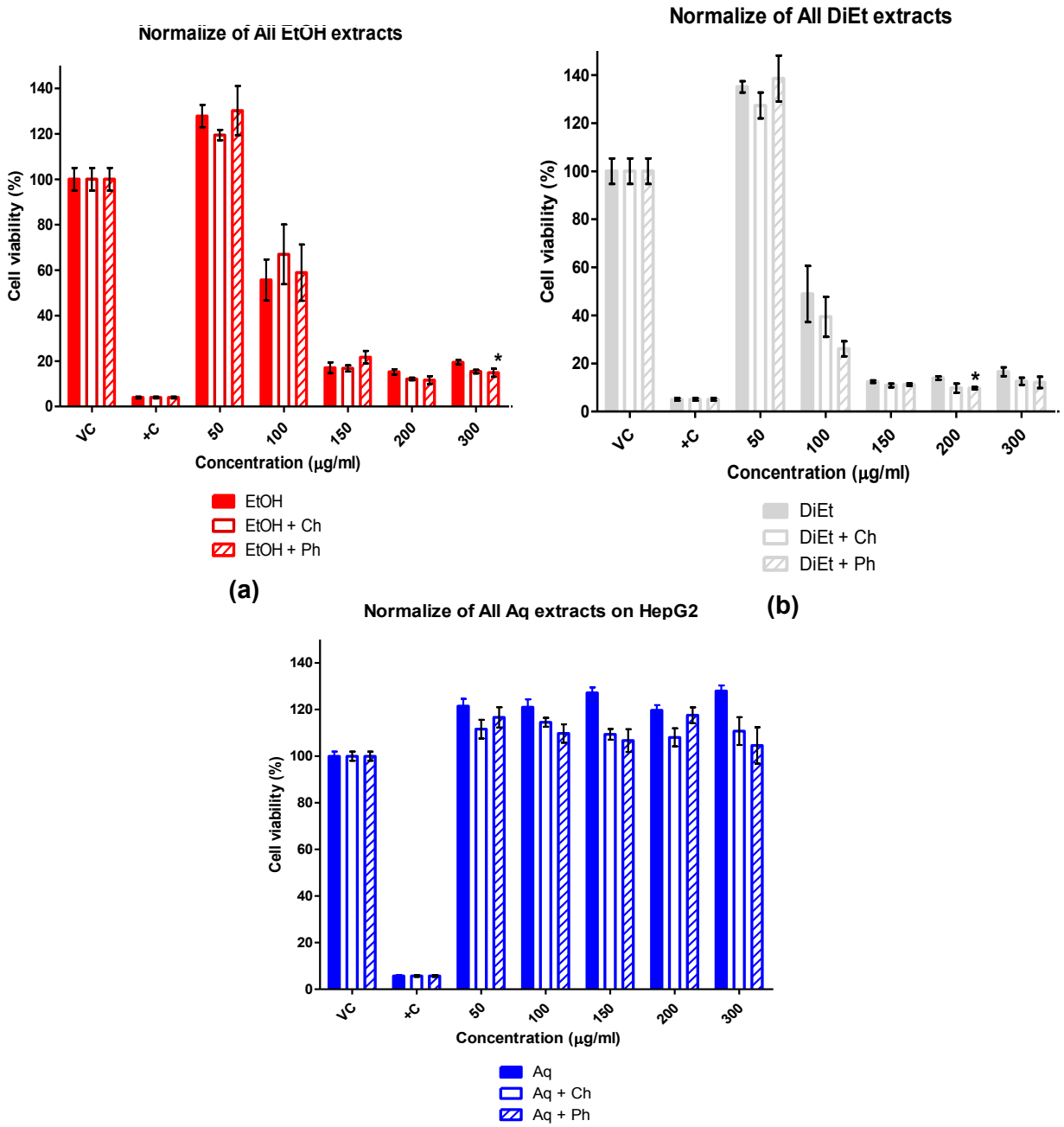


Figure 4.18: *Effects of* ***(a)*** *ethanol (EtOH),* ***(b)*** *diethyl ether (DiEt) and* ***(c)*** *aqueous (Aq)* *S. aethiopicus* *extracts and their various excipient combinations on the cell viability of HepG2 cells as determined by MTT assay. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

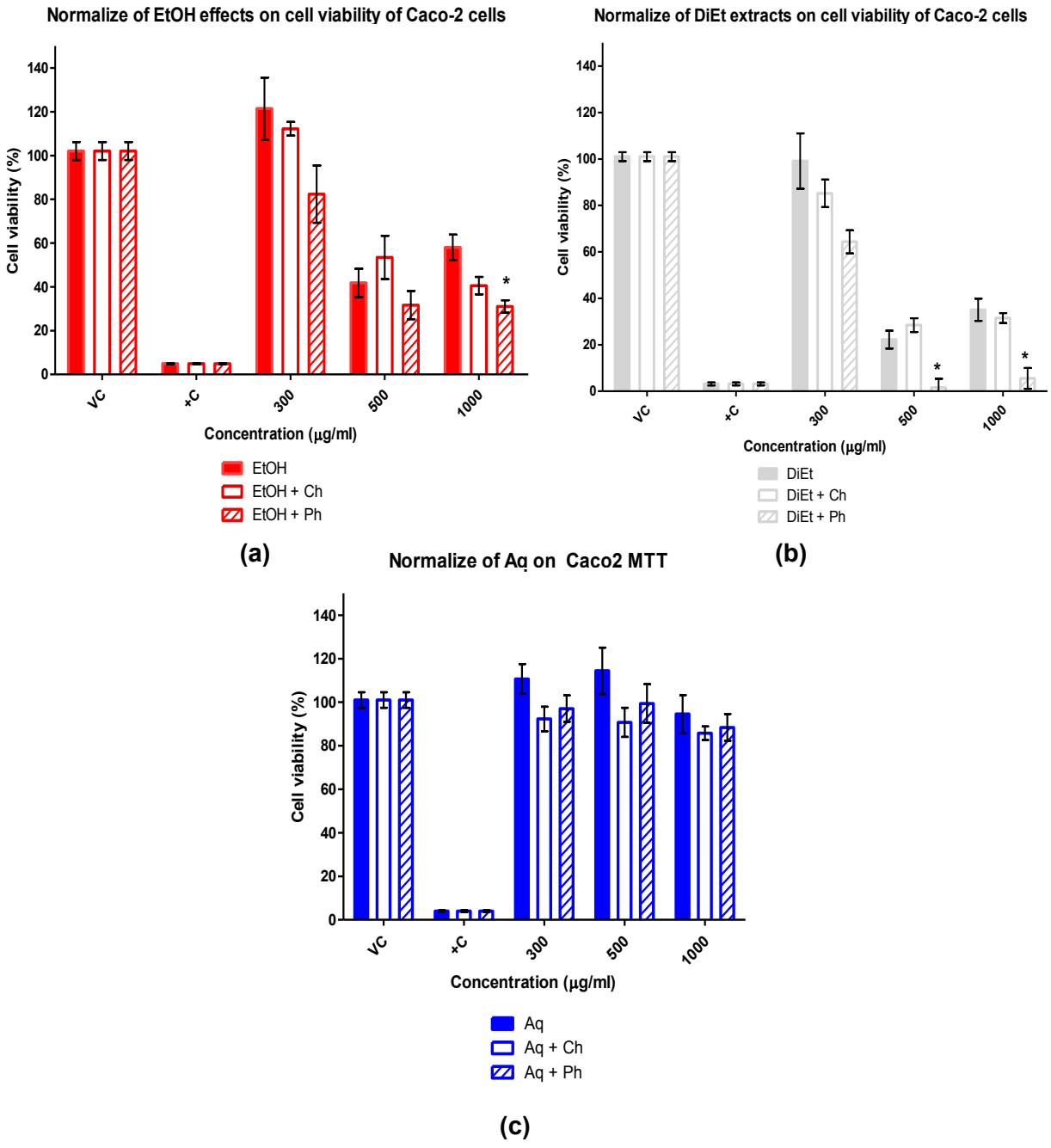


Figure 4.19: *Effects of* ***(a)*** *ethanol (EtOH),* ***(b)*** *diethyl ether (DiEt) and* ***(c)*** *aqueous (Aq)* <u>*S. aethiopicus*</u> *extracts and their various excipient combinations on the cell viability of Caco-2 cells as determined by MTT assay. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

Utilising the data collected from the MTT assays, IC_{50}-values were calculated for ethanol and diethyl ether extracts, as well as their individual combinations with each excipient (Table 4.4; see annexure E for dose-response curves). Each extract (applied to a specific cell line) and its

excipient combinations were simultaneously analysed, constraining them to share certain parameters. This approach permitted more complete information extraction from the data and provided rational estimations of parameters (De Lean *et al.*, 1978). Due to lacking cytotoxic effects, data of aqueous extracts, commercial solutions and traditional infusions were not further analysed. According to Fallahi-Sichani *et al.* (2013) IC_{50}-values are a classical measurement of potency. Congruent with earlier statements, HepG2 cells are most definitely more sensitive to the effects of both organic extracts as indicated by the significantly lower IC_{50}-values, whereas diethyl ether extracts seemed to be more potent than ethanolic extracts. These results furthermore confirmed that the selected excipients rarely altered the effects of each extract, with the exception of the diethyl ether-Pharmacel® 101 combination when applied to Caco-2 cells. However, when focussing on only potency, the biological significance and possible influence of variation on other parameters such as the hill-slope (*HS*) or steepness of the dose-response curve is ignored. Often, these parameters differ systematically from cell line to cell line. Multi-parametric analysis provides the advantage of casting light on aspects of the drug response that are often missed. According to Fallahi-Sichani *et al.* (2013) and Xia *et al.* (2014) a shallow *HS* or dose-response curve, thus a $HS \leq 1$, is associated with cell-to-cell variability. Dose-response curves will be shallower as the *HS* becomes lower and accordingly variability increases (Xia *et al.*, 2014). Through the utilisation of these guidelines, cell-to-cell heterogeneity may be identified from MTT data through accurate analysis of dose-response curves, as more often than not, the cellular response to stimuli is variable. In this investigation though, it was not the case. All *HS*s are higher than 1, indicating most cellular responses to be similar to one another with relatively little heterogeneity. *HS*s observed following ethanol extract exposure were shallower compared to *HS*s following diethyl ether exposure on both cell lines. Comparing the general *HS* trends of HepG2 cells with that of Caco-2 cells, the *HS* of Caco-2 cells (when exposed to ethanol extracts) is relatively lower, indicating less homogeneity in their response to the extract.

Table 4.4: *IC_{50}-values and HSs of the dose-response curves of ethanolic and diethyl ether S. aethiopicus extracts and their extract-excipient combinations on HepG2 and Caco-2 cell lines. IC_{50}-values are presented as means (n=6) with 95% confidence intervals in brackets, whereas HSs are reported as means (n=6) ± standard error*

	IC_{50}-values		**Hill-slope [a]**	
	HepG2	**Caco-2**	**HepG2**	**Caco-2**
EtOH	113.5 (98.82 to 130.40)	876.0 (593.20 to 1294)	-5.293 ± 1.70	-2.502 ± 1.03
EtOH + Ch	115.1 (104.6 to 126.70)	747.9 (587.40 to 952.10)	-5.367 ± 1.18	-2.788 ± 0.74
EtOH + Ph	113.1 (98.23 to 130.30)	473.8 (390.70 to 574.50)	-4.995 ± 1.58	-3.019 ± 0.78

Table 4.4: *IC_{50}-values and HSs of the dose-response curves of ethanolic and diethyl ether S. aethiopicus extracts and their extract-excipient combinations on HepG2 and Caco-2 cell lines. IC_{50}-values are presented as means (n=6) with 95% confidence intervals in brackets, whereas HSs are reported as means (n=6) ± standard error (continued)*

DiEt	**101.2 (89.59 to 114.20)**	**447.3 (310.80 to 643.50)***	**-7.130 ± 4.85**	**-11.21 ± 17.45***
DiEt + Ch	95.70 (84.92 to 107.90)	429.3 (377.40 to 488.40)*	-8.510 ± 8.38	-4.947 ± 1.32*
DiEt + Ph	92.26 (26.53 to 320.80)	~305.6 (Very wide)*	-12.77 ± 96.92	~-32.18 ± ~191296*

* Results indicated by this symbol, are ambiguous and comparison of fits not accurate.

[a] Negative *HS*s are indicative of inhibitory action

In conclusion, when considering the cytotoxicity classification of López-García *et al*. (2014), extracts can be classified as non-cytotoxic, weak, moderately or strongly cytotoxic. *S. aethiopicus* extracts are thus classified in Table 4.5 according to the effects exerted at numerous concentrations on each cell line during this study.

Table 4.5: *Classification of the various concentrations (µg/ml) of all S. aethiopicus extracts used during this study exerting non-cytotoxic, weak, moderately or strong cytotoxic effects on either HepG2 or Caco-2 cell lines. Red (♦) indicates strong cytotoxic effects, orange (♣) indicates moderate cytotoxic effects and green (☻) indicates no cytotoxic acitivity*

	Cytotoxicity category							
	Non (>80% viable)		**Weak (60-80% viable)**		**Moderate (40-60% viable)**		**Strong (<40% viable)**	
Extract	**HepG2**	**Caco-2**	**HepG2**	**Caco-2**	**HepG2**	**Caco-2**	**HepG2**	**Caco-2**
EtOH	50 ☻	50-300 ☻			100 ♣	500-1000 ♣	150-300 ♦	
DiEt	50 ☻	50-300 ☻			100 ♣		150-300 ♦	500-1000 ♦
Aq	All ☻	All ☻						
Commercial	All ☻	All ☻						
Traditional	All ☻	All ☻						

4.7.2 Lactate dehydrogenase assay

Cell viability assays, for example the MTT assay, have two main drawbacks: first of all, false positive results are a possibility (as observed during this study) and secondly, they do not allow distinction between cellular death and cell cycle inhibition (Smith *et al.*, 2011). One of the most conclusive assays for determining cell death is to quantify the effluence of intracellular components, especially cellular enzymes, from jeopardised cells into culture medium. During the LDH assay, sample wells are exposed to a combined enzymatic reagent containing NAD^+, lactate, diaphorase and a redox dye (resazurin) that produces a change in absorbance (refer to Figure 3.3; section 3.9.2), which can be quantified by spectrophotometric analysis (Niles *et al*., 2008).

The LDH assay was therefore used to not only confirm the MTT results, but also determine whether the observed loss in cell viability was caused by loss of cellular membrane integrity. Figure 4.20 illustrates the LDH release percentage of (a) HepG2 and (b) Caco-2 cells following 4 h exposure to ethanol, diethyl ether and aqueous *S. aethiopicus* extracts at several concentrations. Due to the interference of the organic extracts with the MTT reagent, it was decided to use the exact same concentration range for LDH assays. LDH Lysis solution (included in the assay kit) was used as a positive control for maximum LDH release. Aqueous extracts (Figure 4.20) as well as the commercial solution and traditional infusion (Figure 4.21) did not cause any LDH release on either HepG2, nor Caco-2 cells at the concentrations tested, indicating intact cell membranes. Considering the increased cell viability observed during the MTT assay (Figures 4.15 and 4.17), the LDH results are coherent and, consequently, confirm that these extracts do not possess cytotoxic effects.

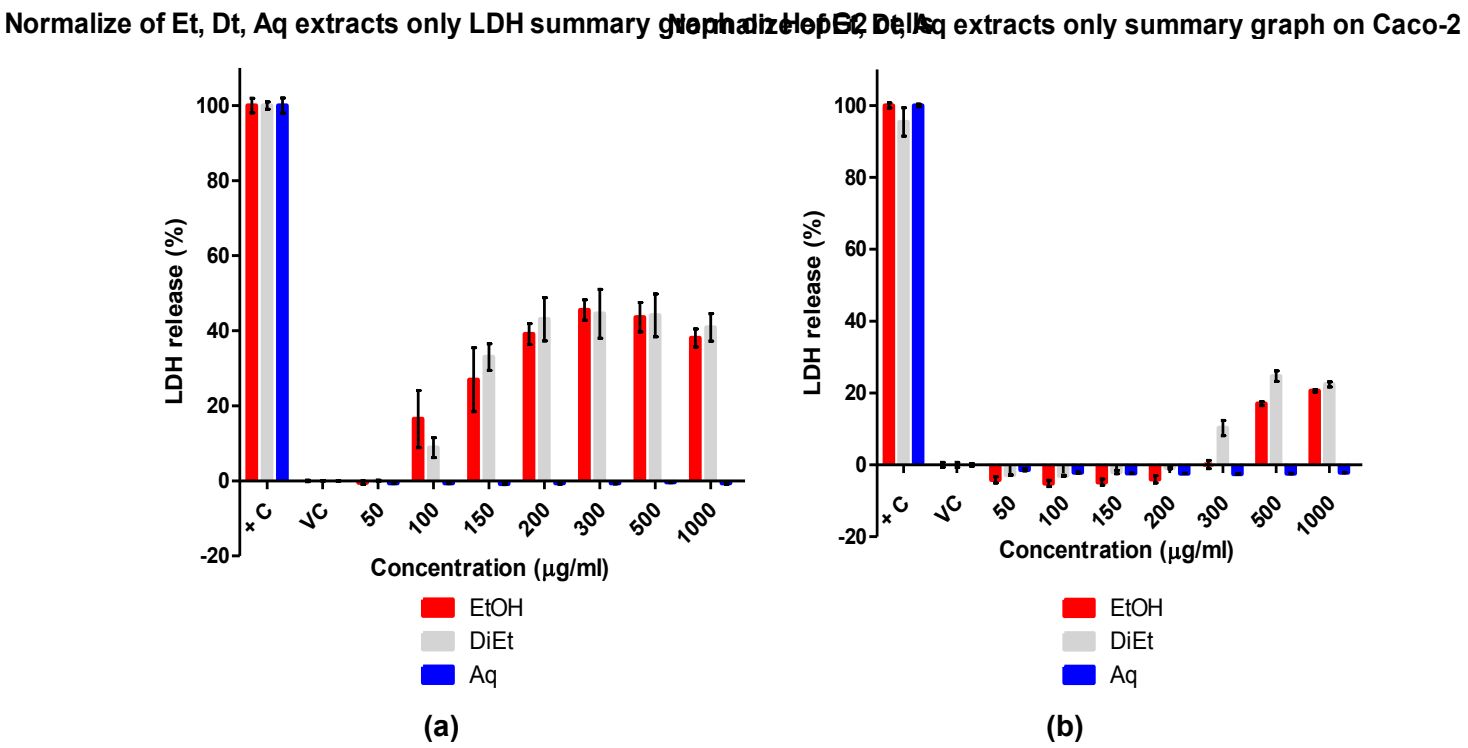


Figure 4.20: *Comparison of effects of various concentrations of ethanolic, diethyl ether and aqueous S. aethiopicus extracts on the LDH release of* ***(a)*** *HepG2 and* ***(b)*** *Caco-2 cells as determined with LDH assay following 4 h of exposure. +C indicates positive controls (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are represented as mean (n=6) ± SEM*

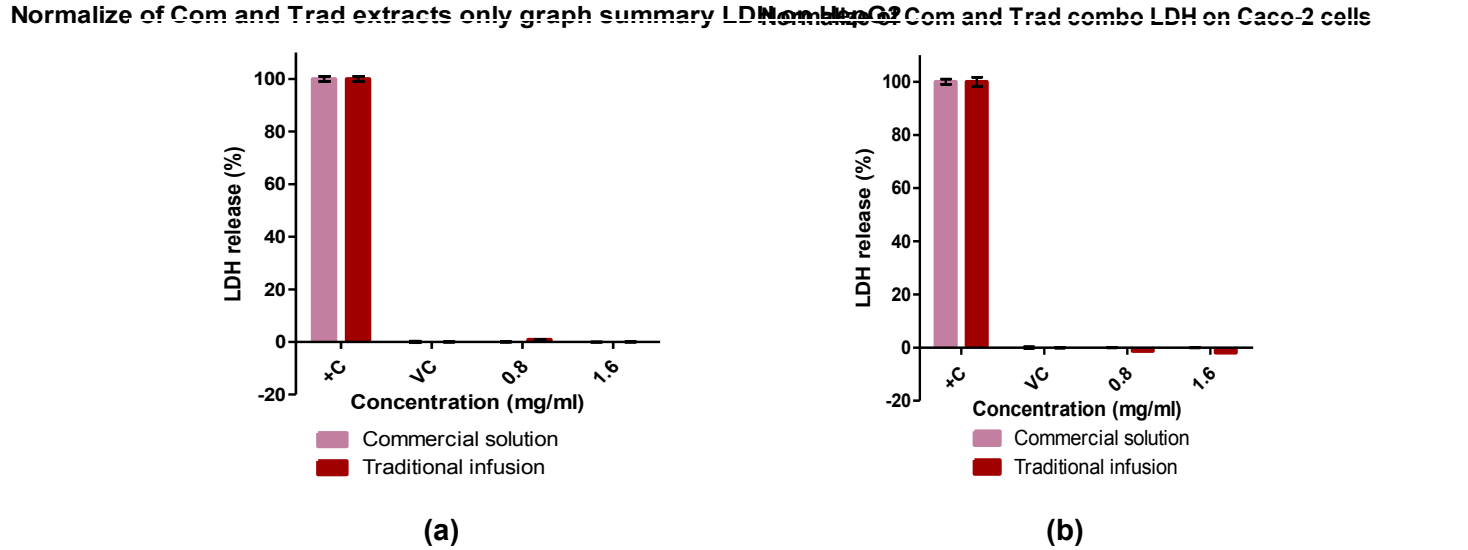


Figure 4.21: *Comparison of effects of various concentrations of a commercial solution and traditional infusion of S. aethiopicus on the LDH release of* ***(a)*** *HepG2 and* ***(b)*** *Caco-2 cells as determined with LDH assay following 4 h of exposure. +C indicates positive controls (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are represented as mean (n=6) ± SEM*

The resilience of Caco-2 cells observed with the MTT assay were again observed with the LDH assay. LDH release was only observed at a concentration of 300 µg/ml, compared to 100 µg/ml

for HepG2 cells when exposed to organic extracts. These concentrations are consistent with the concentrations at which reduced cell viability was observed during MTT assays. Therefore, the cell membranes of Caco-2 cells remained intact up to a concentration of 200 µg/ml. It seemed as though the cell lines responded similarly to ethanol and diethyl ether extracts, granting in some instances diethyl ether extracts resulted in slightly higher LDH release. On both cell lines a slight concentration-dependent increase in LDH release (Table 4.6) was observed following exposure to both ethanol and diethyl ether extracts, however, the release seizes to increase on HepG2 cells after exposure to 200 µg/ml and plateaus. On Caco-2 cells the LDH release is only observed at high concentrations and congruently the plateau effect could not be verified for these cells. For comparability purposes with HepG2 cells, higher concentrations were not investigated on Caco-2 cells during this study. In accordance with the ISO 10993-5 (2009) guidelines, the ethanol and diethyl ether extracts can be considered cytotoxic for HepG2 cells since exposure to these extracts resulted in LDH release (consequently reduced cell viability) higher than 30%; a LDH release of 39.14% was caused by ethanol extracts (200 µg/ml), whereas diethyl ether extracts (150 µg/ml) caused a release of 33%.

Table 4.6: *Mean LDH release (±SEM; n=6) of HepG2 and Caco-2 cells following exposure to ethanol and diethyl ether S. aethiopicus extracts at certain concentrations. Only values indicative of cell membrane damage are indicated*

	HepG2		Caco-2	
Concentration (µg/ml)	**Ethanol**	**Diethyl ether**	**Ethanol**	**Diethyl ether**
100	16.49 ± 7.60	8.85 ± 2.63	-	-
150	26.95 ± 8.53	33.00 ± 3.54	-	-
200	39.14 ± 2.79	43.04 ± 5.77	-	-
300	45.51 ± 2.71	44.52 ± 6.51	0.10 ± 1.15	10.25 ±2.14
500	43.58 ± 3.91	44.10 ± 5.70	17.04 ± 0.54	24.67 ±1.45
1000	38.07 ± 2.39	40.86 ± 3.66	20.58 ± 0.35	22.41 ± 0.71

Ethanol and diethyl ether extracts were furthermore individually combined with chitosan and Pharmacel® 101 in order to investigate whether these excipients altered the cytotoxic effect induced by each respective extract. As observed in Figures 4.22 and 4.23, the extract-excipient combinations did not possess cytotoxic effects that differed significantly ($p \leq 0.05$) from the effects of the extract only on either HepG2 or Caco-2 cells. For Caco-2 cells only relevant concentrations of extracts are displayed in Figure 4.23; for the particulars of the full concentration range on Caco-2 cells, refer to Figure D5 (Annexure D). It should likewise be noted that the results of the aqueous extract-excipient combinations on both cell lines are not displayed here since they did not cause any LDH release. These results are nonetheless presented in Annexure D, Figure D4.

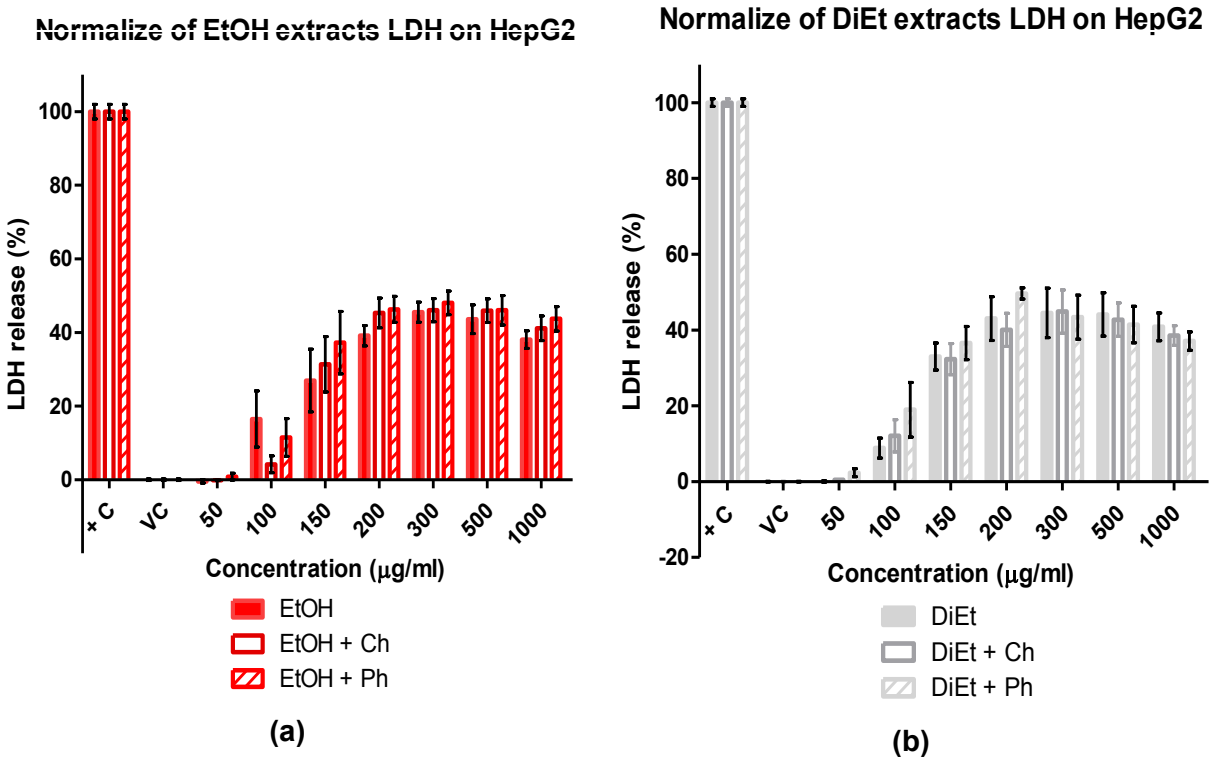


Figure 4.22: *Effects of **(a)** ethanol (EtOH) and **(b)** diethyl ether (DiEt) S. aethiopicus extracts and their various excipient combinations on the LDH release of HepG2 cells as determined by LDH assay. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. Positive controls are indicated by +C (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

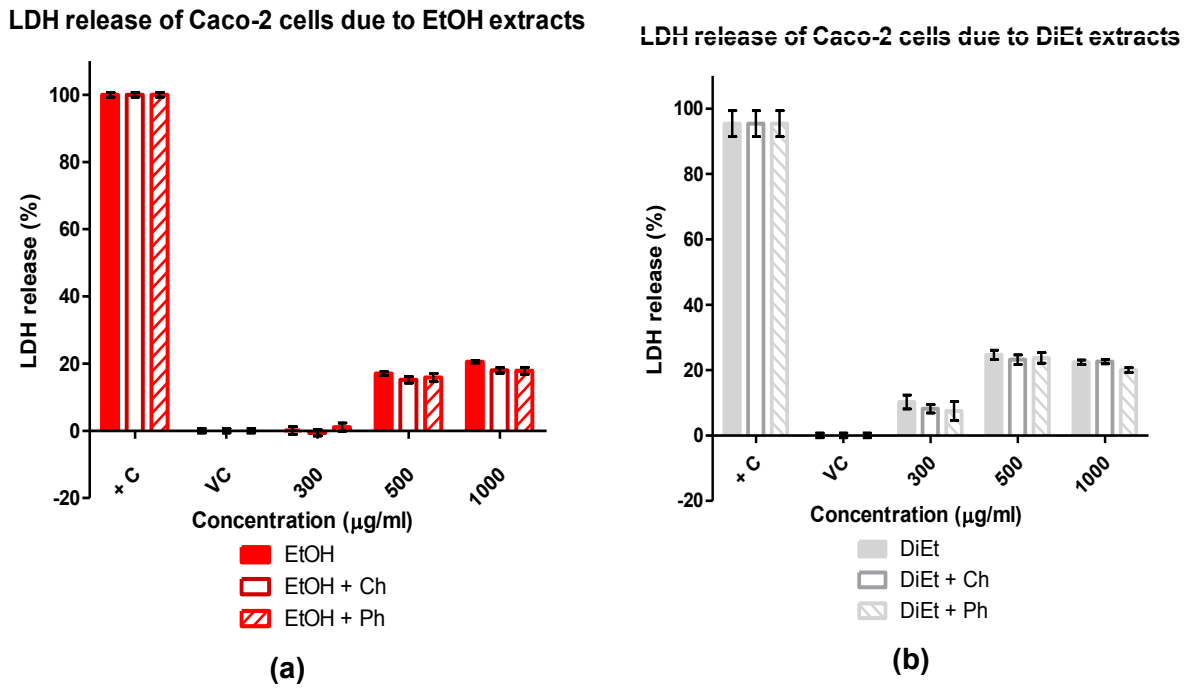


Figure 4.23: *Effects of **(a)** ethanol (EtOH) and **(b)** diethyl ether (DiEt) S. aethiopicus extracts and their various excipient combinations on the LDH release of Caco-2 cells as determined by LDH assay. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. Positive controls are indicated by +C (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

In order to determine whether the exposure of HepG2 and Caco-2 cells to ethanol and diethyl ether *S. aethiopicus* extracts resulted in cell membrane damage and, subsequently, cytotoxic effects, the LDH assay was employed. One of the most reliable markers for cell death is plasma membrane integrity or permeability, which can be evaluated with LDH release as well as propidium iodide (PI) (section 4.7.3) (Cummings *et al.*, 2004). During necrosis, cells undergo oncosis, intracellular organelles start to swell, the cellular membrane ruptures early on (as indicated visually in Figure 2.11, section 2.5.4.5) and intracellular contents are spilled into the extracellular media. However, during apoptosis the plasma membrane remains intact until the final stages of cell death (Kroemer *et al.*, 2009). Correspondingly, when LDH is detected in the extracellular fluid or media, it is indicative of a loss of cell viability, diminished cell membrane integrity and necrosis (Cummings *et al.*, 2004; Chan *et al.*, 2013). Exposure to ethanol and diethyl ether *S. aethiopicus* extracts resulted in LDH release from HepG2 and Caco-2 cells, subsequently causing membrane damage and necrotic cell death. For HepG2 cells, LDH release (16.48% and 8.85% for ethanol and diethyl ether extracts, respectively) occurs at an initial concentration of 100 µg/ml; the results are consistent with MTT data which indicated a radical reduction in HepG2 cell viability at this concentration. For Caco-2 cells, again, the concentration at which LDH release was observed, was similar to that which caused a reduction in cell viability during the MTT assay.

Ethanol extract exposure at a concentration of 500 µg/ml resulted in definite LDH release (17.04%), whereas diethyl ether extract exposure resulted in 10.25% LDH release at a 300 µg/ml concentration. Close examination of MTT results on Caco-2 cells following diethyl ether extract exposure (Figures 4.15 and 4.19), revealed that this extract indeed caused slightly reduced cell viability at this concentration when compared to lower concentrations, though still not below 90%. Subsequently, the organic extracts of *S. aethiopicus* can be classified as cytotoxic according to the ISO 10993-5 (2009) guidelines and can be further categorised as weak cytotoxic according to López-García *et al*. (2014). Thus, the reduction in cell viability caused by organic extracts was partly due to cell membrane damage, resulting in necrotic cell death to some degree, but since LDH release was not nearly as high as the reduction in cell viability at lower concentrations of both extracts, potential cell cycle arrest and/or apoptotic cell death may also be involved. In agreement with the MTT assay, aqueous extracts, commercial solutions and traditional infusions did not result in any cell membrane damage and consequently do not possess cytotoxic effects.

These results again stress the possibility that compounds AG 3 and AG 4 may be responsible for the cytotoxic effects, although absolute estimations and conclusions cannot be drawn. It is therefore again suggested that all biomarker compounds be isolated and screened individually in order to determine their individual role in the cytotoxic effects observed during the LDH assay. It would also be wise to investigate specific compounded fractions of the extracts (in other words specific compound combinations). The reason for this suggestion is that often times interacting substances contained within the extract may influence the pharmacodynamic and pharmacokinetic profiles of the compounds of interest (Dash & Nivsarkar, 2016).

4.7.3 Detection of apoptosis and/or necrosis with flow cytometry using FITC conjugated annexin V and propidium iodide double staining

Procedures designed to differentiate between necrosis and apoptosis should assess at least two selective indicators for each of these different modes of cell death (Cummings *et al.*, 2004). The first marker used in this study was the release of intracellular contents (LDH) into the extracellular fluid, indicative of necrotic cell death. Simultaneous testing for PS externalisation and PI staining, subsequently distinguishing apoptotic cell death form necrotic cell death, was the second marker(s) employed to investigate the cytotoxicity of *S. aethiopicus* extracts (Cummings *et al.*, 2004). When PI is applied alone, it is only indicative of necrotic cell death since it is a membrane impermeable dye, however when combined with annexin V it can be used to distinguish apoptosis from necrosis as PS exposure is an early occurrence during apoptosis (Cummings *et al.*, 2004; Chan *et al*., 2013; Demchenko, 2013). Only extracts that exhibited some cytotoxicity during MTT and LDH assays, i.e. ethanol and diethyl ether extracts, were selected for flow cytometric analysis. Since the selected excipients did not seem to affect the baseline cytotoxicity of extracts, extract-excipient combinations were not considered. Based on microscopic observations which indicated

organic extracts to induce morphological changes at 50 µg/ml as well as MTT and LDH results that showed reduced cell viability and LDH release at 100 µg/ml (for HepG2 cells), it was decided to use 50, 100 and 150 µg/ml as exposure concentrations for both HepG2 and Caco-2 cells during flow cytometric analysis. The same concentration range was utilised for Caco-2 cells for comparative purposes, though they were not as susceptible as HepG2 cells during MTT and LDH assays at these concentrations.

Figure 4.24 illustrates the figure key that is applicable to the interpretation of results illustrated in the dot-plots of (a) and (b) in Figures 4.25 and 4.26. It indicates in what quadrant of the dot-plots viable, apoptotic and late apoptotic and/or necrotic cells can be found. Typically, viable cells can be found in quadrant 4 (Q4) with little FITC annexin V (x-axis) or PI (y-axis) fluorescence; apoptotic cells in quadrant 3 (Q3) with high FITC annexin V but little PI fluorescence; and necrotic and/or late apoptotic cells in quadrant 2 (Q2) with high FITC annexin V and PI fluorescence (Van Engeland *et al*., 1996; Hansakul *et al.,* 2014; Behzad *et al.*, 2016; Wentzel *et al.*, 2017).

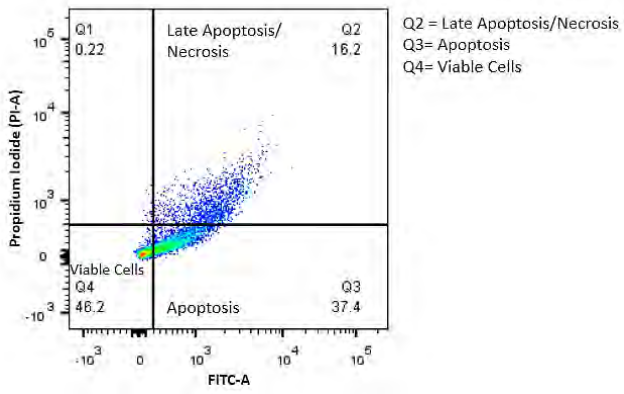


Figure 4.24: *Figure key for the interpretation of FITC annexin V and PI flow cytometry dot-plots. Quadrant 2 (Q2) represents cells in late-stage apoptosis and/or necrosis, quadrant 3 (Q3) represents cells undergoing apoptosis and quadrant 4 (Q4) represents viable cells*

Flow cytometric analysis of HepG2 cells revealed the following results (Figure 4.25) after incubation with ethanol and diethyl ether *S. aethiopicus* extracts, separately, for 4 h. HepG2 cells exposed to 50 µg/ml ethanol *S. aethiopicus* extract for 4 h were 71.100% (±1.7%) viable, 15.467% (±0.9%) apoptotic and 11.333% (±2.1%) necrotic or late apoptotic. Following exposure to 100 µg/ml ethanol extract, cells were 60.867% (±1.5%) viable, 12.533% (±0.7%) apoptotic and 24.233% (±1%) necrotic or late apoptotic. Exposure to 150 µg/ml ethanol extract, however, revealed cells to be only 30.867% (±1.9%) viable, whereas 32.800% (1.3%) were undergoing apoptosis and a surprising 35.200% (±1.4%) were necrotic. HepG2 cell exposure to the diethyl ether extract revealed similar results. Succeeding incubation with 50 µg/ml, 87.767% (±1.8%) of cells were viable, slightly higher compared to the observed results of the ethanolic extracts, where

4.467% (±0.6%) were apoptotic and 4.067% (±0.4%) were necrotic or late apoptotic; both somewhat lower than detected for ethanol extracts. Approximately 58.600% (±1.5%) of HepG2 cells were viable, 13.467% (±1.2) were in early apoptosis and 25.4675 (±1.0%) were in necrosis or late apoptosis after exposure to 100 µg/ml diethyl ether extract. Approximately 30.167% (±2.6%) of HepG2 cells were viable subsequent to exposure to 150 µg/ml diethyl ether *S. aethiopicus* extract, 33.967% (±1.1%) were apoptotic and 33.667% (±2.1%) were necrotic or late apoptotic.

As illustrated in Figure 4.26, FITC annexin V/PI double labelling revealed surprising flow cytometric results for Caco-2 cells following 4 h exposure to ethanol and diethyl ether extracts of *S. aethiopicus*, respectively. Approximately 60.067% (±1.2%) and 51.767% (±2.1%) of Caco-2 cells were viable; 20.167% (±1.4%) and 21.300% (±1.8%) were apoptotic; whereas only 16.033% (±1.5%) and 25.900% (±3.0%) of Caco-2 cells were necrotic or late stage apoptotic following respective incubation with ethanol and diethyl ether *S. aethiopicus* extracts. Succeeding exposure of Caco-2 cells to 100 µg/ml ethanol and diethyl ether extracts, individually, 54.233% (±0.1%) and 46.500% (±1.5%) of cells were viable with 19.867% (±1.7%) and 10.333% (±0.9) being in early apoptosis as well as 20.073% (±1.2) and 42.6% (±1.3%) in late apoptosis or necrosis, correspondingly. An estimated 51.967% (±2.0%) and 49.200% (±1.4) of Caco-2 cells were viable; 12.567% (±1.3%) and 11.700%) (±1.4%) were undergoing apoptosis; and 27.967% (±1.7%) and 35.367% (±3.0%) were experiencing necrosis or late apoptosis after exposure to 150 µg/ml ethanol and diethyl ether extracts, respectively.

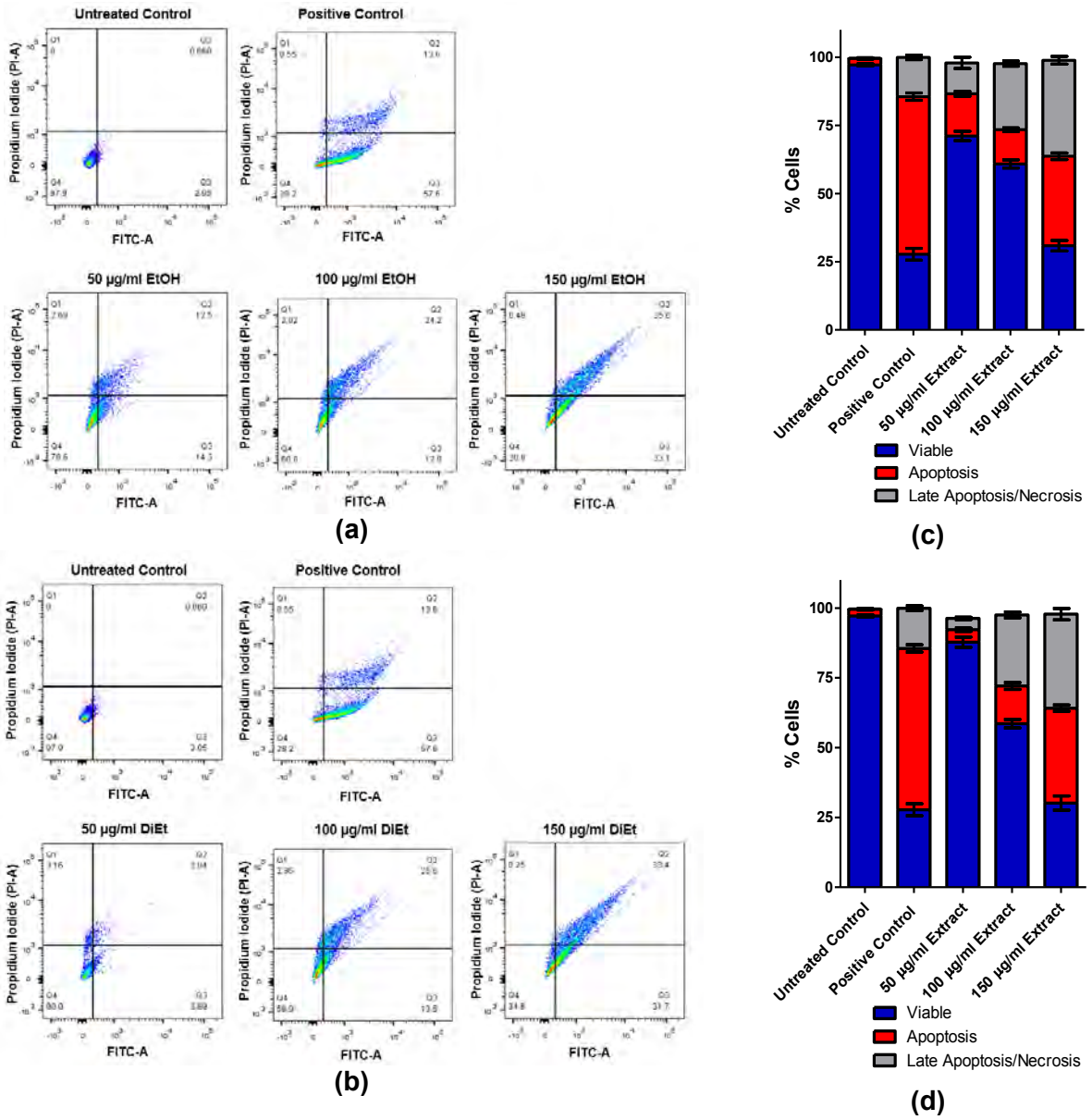


Figure 4.25: *Dot-plots representing the amount of apoptotic and necrotic HepG2 cells following exposure to* ***(a)*** *ethanol and* ***(b)*** *diethyl ether extracts of S. aethiopicus at 50, 100 as well as 150 µg/ml for 4 h. FITC conjugated annexin V and PI double staining was used for the flow cytometric analysis. Every dot represents a single cell or event, 10 000 events were counted per sample. Untreated controls were not treated with extracts, whereas positive controls were exposed to 1 mM staurosporine for 4 h. All experiments were performed in triplicate and independently repeated. See Figure 4.24 for the figure key applicable in dot-plots. Bar graphs demonstrate the percentage viable, apoptotic, as well as late-stage apoptotic and/or necrotic HepG2 cells following 4 h exposure to (c) ethanol and (d) diethyl ether S. aethiopicus extracts. Data are represented as the mean (n=6) ±SEM*

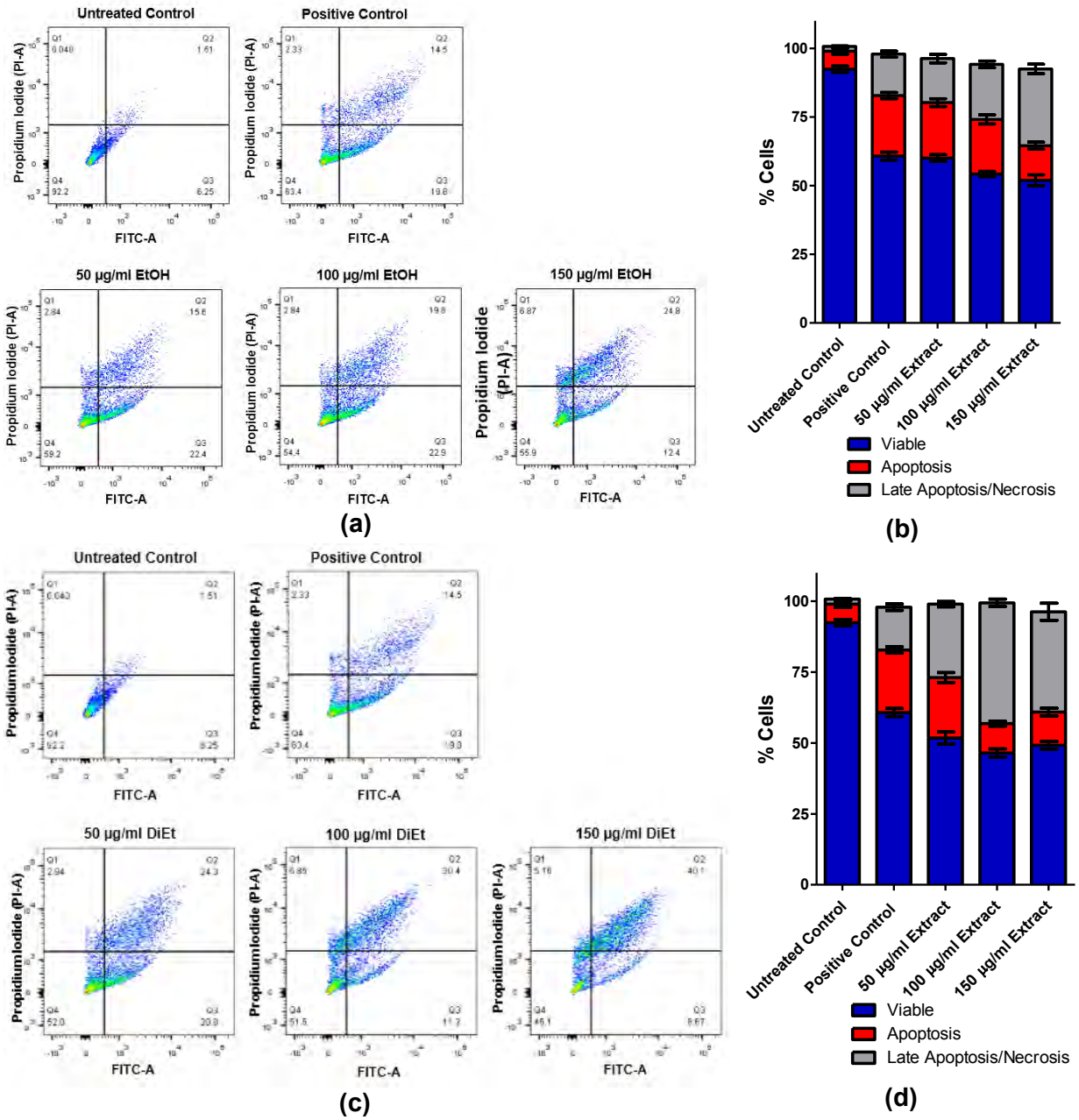


Figure 4.26: *Dot-plots representing the amount of apoptotic and necrotic Caco-2 cells following exposure to* ***(a)*** *ethanol and* ***(b)*** *diethyl ether extracts of S. aethiopicus at 50, 100 as well as 150 µg/ml for 4 h. FITC conjugated annexin V and PI double staining was used for the flow cytometric analysis. Every dot represents a single cell or event, 10 000 events were counted per sample. Untreated controls were not treated with extracts, whereas positive controls were exposed to 1 mM staurosporine for 4 h. All experiments were performed in triplicate and independently repeated. See Figure 4.24 for the figure key applicable in dot-plots. Bar graphs demonstrate the percentage viable, apoptotic, as well as late-stage apoptotic and/or necrotic Caco-2 cells following 4 h exposure to (c) ethanol and (d) diethyl ether S. aethiopicus extracts. Data are represented as the mean (n=6) ±SEM (continued)*

Membrane asymmetry is lost during early apoptosis and PS residues translocated to the extracellular membrane surface, without disrupting the barrier function of the plasma membrane. Consequently, positively charged annexin V will bind to negatively charged PS residues with high selectivity and affinity. However, necrotic or dead cells lose their cell membrane integrity early on, resulting in inner leaflet PS residues being available for annexin V binding and hence, cannot alone, distinguish apoptotic from necrotic cells. The addition of PI, a membrane impermeable dye, enables researchers to discriminate between apoptotic and necrotic cells through flow cytometric analysis (Demchenko, 2013; Badmus *et al.*, 2015; Behzad *et al.*, 2016).

Results indicated the activation of both apoptotic and necrotic cell death pathways in both cell lines following exposure to ethanol and diethyl ether *S. aethiopicus* extracts at all concentrations used. Due to the short incubation period (4 h) it can be concluded that the cells in Q2 are necrotic rather than late apoptotic (or post-apoptotic necrosis), since this phenomena only occurs *in vitro* much later; see Figure 2.11 (Niles *et al.*, 2008). Overall, the induction of apoptosis and/or necrosis were concentration-dependent for HepG2 cells; cell viability decreased with a concomitant proportional increase in apoptotic and necrotic cells as extract concentrations increased when compared to untreated control cells. The proportion of apoptotic to necrotic cells definitely increased as concentration increased, resulting in almost a 1:1:1 (viable: apoptotic: necrotic cells) ratio at 150 µg/ml for both extracts. Only a concentration of 50 µg/ml for ethanol extracts depicted a slightly higher incidence of apoptosis compared to necrosis for HepG2 cells. All other concentrations, irrespective of the extract, concentration, or cell line showed a higher or relatively comparable occurrence of necrosis compared to apoptosis. On HepG2 cells, ethanol and diethyl ether extracts induced apoptotic as well as necrotic effects that are fairly similar at concentrations of 100 and 150 µg/ml, however ethanolic extracts seem to be more pro-apoptotic and -necrotic (15.467 ± 0.9% apoptotic; 11.333 ±2.1% necrotic) than diethyl ether extracts (4.467 ± 0.6% apoptotic; 4.067 ± 0.4% necrotic) at 50 µg/ml, respectively. Subsequently, HepG2 cells seem to be more sensitive to the apoptotic effects of ethanol extracts at lower concentrations, however, this effect dissipates at higher concentrations. Both extracts revealed similar results at higher concentrations (100 and 150 µg/ml) irrespective of the cellular condition (viable, necrotic or apoptotic).

The reduction in cell viability of Caco-2 cells was marginally concentration-dependent. Interestingly, exposure to 50 µg/ml ethanol extracts, revealed results very analogous to that of the positive apoptotic control. Diethyl ether extract exposure at this concentration even resulted in cell viability lower than that caused by the positive control. Furthermore, an increase in diethyl ether extract concentration ensued in only a slightly reduced cell viability, but the percentage necrotic cells increased in a definite concentration-dependent manner, resulting in a smaller percentage apoptotic cells. However, for the ethanol extract a concomitant decrease in cell viability, caused a proportional increase in apoptotic and necrotic cells. Similarly, Caco-2 cells

were more susceptible to the necrotic effects induced by the diethyl ether extract compared to the ethanol extract at especially 50 µg/ml (16.033 ± 1.5 for ethanol extract; 25.900 ± 3.0% for diethyl ether extract) and 100 µg/ml (20.073 ± 1.2% for ethanol extract; 42.600 ± 1.3% for diethyl ether extract). The high incidence of apoptosis and necrosis observed with Caco-2 cells were surprising, as cell viability only started to diminish at 500 µg/ml during the MTT assay; and LDH release only occurred at 300 µg/ml for both extracts.

Recognising the effects of ethanol and diethyl ether extracts on the viability of HepG2 cells (Figure 4.27), a concentration of 150 µg/ml caused an obvious statistically significant decrease in the cell viability of these cells. The effects are, as previously stated, clearly concentration-dependent. With Caco-2 cells (Figure 4.28), the ethanol extract (150 µg/ml) again caused a substantial decrease in cell viability, however not as apparent as with HepG2 cells. Interestingly, cell viability of Caco-2 cells was meaningfully decreased by a 100 µg/ml diethyl ether extract but not by the 150 µg/ml concentration, as observed on HepG2 cells. As specified previously, the Caco-2 cells are not as susceptible to the effects of organic extracts compared to the HepG2 cells, considering their cell viability is not decreased in such a concentration dependent manner.

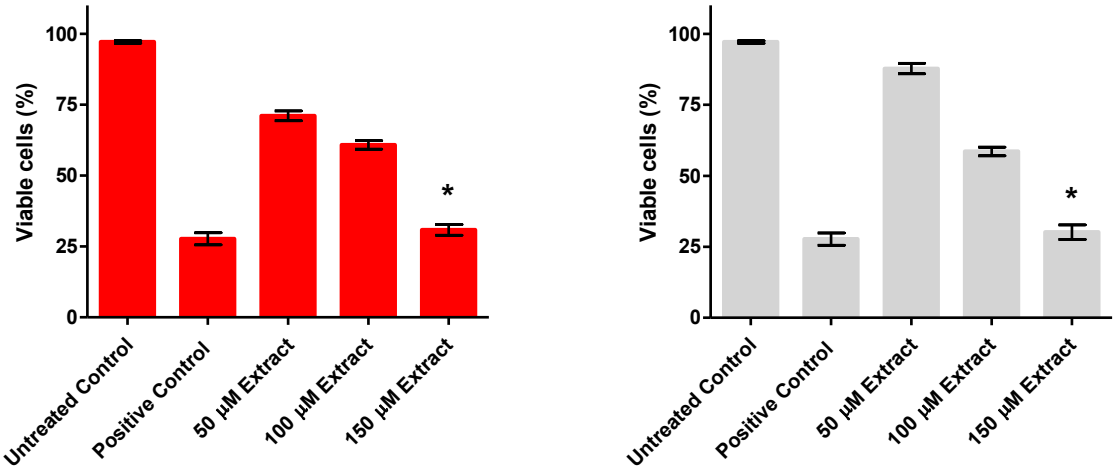


Figure 4.27: *Bar graphs illustrating the decrease in cell viability of HepG2 cells as caused by **(a)** ethanol and **(b)** diethyl ether S. aethiopicus extracts. Data are presented as mean (n=6) ±SEM. * represents a statistically meaningful decrease in cell viability with p ≤ 0.05*

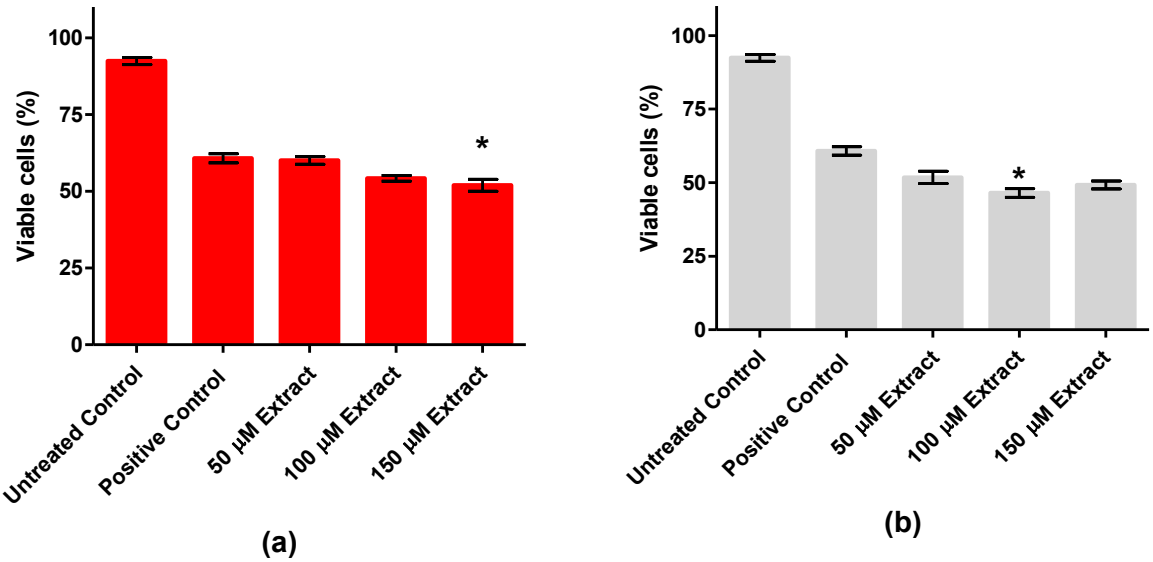


Figure 4.28: *Bar graphs illustrating the decrease in cell viability of Caco-2 cells as caused by* ***(a)*** *ethanol and* ***(b)*** *diethyl ether S. aethiopicus extracts. Data are presented as mean (n=6) ±SEM. * represents a statistically meaningful decrease in cell viability with p ≤ 0.05*

Data from FITC conjugated annexin V/PI double labelling consequently confirm earlier suspicions that necrotic cell death (as indicated in LDH release) was accompanied by apoptotic cell death, which cannot be detected by the LDH release assays due to the intactness of the plasma membranes of cells, but may result in reduced cell viability as detected by the MTT assay. On HepG2 cells, all extracts revealed at least 30% apoptotic and necrotic cells, respectively, at 150 µg/ml, whereas Caco-2 cells were at least 10% apoptotic, however more than 27% necrotic at the same concentration. Once again, a relatively higher resilience of Caco-2 cells was revealed, coinciding with LDH and MTT results.

4.8 CONCLUSIONS

In this study several extracts of *S. aethiopicus* were prepared, namely: ethanol, diethyl ether and aqueous extracts; as well as a commercial solution and traditional infusion. Solvents were chosen due to their differences in polarity, consequently resulting in the extraction of varying amounts and types of compounds in each extract. These extracts were characterised by means of UPLC-Q-TOF/MS based on methods previously described by Bergh (2016). Four biomarker molecules (AG 1–4), previously identified by Holzapfel *et al.* (2002), Viljoen *et al.* (2002), Lategan *et al.* (2009), Fouche *et al.* (2011) and Bergh (2016), were used as reference markers to recognise possible differences in the chemical composition of the various extracts. Ethanol and diethyl ether extracts revealed relatively similar chemical profiles containing all four compounds; whereas aqueous extracts and commercial solutions did not contain AG 3 or AG 4; and AG 1 and AG 2 were only present in comparatively small amounts. The ethanol, diethyl ether and aqueous

extracts were furthermore combined with certain excipients, i.e. chitosan and Pharmacel® 101, in order to determine whether these selected excipients altered the baseline effect of extracts during cytotoxicity assays, indicating possible physical or chemical interactions.

MTT and LDH assays were performed to investigate whether the extracts caused cytotoxic effects; and FITC conjugated annexin V/PI was used to determine whether these effects were caused by apoptotic or necrotic cell death pathways. The difference in chemical composition proved to be noteworthy, as aqueous extracts, commercial solutions and traditional infusions did not cause any cytotoxic effects on either HepG2 or Caco-2 cells during MTT or LDH assays, but rather resulted in a slight increase in cell viability. Practically the exact opposite effects were evident for organic extracts. Ethanol and diethyl ether extracts caused a notable reduction in HepG2 and Caco-2 cell viability during MTT assays; increased enzyme release during the LDH assay; and caused not only apoptotic, but also necrotic cell death during flow cytometric analysis using FITC conjugated annexin V/PI double labelling. Therefore, the possibility exists that AG 3 and AG 4 might be partially responsible for these effects. During MTT and LDH assays, the excipients did not cause altered cytotoxicity compared to baseline effects of extracts only, possibly indicating no significant interactions between extracts and these selected excipients. It should be noted, at this point, that these assays were performed without further mechanistic elucidation. Our investigation should thus only be considered preliminary and more in depth analyses, eventually involving mammalian *in vivo* studies are needed before final conclusions can be reached (Taylor *et al.*, 2003).

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CHAPTER 5: SUMMARY AND FUTURE PROSPECTS

5.1 SUMMARY

The initial aim of this study was to investigate the cytotoxicity of numerous *S. aethiopicus* extracts with the purpose of establishing a baseline cytotoxic profile for each extract. These extracts were furthermore compounded with two popular excipients for tableting, namely chitosan and Pharmacel® 101, in order to determine whether they would influence the observed cytotoxic effects of the respective extracts, and hence to establish the occurrence of any possible physical-, or chemical extract-excipient interactions. The chemical compositions of the extracts were also determined with the purpose of possibly linking the presence, or absence of certain compounds to the observed cytotoxic effects. To achieve this aim, several objectives were identified.

First, crude extracts were prepared from the rhizomes of *S. aethiopicus*. These rhizomes were grated, dried and powdered. The dry plant powder was used to prepare crude ethanol, diethyl ether and aqueous extracts. A commercial solution was prepared using Phyto nova African Ginger® tablets, a commercially available product, along with a traditional infusion. Next, these extracts were chemically profiled by means of UPLC-Q-TOF/MS, based upon methods previously described by Bergh (2016). The four marker compounds, AG 1–4, were used to qualitatively compare the chemical compositions of the prepared extracts and identify possible anomalies. Interestingly, neither the aqueous extracts, nor commercial solutions contained compounds AG 3 and AG 4; whereas only small amounts of AG 1 and AG 2 were detected. Organic extracts, i.e. ethanol and diethyl ether, contained all four compounds, though quantitative differences among the extracts were evident. It appeared as though diethyl ether, the least polar solvent used, had been more efficient in extracting the four compounds, since their peak intensities were noticeably higher. Interestingly, in previous studies (Holzapfel *et al.*, 2002; Viljoen *et al.*, 2002; Fouche *et al.*, 2011; Katele Zongwe, 2015; Bergh, 2016), AG 4 had been identified as the main compound present in *S. aethiopicus*, whereas, this study revealed the peak intensity of AG 3 to be dominant, instead. The abundance of AG 3 could not be attributed to the autoxidation of AG 4, as it had not been identified as an oxidation product in a previous study (Katele Zongwe, 2015). This observation confirmed the variability in composition that may exist among different plant extracts, due to several factors, such as geographical location, season, harvest time, and extraction solvents and methods used. Variability among herbal preparations is the one major concern with regards to herbal products, as it often limits standardisation of such products, results in poor reproducibility, and consequently impedes herbal product acceptance (Kunle *et al.*, 2012; Dash & Nivsarkar, 2016).

Several routine cytotoxicity assays, including the MTT and LDH assays, as well as FITC conjugated annexin V/PI using flow cytometry, were employed to determine the *in vitro* cytotoxic effects of the crude *S. aethiopicus* extracts and to establish a baseline cytotoxic profile (this was the third objective). Traditionally, the rhizomes are administered orally, either through chewing, or drinking as an infusion/tea (Hartzell, 2011; Fouche *et al.,* 2013). HepG2 and Caco-2 cell lines were subsequently utilised as representations of liver hepatocytes and small intestine cells, respectively (Wentzel *et al.*, 2017). During these assays, the differences in chemical compositions of the aqueous and organic extracts indeed proved noteworthy. Aqueous extracts, commercial solutions and traditional infusions did not cause any reduction in cell viability during MTT assays, but rather increased cell proliferation. LDH assays also revealed that exposure to these extracts had not resulted in any cell membrane damage. Therefore, these extracts were not considered for flow cytometric analysis.

Organic extracts displayed a completely opposite profile. Ethanol and diethyl ether extracts resulted in a clear reduction in cell viability of both the HepG2 and Caco-2 cells during the MTT assays, despite observed plant extract interference. LDH assays confirmed that these extracts had caused damage to the cellular plasma membranes, resulting in necrotic cell death. The fourth and fifth objectives of this study were to combine the individual crude extracts (ethanol, diethyl ether and aqueous extracts) with the two selected excipients, chitosan and Pharmacel® 101, in order to determine whether these excipients would affect the cytotoxicity of each extract during MTT and LDH assays. Not only was this area investigated statistically, but IC_{50}-values (objective six) were also calculated for extracts only, as well as for extract-excipient combinations. Congruently, these excipients rarely significantly affected the baseline cytotoxic properties of the selected extracts, whereas extract-excipient combinations resulted in relatively similar IC_{50}-values, indicative of a reasonably low probability of the occurrence of physical or chemical extract-excipient interactions.

Moreover, two distinct observations were perceived following MTT and LDH assays. Firstly, Caco-2 cells were not as susceptible to the deleterious effects of the extracts when compared to HepG2 cells, with reduced cell viability and increased LDH release only observed at significantly higher concentrations. Secondly, LDH release did not correspond with the degree of reduced cell viability as detected with the MTT assays, leading us to the belief that necrotic cell death had probably not been the sole cell death pathway activated. This suspicion was confirmed by flow cytometric analysis, using FITC conjugated annexin V/PI double labelling. Flow cytometry results indicated the presence of apoptotic and late apoptotic, or necrotic HepG2 and Caco-2 cells, following exposure to ethanolic and diethyl ether extracts (study objective seven). Due to the short incubation period (4 h), it was concluded that annexin V and PI positive cells most likely underwent necrotic cell death, since late apoptosis, or post-apoptotic necrosis, are only evident following longer incubation periods (> 24 h). The effects of the extracts were clearly

concentration-dependent with HepG2 cells, resulting in a decrease in cell viability as the concentration increased, with a concomitant increase in apoptosis and necrosis. Exposure to both extracts resulted in a decrease in Caco-2 cell viability, which resembled staurosporine exposure, however, this was not concentration-dependent. In some instances, the percentage of necrotic cells increased, without decreasing the viable cell population, subsequently resulting in a reduced apoptotic cell population. The presence of apoptotic and necrotic cells at the concentrations used for flow cytometry was surprising, since MTT and LDH assays only indicated cellular damage at significantly higher concentrations.

When considering the chemical compositions of the extracts, it was possible that compounds AG 3 and AG 4, which were completely absent in aqueous extracts, but rather abundantly present in organic extracts, might have been largely responsible for the observed cytotoxic effects. These findings supported the fact that contrasting effects may have been found for differing extracts of the same plant, due to the differences in individual compounds and their quantities present therein (Stafford *et al.,* 2005; Fouche *et al.*, 2011; Złotek *et al.*, 2015).

5.2 FUTURE PROSPECTS AND CONSTRAINTS

Prior to this study, only superficial information was available regarding the possible cytotoxic potential of *S. aethiopicus*, despite numerous investigations that had been conducted before, aimed at elucidating the potential pharmacological efficacy thereof for the possible treatment of various ailments (Lindsey *et al.*, 1999; Light *et al.*, 2002; Motsei *et al.*, 2003; Taylor *et al.*2003; Fennell *et al.*, 2004; Jäger & Van Staden, 2005; Fouche *et al.*, 2008; Lategan *et al.*, 2009; Coopoosamy *et al.*, 2010; Fouche *et al.*, 2011; Igoli *et al.*, 2012; Fouche *et al.*, 2013). The time-honoured usage of plants in traditional health care systems, however, does not guarantee its safety, since many chronic adverse effects may often go undetected. Cancerous potential, carcinogen activity and liver injury are only some of the possible long-term adverse effects reportedly caused by plants (Moreira *et al.*, 2014; Calitz *et al.*, 2015). To the best of our knowledge, this study was the first of its kind, as it aimed at investigating the possible cytotoxicity of several extracts of *S. aethiopicus*, as well as at establishing whether compounding thereof with specific tableting excipients might affect any observed cytotoxic effects.

Several recommendations and precautions can be made in order to assist with future investigations. Valuable information regarding the choice of extraction solvent during formulation was identified. Bagla (2012) stated that extraction solvents may influence the cytotoxic activity of a plant extract; consequently different extracts might induce almost completely contrasting effects (Stafford *et al.,* 2005; Fouche *et al.*, 2011; Złotek *et al.*, 2015). Depending on the solvent used, some extracts may even contain compounds that may induce cellular proliferation, rather than causing cell damage (Bagla, 2012). This statement was proven true during this study, as it was

found that aqueous extracts increased cellular proliferation, whereas organic extracts induced cell damage. These findings could be attributed to the variances in phytochemical compositions of the aqueous extract, compared to the organic extracts. As previously described, organic extracts contained AG 3 and AG 4, whereas aqueous extracts did not. Consequently, the cytotoxic effects observed following organic extract exposure might have been attributed to these two compounds, although no absolute assumptions could be made. It is therefore suggested that the biomarker compounds, AG 1–4, should be isolated and screened individually for any potential cytotoxicity. However, it remains important to keep in mind that the pharmacodynamic and pharmacokinetic activities of active constituents are often influenced by other chemical compounds present in plant extracts, resulting in altered absorption and bioavailability profiles (Dash & Nivsarkar, 2016).

Plant extract interference was identified during the MTT assays, which may have possibly resulted in an under estimation of cytotoxicities being demonstrated by the different extracts. Numerous studies have indicated that plant extracts non-enzymatically convert MTT, due to the presence of reducing or polyphenolic compounds (Bruggisser *et al.*, 2002; Han *et al.*, 2010; Riss 2014; Wang *et al.*, 2015). The adenosine triphosphate (ATP) assay is thus recommended as a possible alternative (Riss *et al.*, 2014). Moreover, in agreement with Cordier and Steenkamp (2015), it is recommended that no less than two cytotoxicity assays should be conducted to limit over- or under estimation of cytotoxicity, as well as to eradicate scientific bias. It is furthermore recommended that assay compatibility be confirmed prior to conducting experiments.

FITC conjugated annexin V/PI double labelling similarly only provided information with regards to whether cell death had been initiated through apoptotic or necrotic pathways, following exposure to *S. aethiopicus* extracts. In order to establish how these extracts might be modulating cell death, further elucidation of the precise biochemical cascades that are being induced, or inhibited, are necessary. Moreover, an expanded cell line range may also be considered in future studies, specifically the inclusion of non-cancerous cell types. This is quite important, as Igoli *et al*. (2012) previously demonstrated that the diterpenes isolated from *S. aethiopicus* had possessed some selectivity towards cancerous cell lines.

Final recommendations relate to the formulation of *S. aethiopicus* extracts, by using natural polymeric nanoparticles. The use of herbal preparations is often limited by their phytochemical complexities, solubility challenges (as was evident from this study), physicochemical instabilities and possible toxicities. Nanoparticle formulations might reduce these limitations and offer a more suitable solution for ensuring the safe and effective use of these extracts through improved solubility, enhanced bioavailability, increased stability, steady plasma levels and reduced dosage-amount. Increasingly more research studies currently focus on the development of nanoparticles, consisting of natural polymeric substances, which encapsulate plant extracts (Armendáriz-Barragán *et al.*, 2016; Sharma *et al*., 2016). Since our study proved chitosan had little effect on

the extracts of *S. aethiopicus*, it might very well be an ideal polymer to start with. The following aspects would require investigation, following nanoparticle formulation, namely: standardising techniques for carriers, physicochemical characterisation (e.g. morphology, particle size, zeta-potential), encapsulation efficacy, drug release efficacy, as well as *in vitro* and *in vivo* evaluations of the crude and encapsulated extract. Moreover, polymeric nanoparticles might protect active constituents from enzymatic degradation, as well as improve their biodistribution, mask unpleasant odours and tastes, provide prolonged release and increase membranous absorption (Armendáriz-Barragán *et al.*, 2016; Sharma *et al*., 2016).

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ANNEXURE A

Figure A1: *The notable colour difference of the grated S. aethiopicus rhizome material. Some grated pieces are a light brown colour, while others are a paler, ivory colour.*

Table A1: *The percentage yield of ethanol, diethyl ether and aqueous crude extracts of S. aethiopicus*

Extract	Plant material weight (g)	Plant extract weight (g)	Percentage yield of extract (%)
Ethanol	50	2.5484	5.097
Diethyl ether	50	2.4926	4.985
Aqueous	50	3.1880	6.376

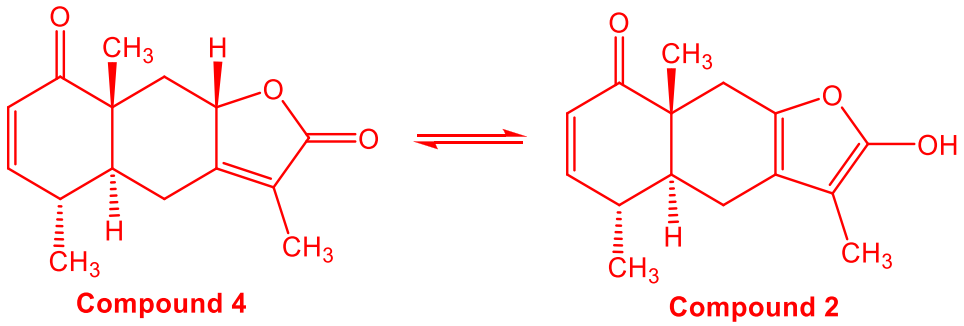


Figure A2: *The tautomeric relation between seperately isolated S. aethiopicus compounds, namely Compound 2 and 4*

Permission from Rogan B. Roth to use photographs he took of *Siphonochilus aethiopicus*

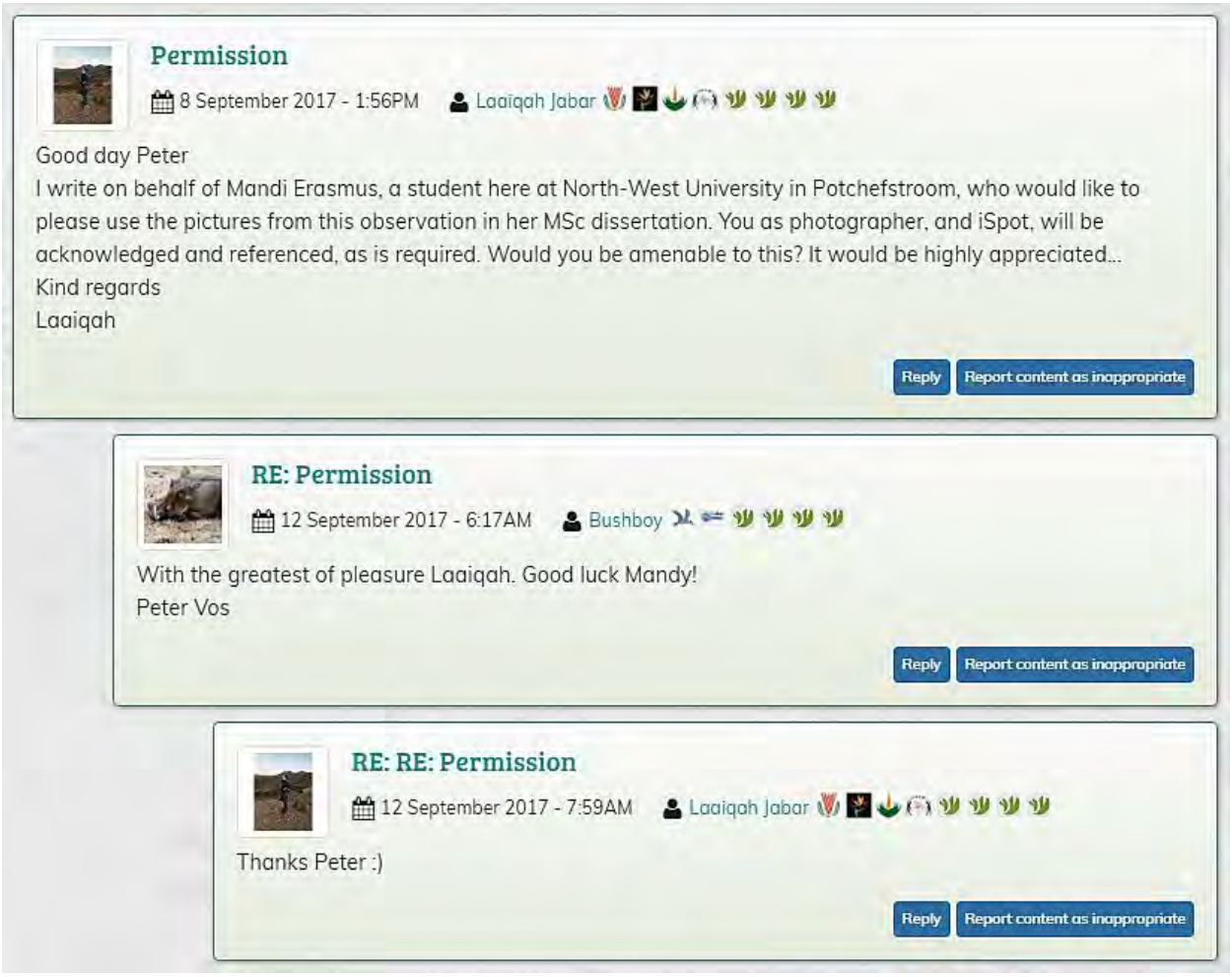
Rogan B. Roth
P.O. Box 344
Suurbraak, 6743
Western Cape

To whom it may concern

I hereby grant Ms Mandi Erasmus permission to use three images of *Siphonochilus aethiopicus* that I photographed in the botanical garden of the University of KwaZulu-Natal, Pietermaritzburg on 26th November, 2010. These images are to be used to illustrate her MSc thesis titled: "*In vitro* cytotoxicity of *Siphonochilus aethiopicus* in combination with selected fillers for tableting".

Signature:  Date: 11th September 2017

Permission from Peter Vos (username: Bushboy) to use photographs he took of *Siphonochilus aethiopicus* and shared on iSpot



ANNEXURE B

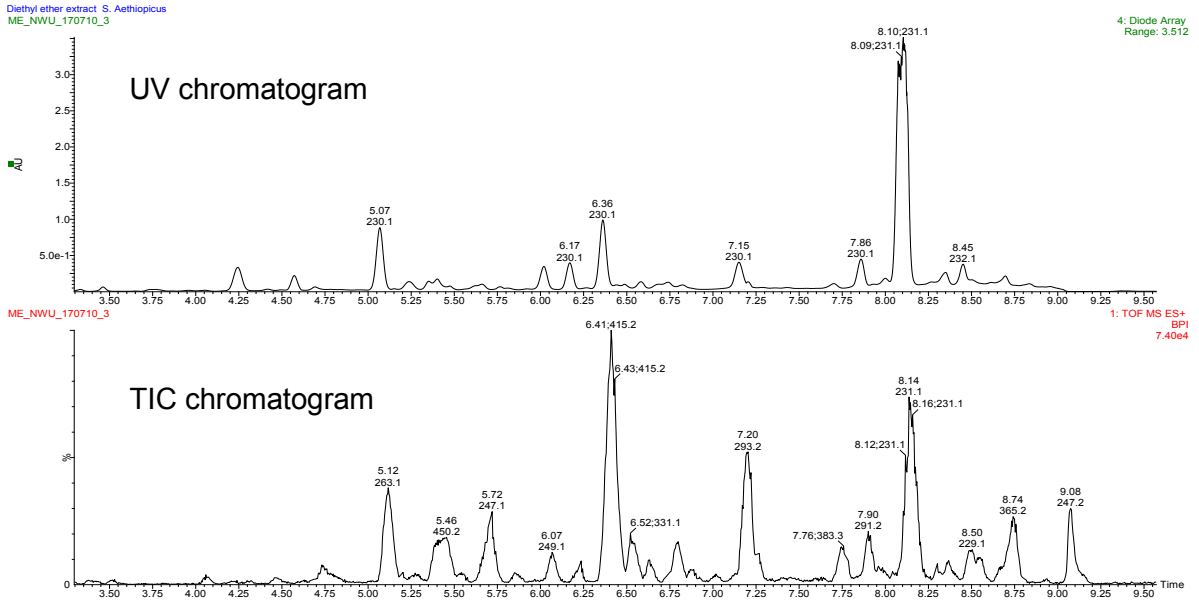


Figure B1: *UPLC chromatogram of the diethyl ether extract of S. aethiopicus; **(a)** UV-DAD chromatogram and **(b)** BPI chromatogram*

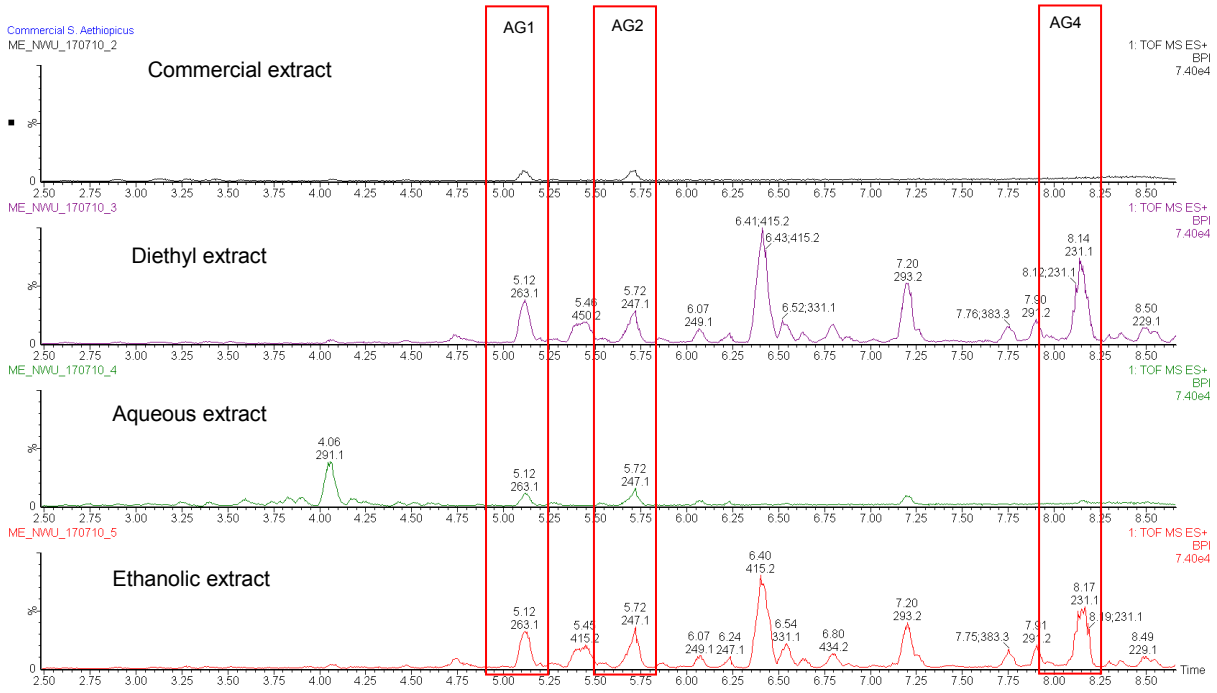
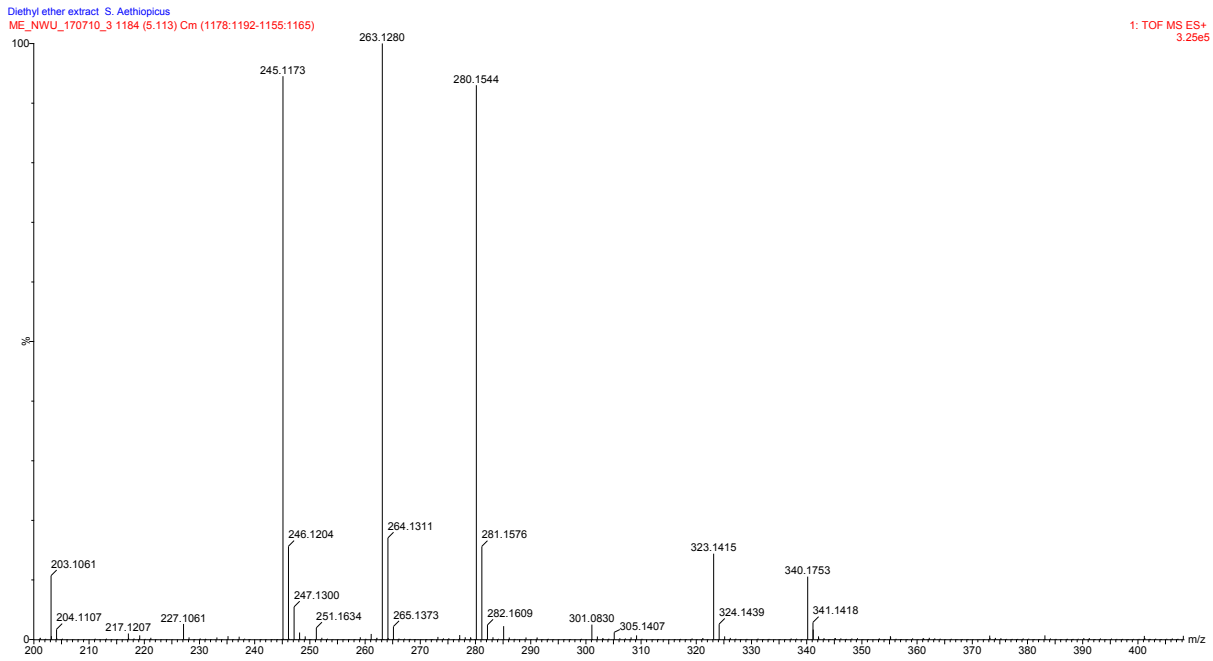
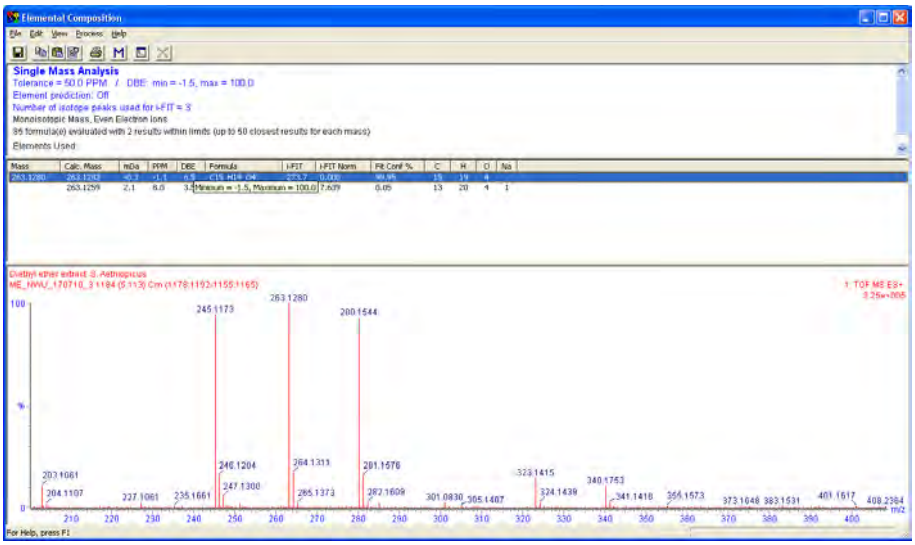


Figure B2: *Comparison of AG1, AG2, AG 3 and AG4 peaks in the numerous extracts of S. aethiopicus used during cytotoxicity assays by means of their individual BPI chromatograms*

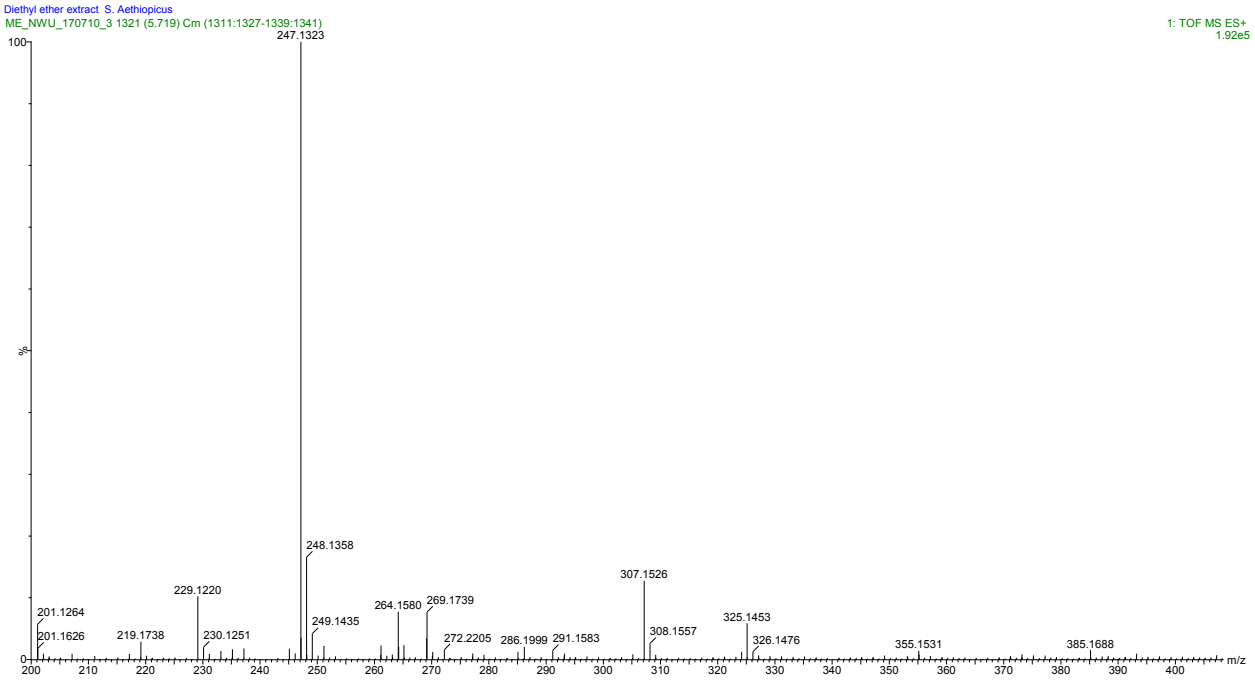


(a)

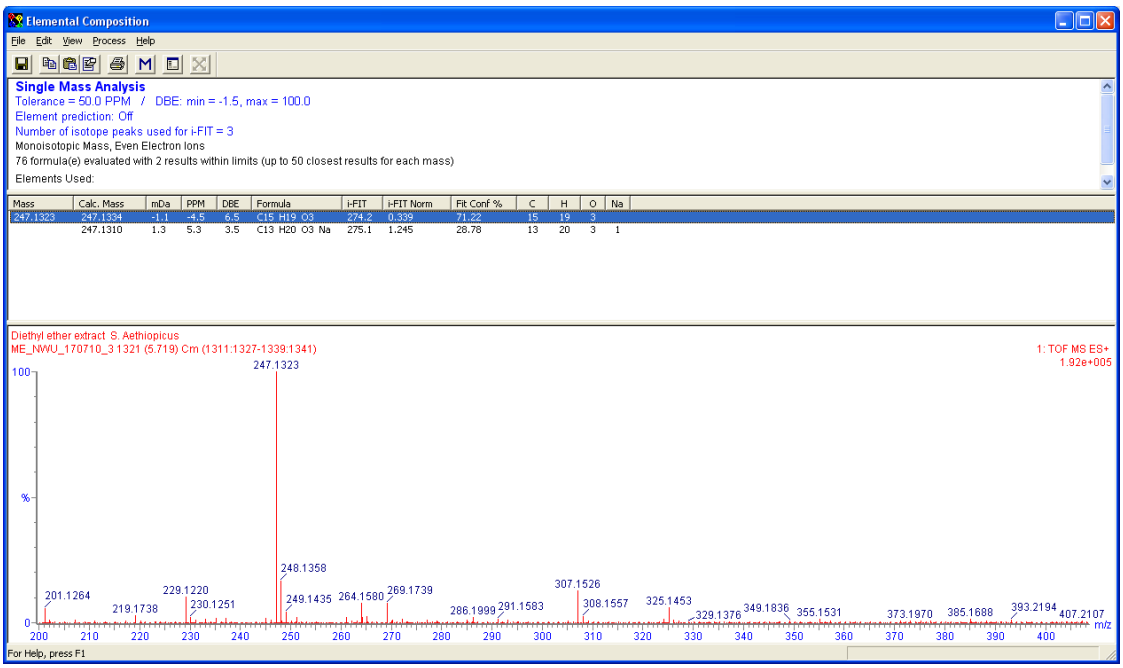


(b)

Figure B3: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of AG 1 (263 m/z) at 5.1 min*

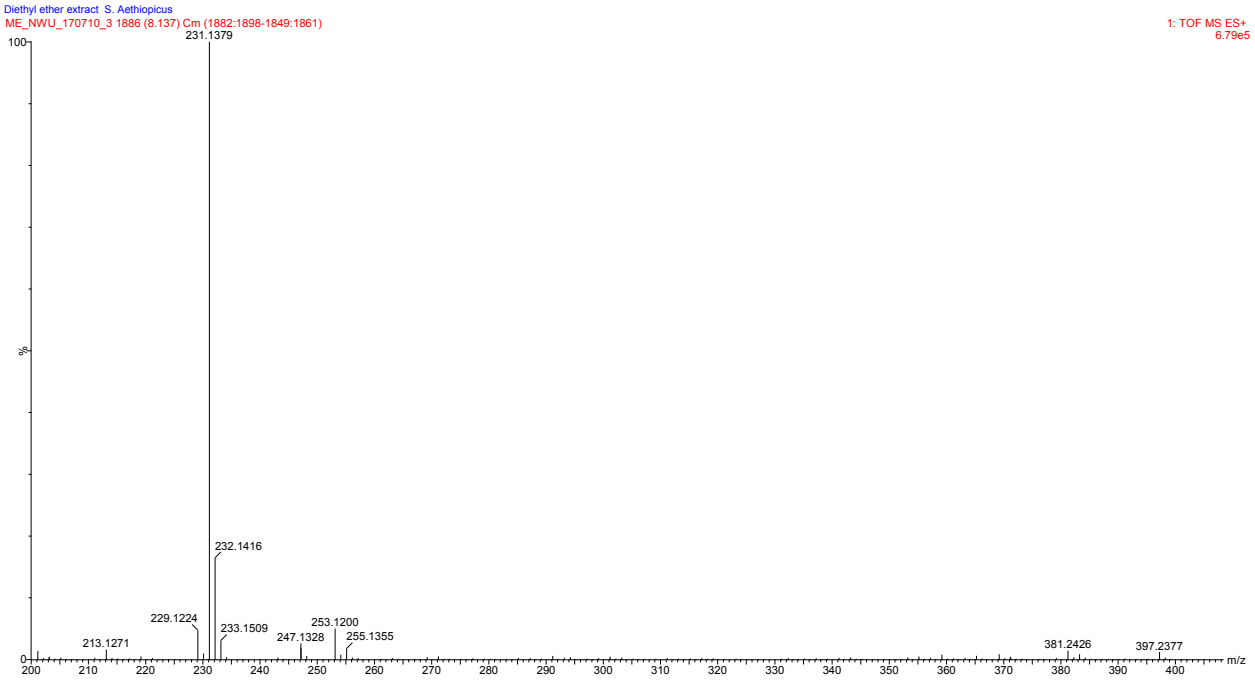


(a)

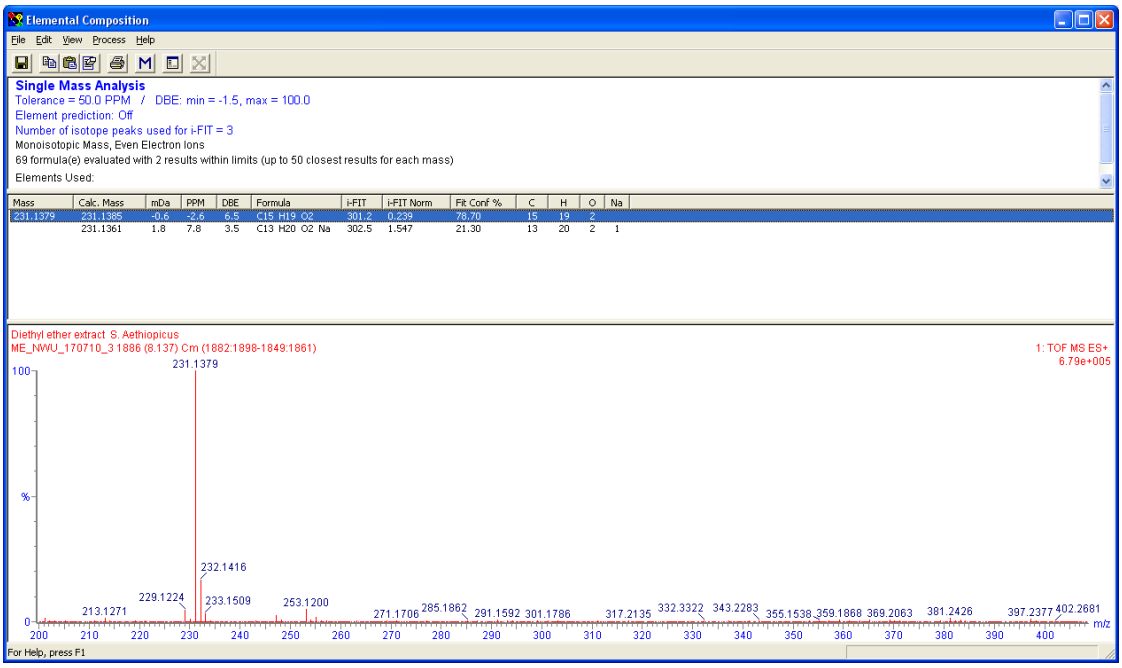


(b)

Figure B4: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of AG 2 (247 m/z) at 5.7 min*



(a)



(b)

Figure B5: *TOF/MS fragmentation spectra* ***(a)*** *and MassLynx 4.1 data* ***(b)*** *of AG 4 (231 m/z peak) at 8.1 min*

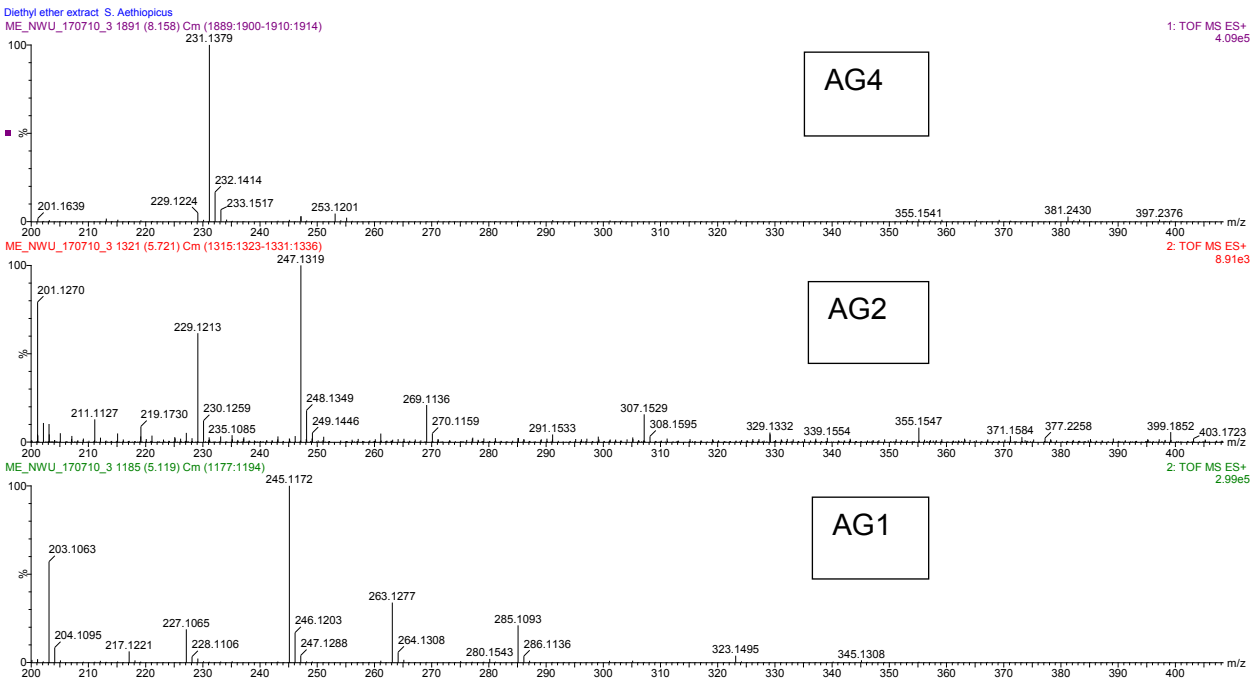
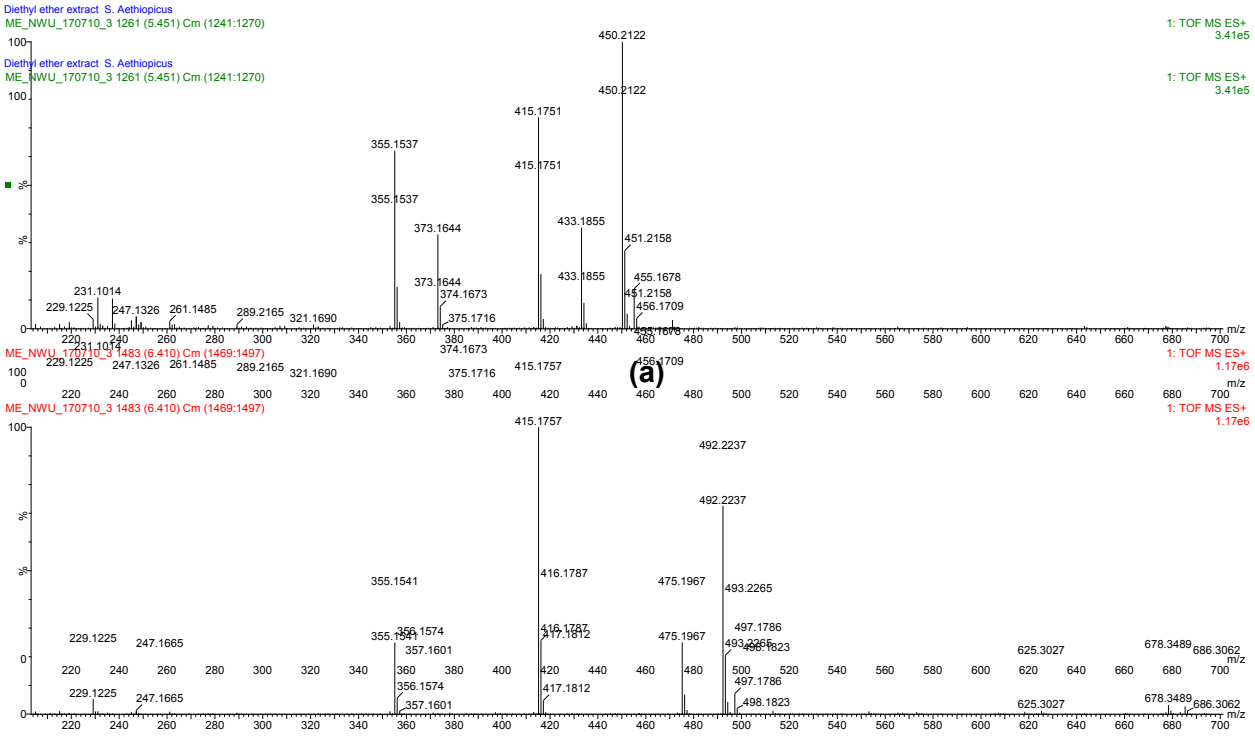
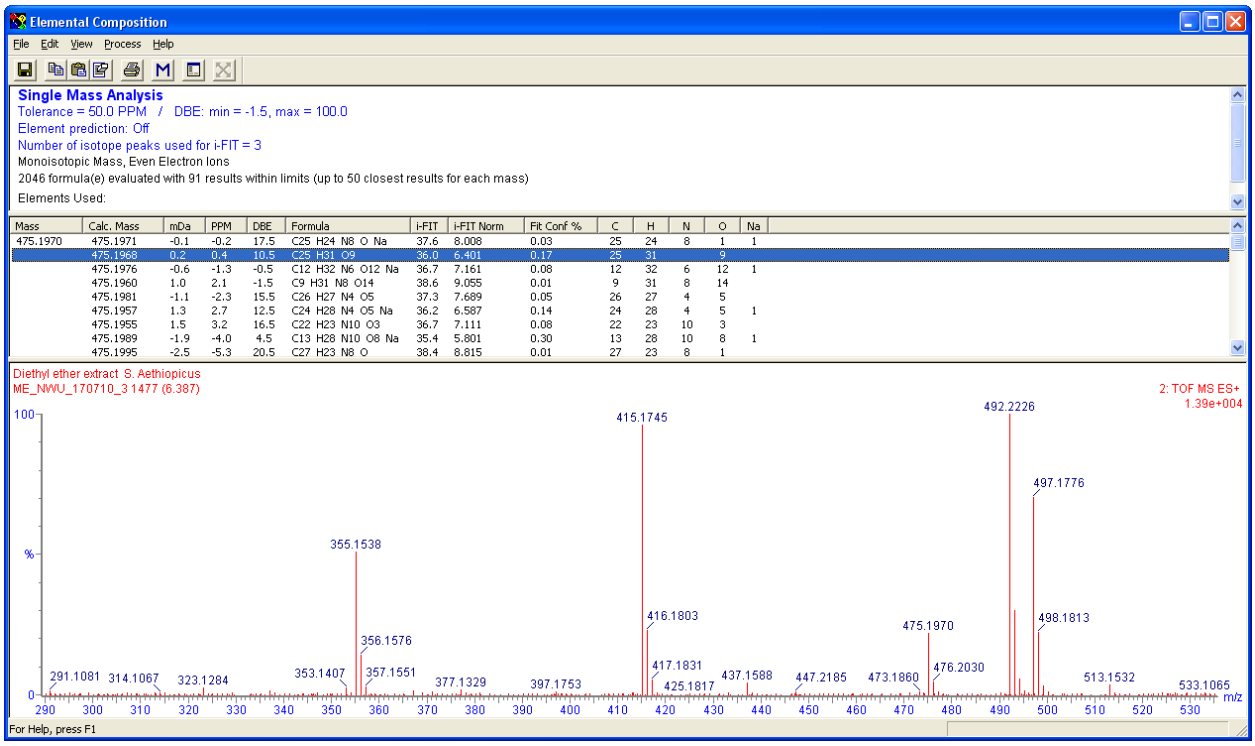


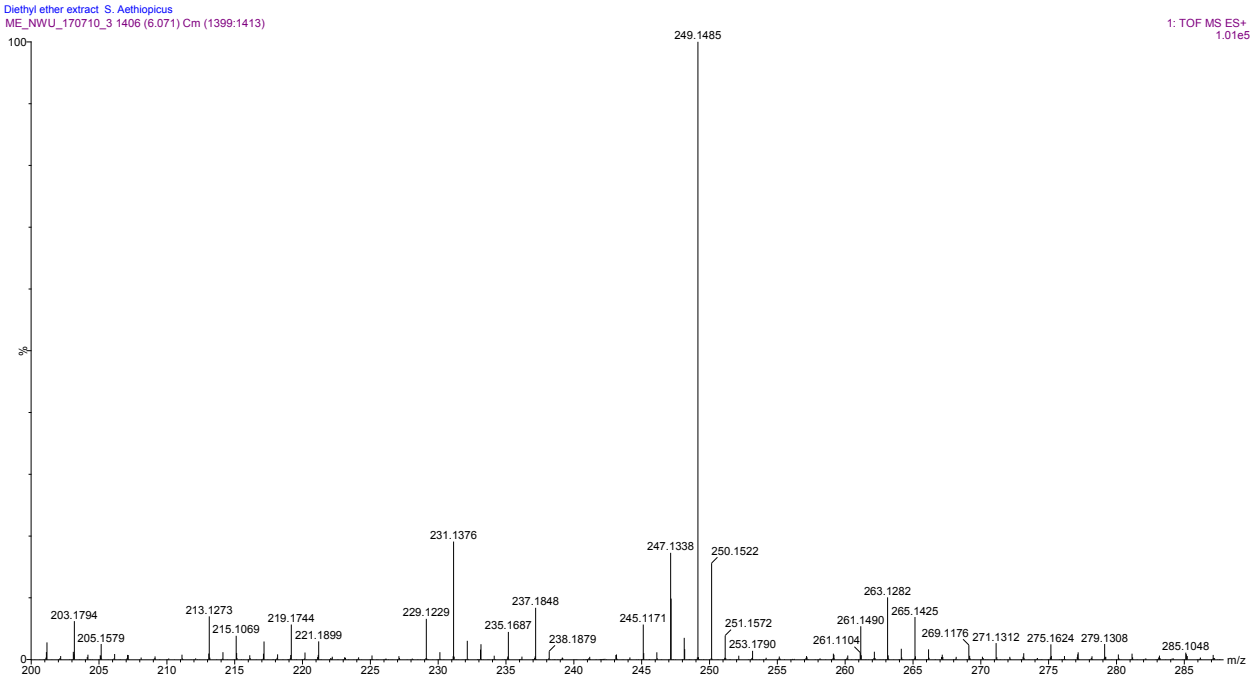
Figure B6: *Fragmentation spectra (MS^E) for the 3 main peaks showing addition and loss of water from AG1 and AG2 due to the presence of hydroxyl groups. AG4 has no hydroxyls and thus cannot lose water during fragmentation*



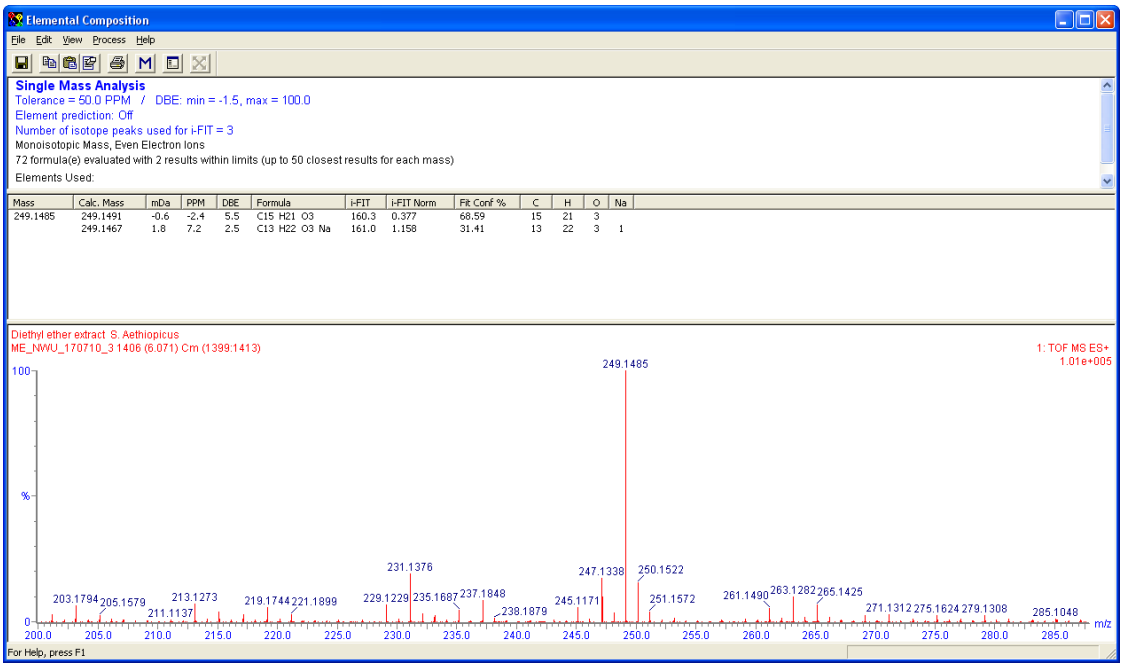


(c)

Figure B7: *Mass spectra for peaks at* ***(a)*** *5.4 and* ***(b)*** *6.4 min (450 m/z and 415 m/z respectively) showing similar fragment ions indicating that they are structurally similar.* **(c)** MassLynx 4.1 data for the peak at 6.4 min, illustrated in (b); 497=M+Na and 492=M+NH_3, repeating loss of *m/z* 60 indicates a polymer.



(a)



(b)

Figure B8: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of 249 m/z peak at 6.07 min*

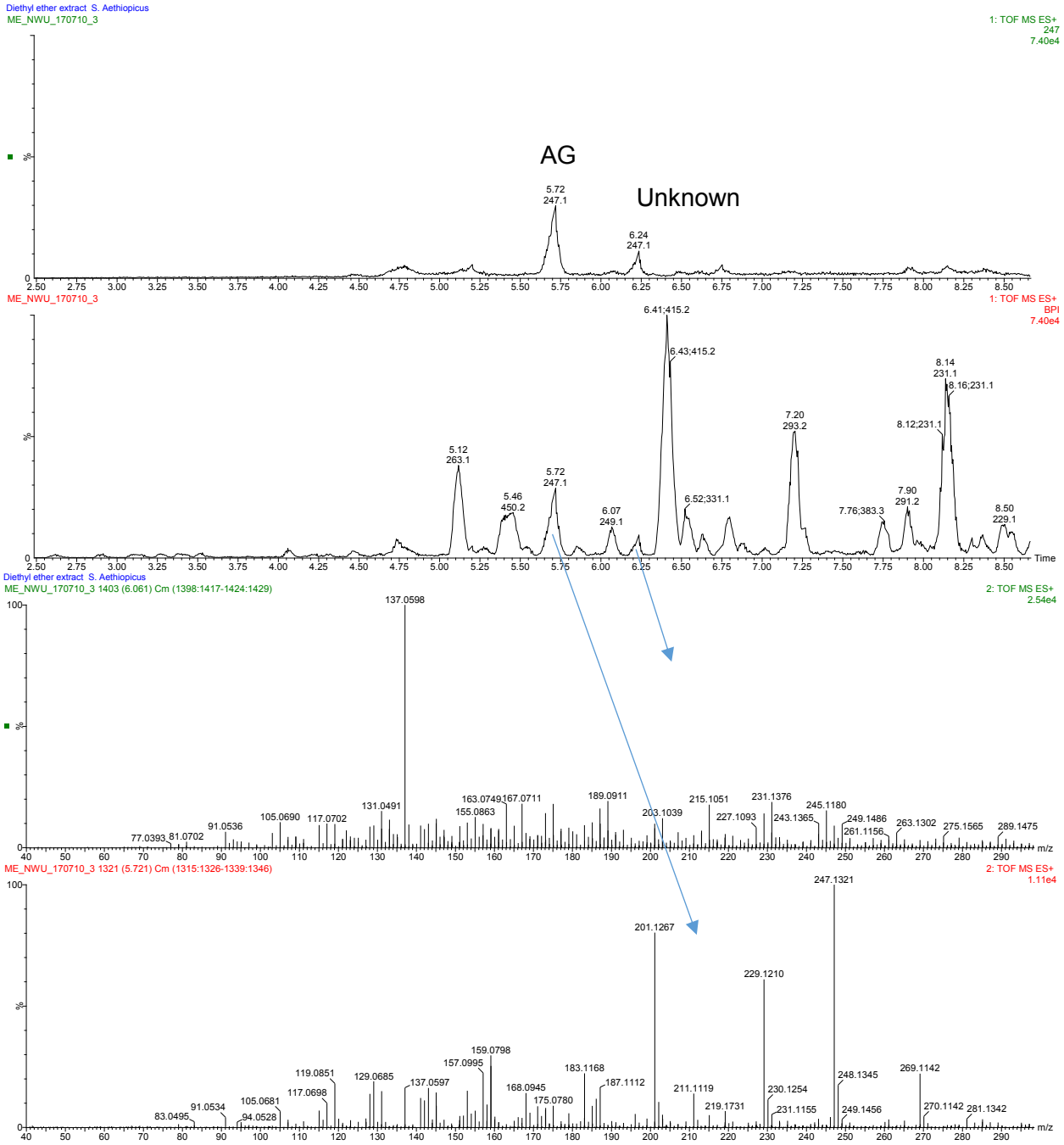
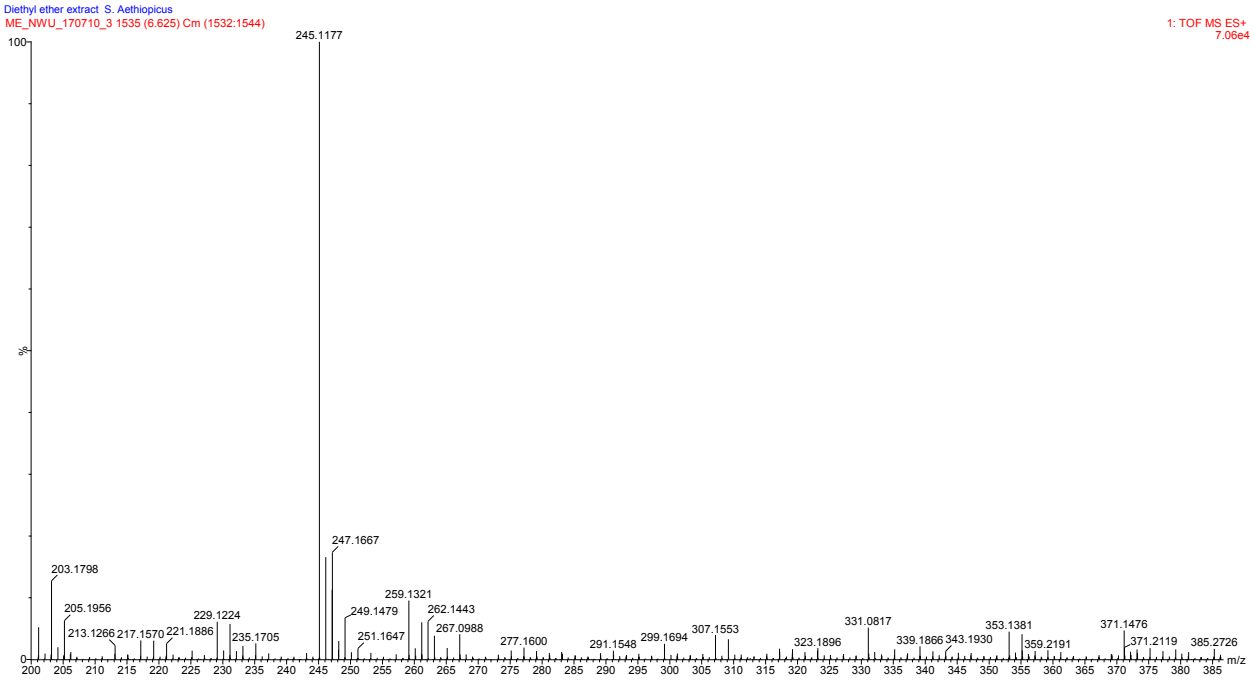
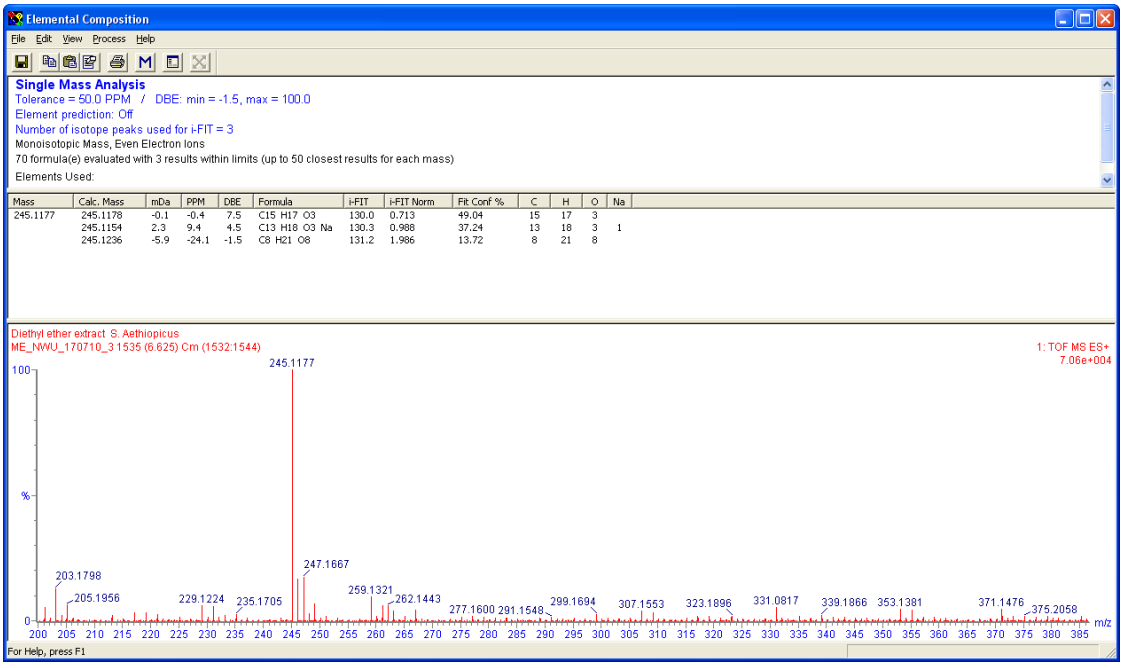


Figure B9: *The extracted mass chromatogram of peak 247 m/z revealed two peaks, AG 2 and that of an unknown compound. Comparison of the fragmentation spectra for the two 247 m/z peaks, indicated that the second peak was not structurally related to the first at 5.72 min*

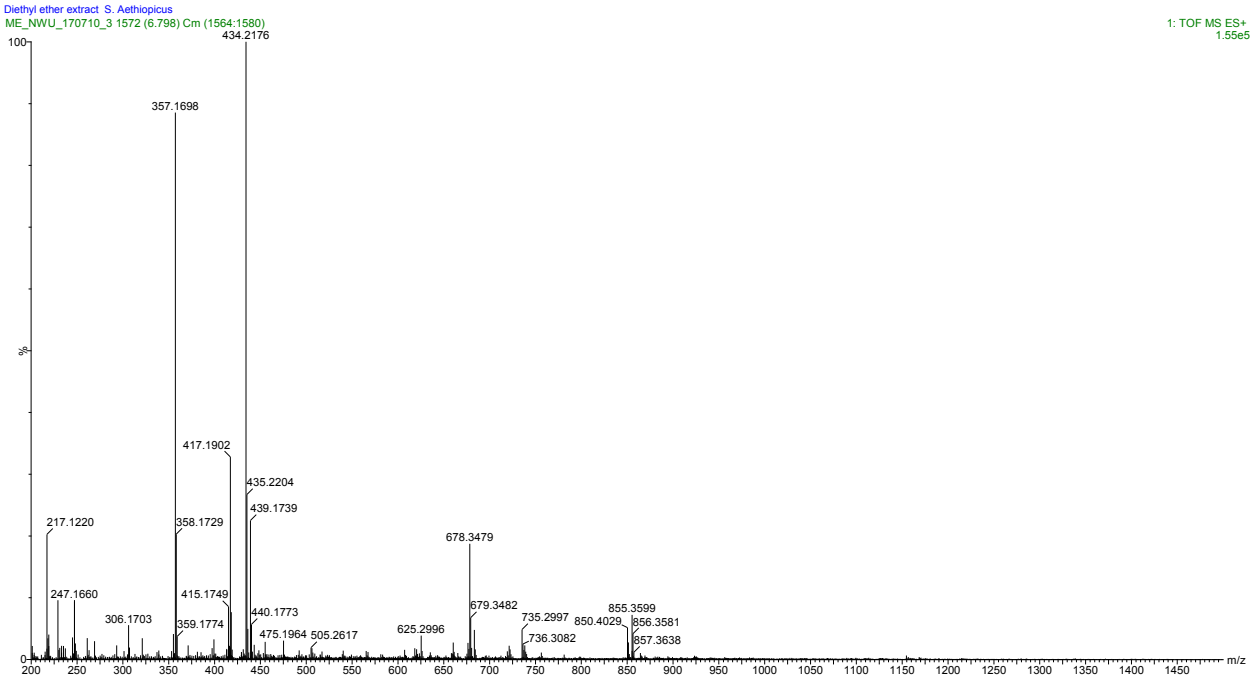


(a)

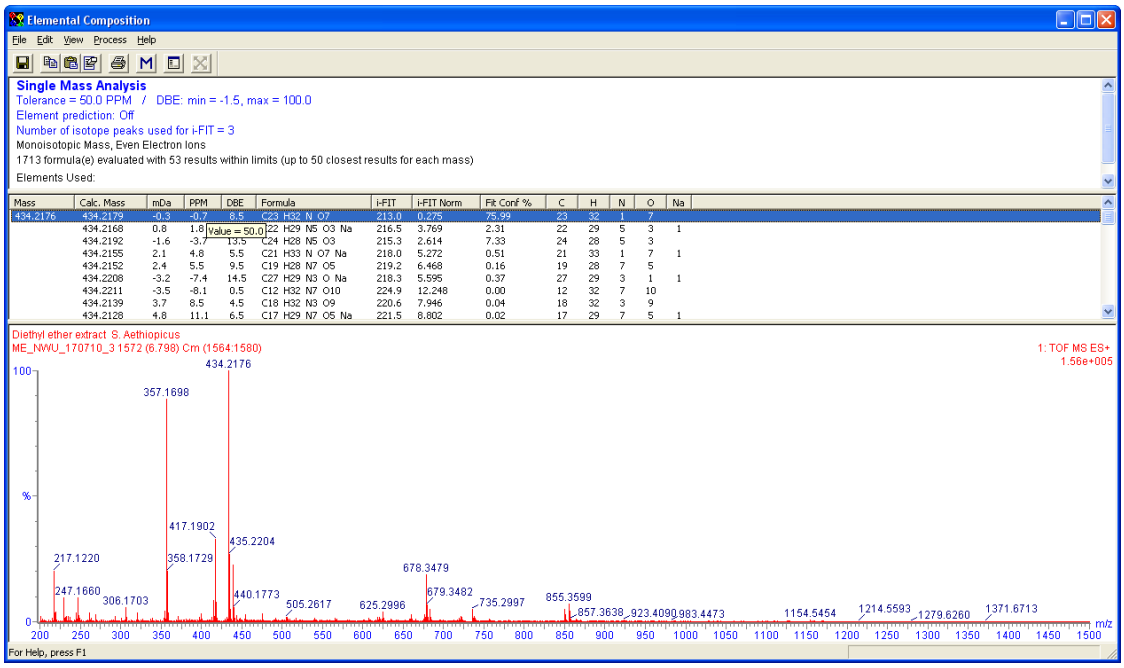


(b)

Figure B10: *TOF/MS fragmentation spectra* ***(a)*** *and MassLynx 4.1 data* ***(b)*** *of 245 m/z peak at 6.6 min*

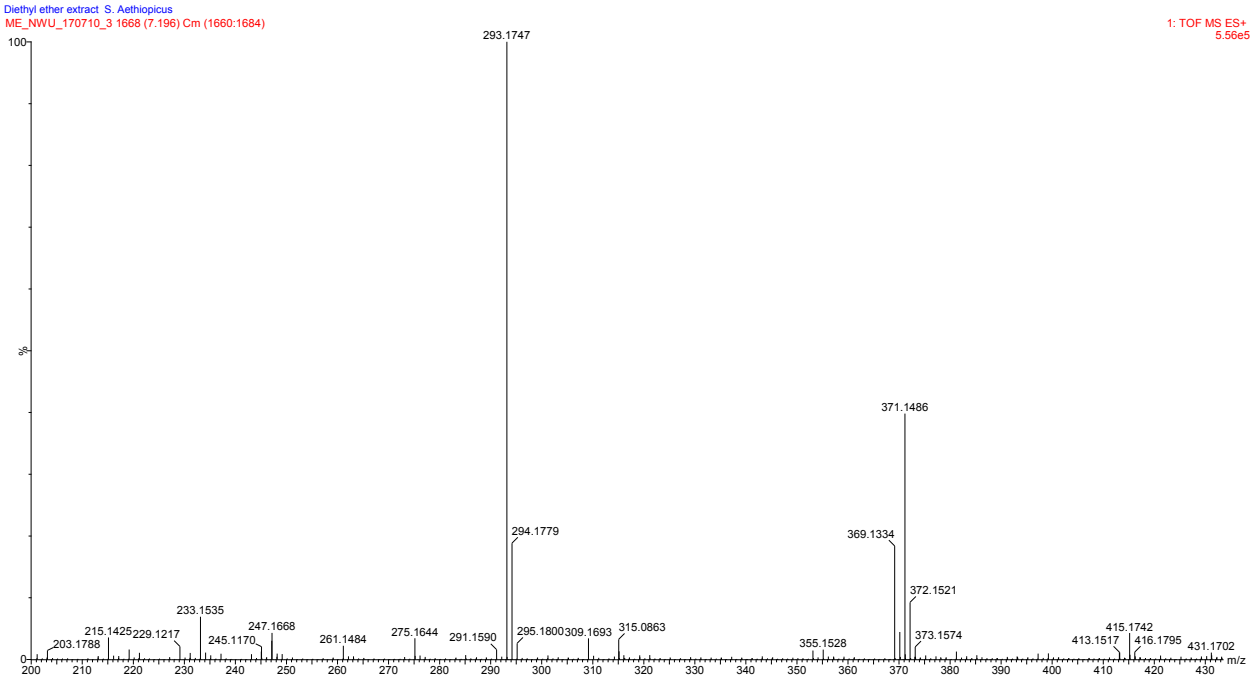


(a)

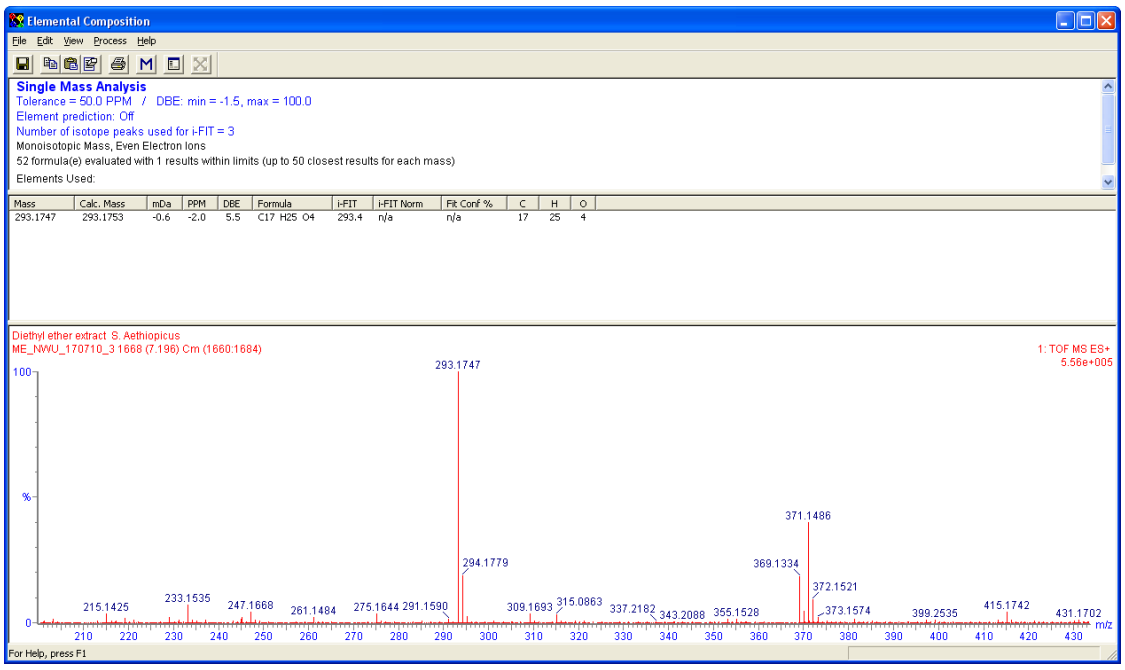


(b)

Figure B11: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of 434 m/z peak at 6.8 min*

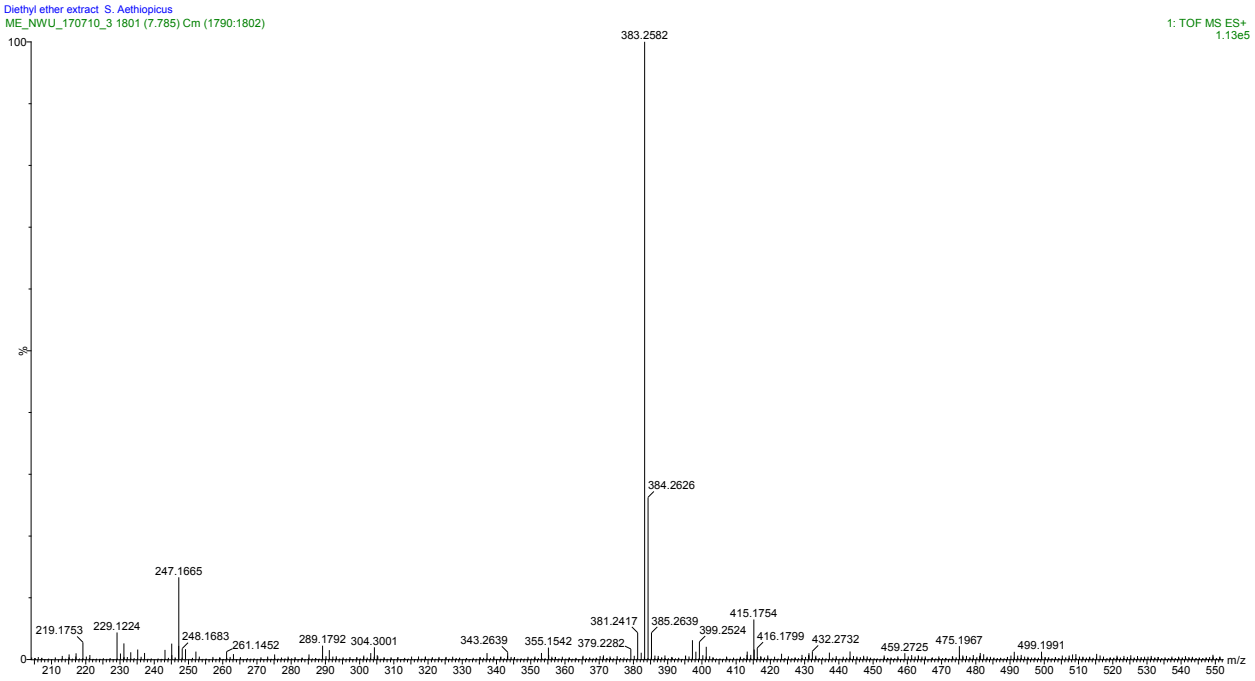


(a)

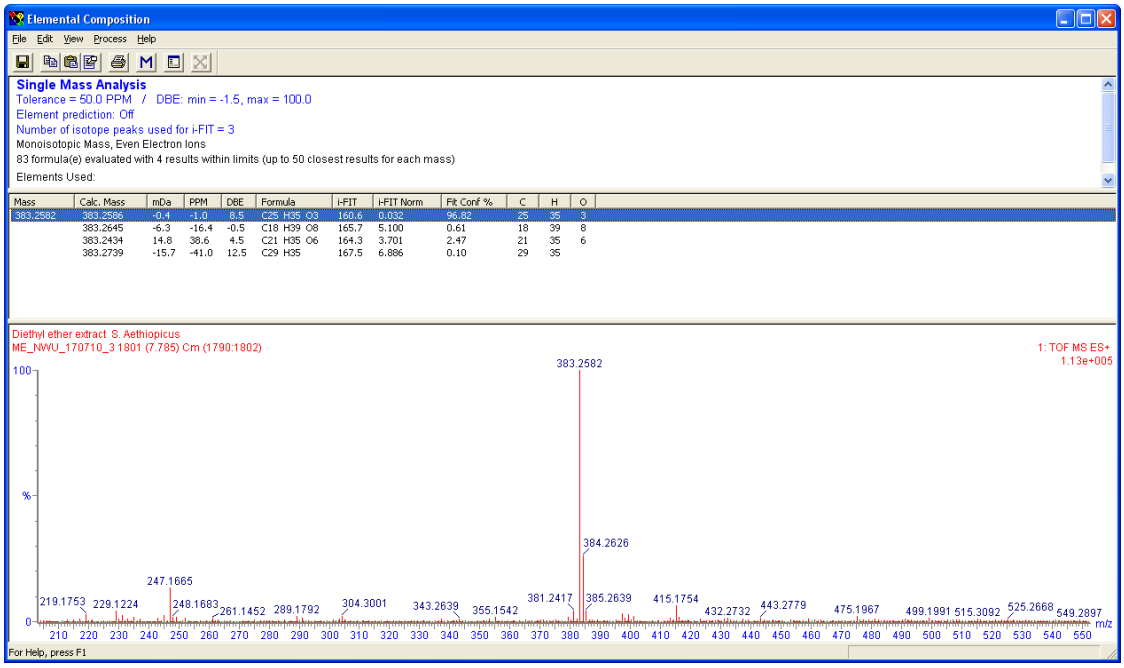


(b)

Figure B12: *TOF/MS fragmentation spectra* ***(a)*** *and MassLynx 4.1 data* ***(b)*** *of 293 m/z peak at 7.2 min*

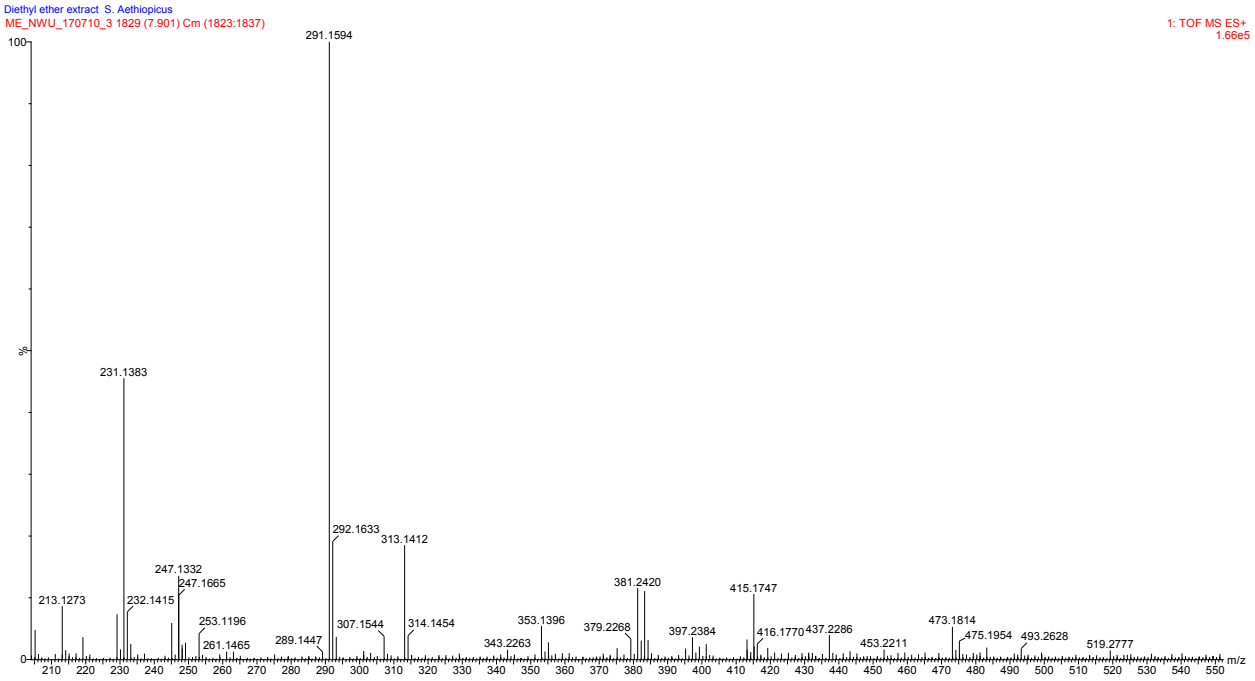


(a)

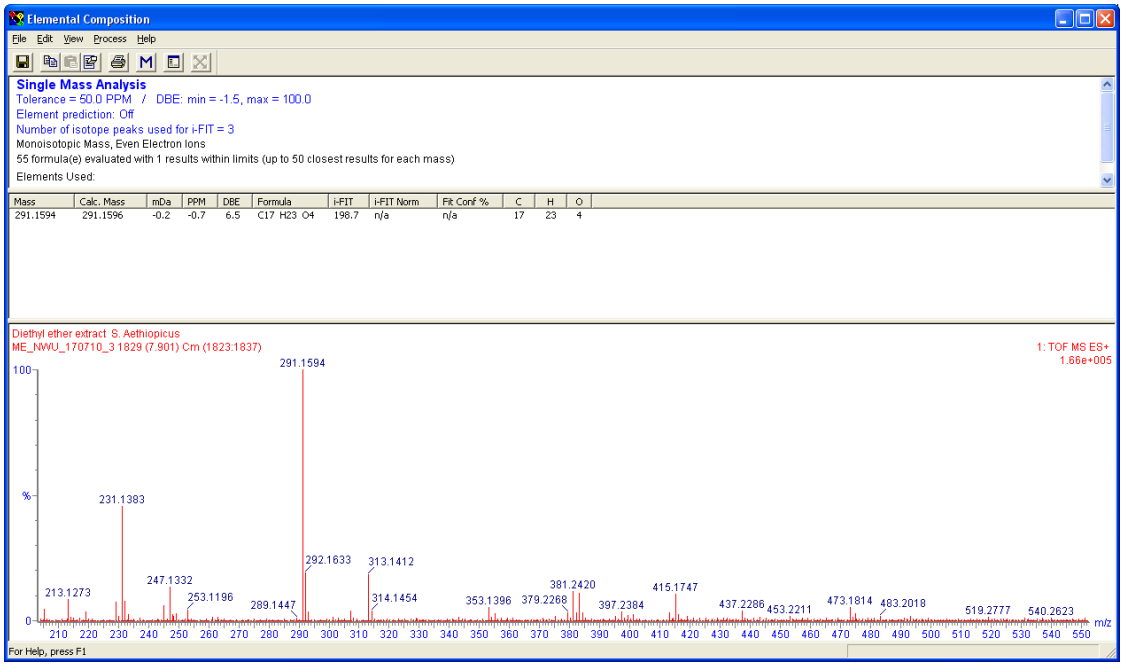


(b)

Figure B13: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of 383 m/z peak at 7.8 min*

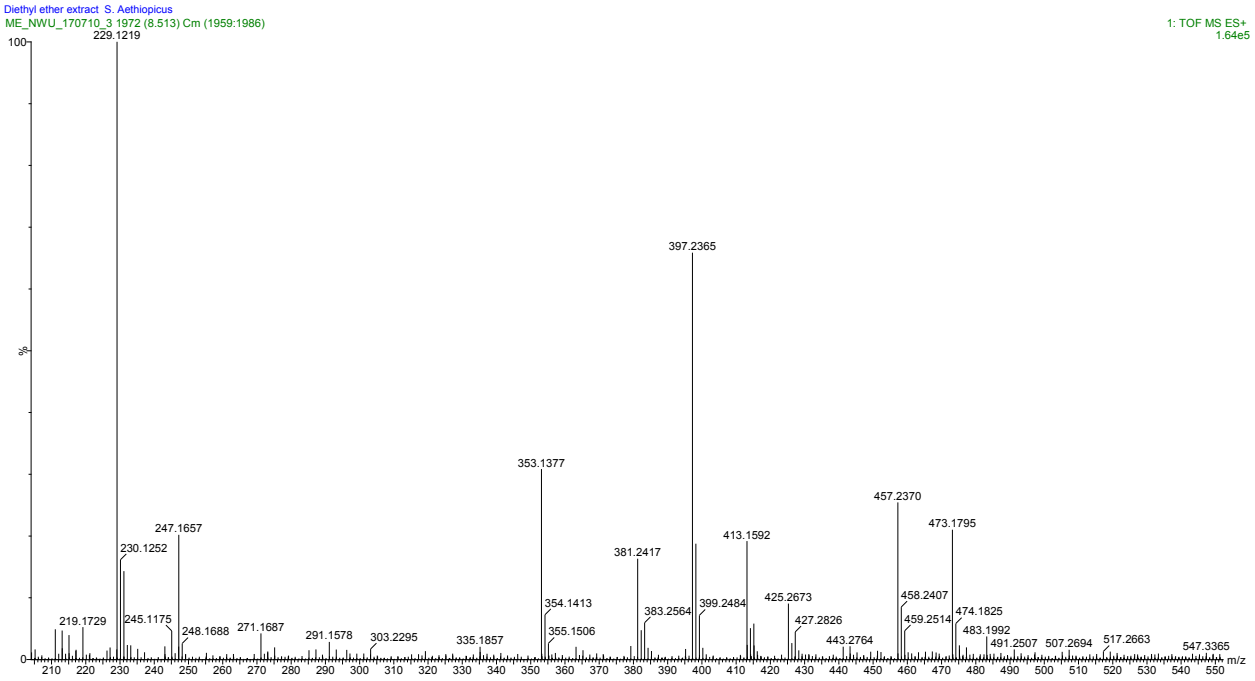


(a)

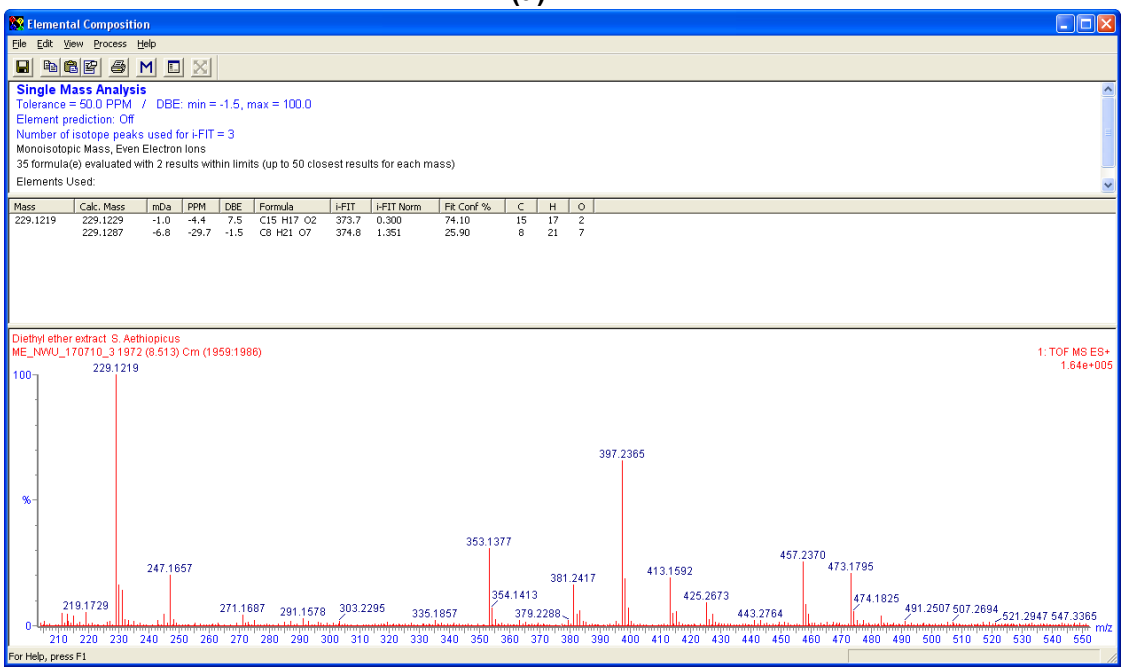


(b)

Figure B14: *TOF/MS fragmentation spectra **(a)** and MassLynx 4.1 data **(b)** of 291 m/z peak at 7.9 min*



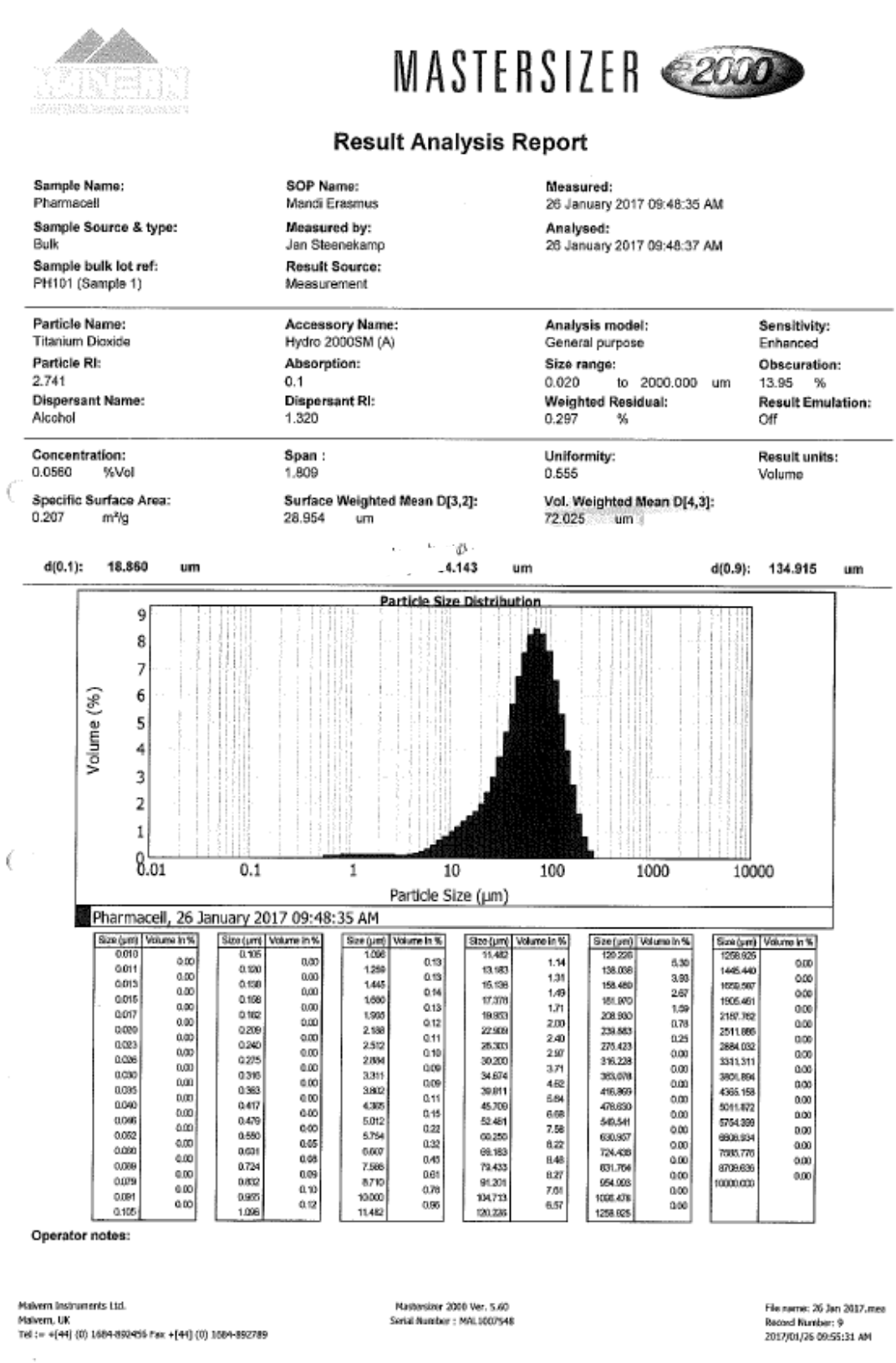
(a)



(b)

Figure B15: *TOF/MS fragmentation spectra* ***(a)*** *and MassLynx 4.1 data* ***(b)*** *of 229 m/z peak at 8.5 min*

ANNEXURE C



MASTERSIZER 2000

Result Analysis Report

Sample Name: Pharmacell	**SOP Name:** Mandi Erasmus	**Measured:** 26 January 2017 09:48:35 AM	
Sample Source & type: Bulk	**Measured by:** Jan Steenekamp	**Analysed:** 26 January 2017 09:48:37 AM	
Sample bulk lot ref: PH101 (Sample 1)	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 13.95 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.297 %	**Result Emulation:** Off
Concentration: 0.0560 %Vol	**Span :** 1.809	**Uniformity:** 0.555	**Result units:** Volume
Specific Surface Area: 0.207 m²/g	**Surface Weighted Mean D[3,2]:** 28.954 um	**Vol. Weighted Mean D[4,3]:** 72.025 um	

d(0.1): 18.860 um _4.143 um **d(0.9):** 134.915 um

Size (µm)	Volume In %
0.010	0.00
0.011	0.00
0.013	0.00
0.015	0.00
0.017	0.00
0.020	0.00
0.023	0.00
0.026	0.00
0.030	0.00
0.035	0.00
0.040	0.00
0.046	0.00
0.052	0.00
0.060	0.00
0.069	0.00
0.079	0.00
0.091	0.00
0.105	0.00

Size (µm)	Volume In %
0.105	0.00
0.120	0.00
0.138	0.00
0.158	0.00
0.182	0.00
0.209	0.00
0.240	0.00
0.275	0.00
0.316	0.00
0.363	0.00
0.417	0.00
0.479	0.00
0.550	0.00
0.631	0.05
0.724	0.08
0.832	0.09
0.955	0.10
1.096	0.12

Size (µm)	Volume In %
1.096	0.13
1.259	0.13
1.445	0.14
1.660	0.13
1.905	0.12
2.188	0.11
2.512	0.10
2.884	0.09
3.311	0.09
3.802	0.09
4.365	0.11
5.012	0.15
5.754	0.22
6.607	0.32
7.586	0.45
8.710	0.61
10.000	0.78
11.482	0.96

Size (µm)	Volume In %
11.482	1.14
13.183	1.31
15.136	1.49
17.378	1.71
19.953	2.00
22.909	2.40
26.303	2.97
30.200	3.71
34.674	4.62
39.811	5.64
45.709	6.68
52.481	7.58
60.256	8.22
69.183	8.48
79.433	8.27
91.201	7.61
104.713	6.57
120.226	5.30

Size (µm)	Volume In %
120.226	5.30
138.038	3.93
158.489	2.67
181.970	1.59
208.930	0.78
239.883	0.25
275.423	0.00
316.228	0.00
363.078	0.00
416.869	0.00
478.630	0.00
549.541	0.00
630.957	0.00
724.436	0.00
831.764	0.00
954.993	0.00
1096.478	0.00
1258.925	0.00

Size (µm)	Volume In %
1258.925	0.00
1445.440	0.00
1659.587	0.00
1905.461	0.00
2187.762	0.00
2511.886	0.00
2884.032	0.00
3311.311	0.00
3801.894	0.00
4365.158	0.00
5011.872	0.00
5754.399	0.00
6606.934	0.00
7585.776	0.00
8709.636	0.00
10000.000	

Operator notes:

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Malvern, UK
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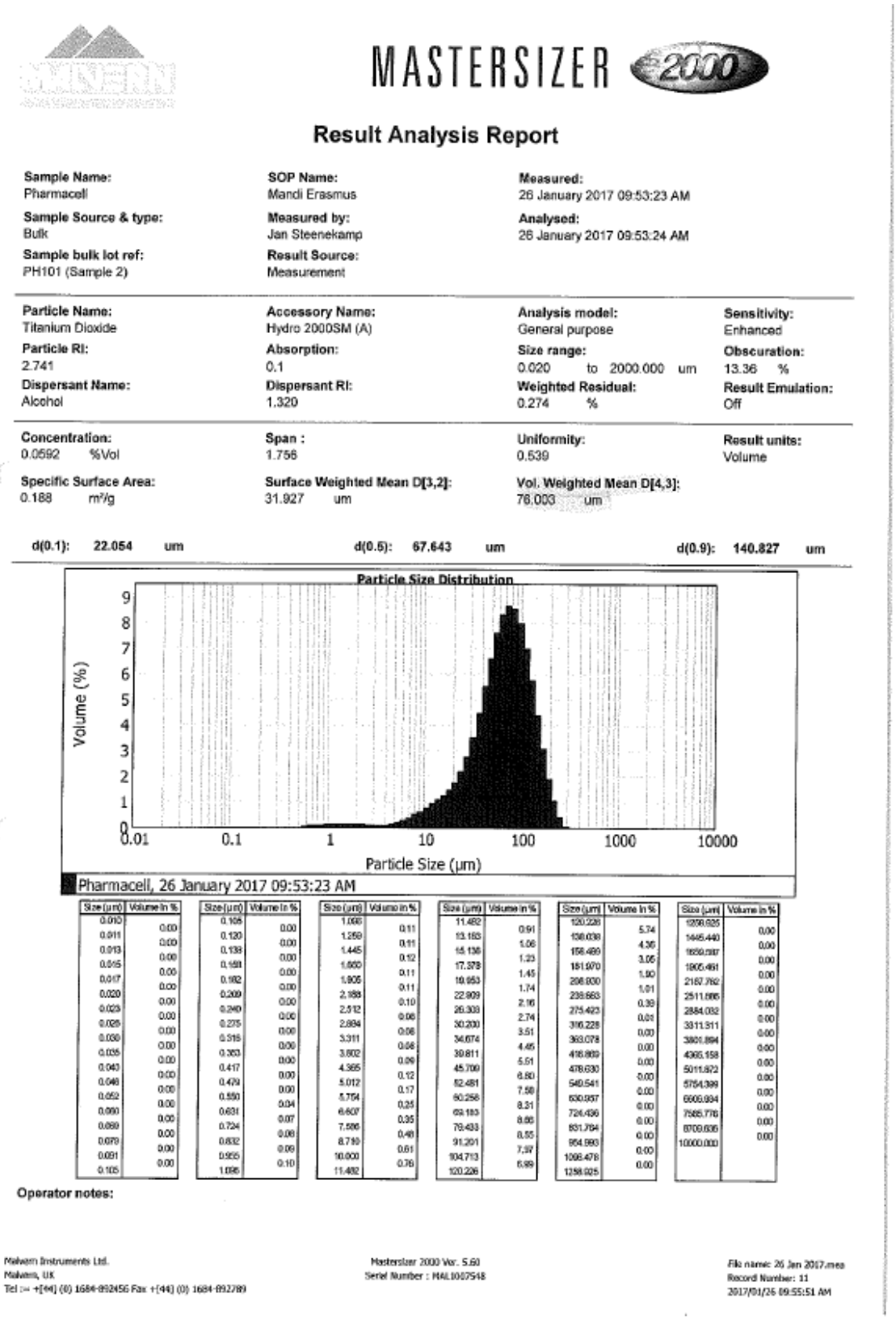
Mastersizer 2000 Ver. 5.60
Serial Number : MAL1007548

File name: 26 Jan 2017.mea
Record Number: 9
2017/01/26 09:55:31 AM

Figure C1: *Mastersizer 2000 analysis report for Pharmacel® 101, A*



MASTERSIZER 2000

Result Analysis Report

Sample Name: Pharmacell	**SOP Name:** Mandi Erasmus	**Measured:** 26 January 2017 09:53:23 AM	
Sample Source & type: Bulk	**Measured by:** Jan Steenekamp	**Analysed:** 26 January 2017 09:53:24 AM	
Sample bulk lot ref: PH101 (Sample 2)	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 13.36 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.274 %	**Result Emulation:** Off
Concentration: 0.0592 %Vol	**Span :** 1.756	**Uniformity:** 0.539	**Result units:** Volume
Specific Surface Area: 0.188 m²/g	**Surface Weighted Mean D[3,2]:** 31.927 um	**Vol. Weighted Mean D[4,3]:** 76.003 um	

d(0.1): 22.054 um **d(0.5):** 67.643 um **d(0.9):** 140.827 um

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.106	0.00	1.096	0.11	11.482	0.91	120.226	5.74	1259.025	0.00
0.011	0.00	0.120	0.00	1.259	0.11	13.183	1.06	138.038	4.36	1445.440	0.00
0.013	0.00	0.139	0.00	1.445	0.12	15.136	1.23	158.489	3.05	1659.587	0.00
0.015	0.00	0.161	0.00	1.660	0.11	17.378	1.45	181.970	1.90	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.11	19.953	1.74	208.930	1.01	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.10	22.909	2.16	239.883	0.39	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.08	26.303	2.74	275.423	0.01	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.08	30.200	3.51	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.08	34.674	4.45	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.09	39.811	5.51	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.12	45.709	6.60	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.17	52.481	7.59	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.04	5.754	0.25	60.256	8.31	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.07	6.607	0.35	69.183	8.66	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.08	7.586	0.48	79.433	8.55	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.09	8.710	0.61	91.201	7.97	954.993	0.00	10000.000	0.00
0.091	0.00	0.955	0.10	10.000	0.76	104.713	6.99	1096.478	0.00		
0.105	0.00	1.096		11.482		120.226		1258.925	0.00		

Operator notes:

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Mastersizer 2000 Ver. 5.60
Serial Number : MAL1007548

File name: 26 Jan 2017.mea
Record Number: 11
2017/01/26 09:55:51 AM

Figure C2: *Mastersizer 2000 analysis report for Pharmacel® 101, B*

MASTERSIZER 2000

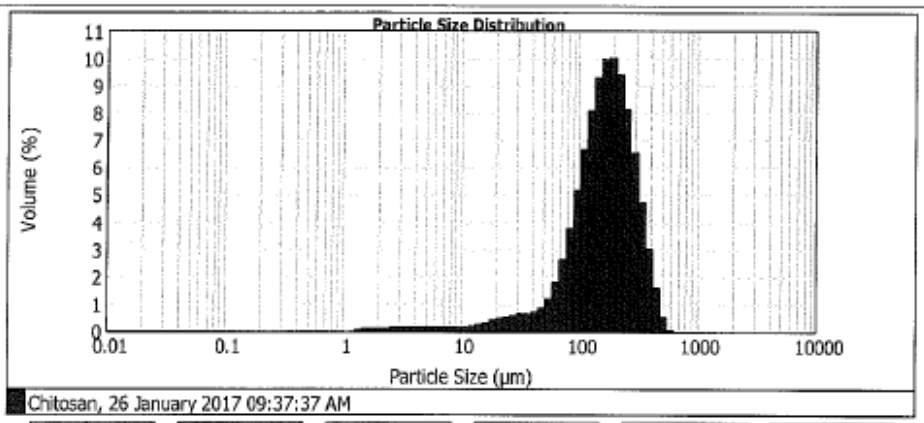
Result Analysis Report

Sample Name: Chitosan	**SOP Name:** Mandi Erasmus	**Measured:** 26 January 2017 09:37:37 AM
Sample Source & type: Bulk	**Measured by:** Jan Steenekamp	**Analysed:** 26 January 2017 09:37:38 AM
Sample bulk lot ref: CH (Sample 1)	**Result Source:** Measurement	

Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 12.00 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.667 %	**Result Emulation:** Off

Concentration: 0.1323 %Vol	**Span :** 1.480	**Uniformity:** 0.458	**Result units:** Volume
Specific Surface Area: 0.078 m²/g	**Surface Weighted Mean D[3,2]:** 76.918 um	**Vol. Weighted Mean D[4,3]:** 180.914 um	

d(0.1): 67.129 um **d(0.5):** 167.504 um **d(0.9):** 315.072 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.00	11.482	0.19	120.226	8.10	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.01	13.183	0.25	138.038	9.29	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.06	15.136	0.32	158.489	9.97	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.07	17.378	0.39	181.970	10.04	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.09	19.953	0.47	208.930	9.41	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.10	22.909	0.54	239.883	8.15	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.12	26.303	0.59	275.423	6.51	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.13	30.200	0.61	316.228	4.69	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.14	34.674	0.64	363.078	2.99	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.15	39.811	0.66	416.869	1.58	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.15	45.709	0.70	478.630	0.54	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.15	52.481	0.87	549.541	0.04	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.15	60.256	1.20	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.15	69.183	1.77	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.14	79.433	2.62	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.14	91.201	3.77	954.993	0.00	10000.000	
0.091	0.00	0.955	0.00	10.000	0.14	104.713	5.15	1096.478	0.00		
0.105	0.00	1.096	0.00	11.482	0.16	120.226	6.67	1258.925	0.00		

Operator notes:

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Serial Number : MAL1007548

File name: 26 Jan 2017.mea
Record Number: 5
2017/01/26 09:54:32 AM

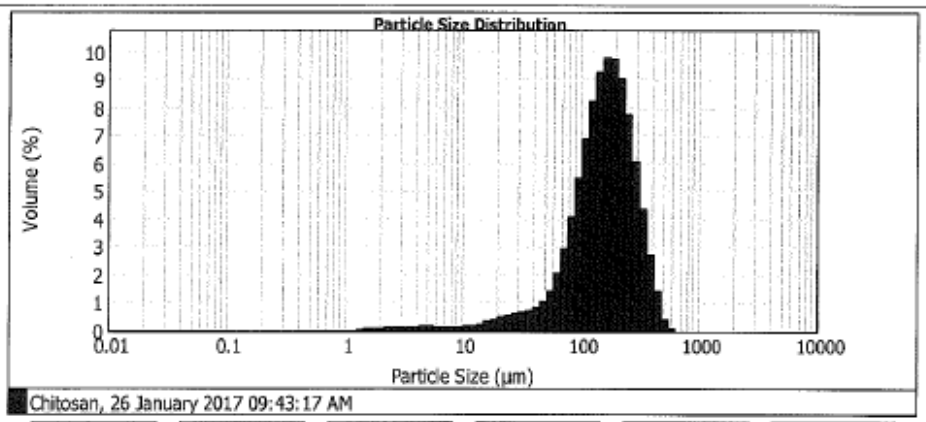
Figure C3: *Mastersizer 2000 analysis report for chitosan, A*

MASTERSIZER 2000

Result Analysis Report

Sample Name: Chitosan	**SOP Name:** Mandi Erasmus	**Measured:** 26 January 2017 09:43:17 AM	
Sample Source & type: Bulk	**Measured by:** Jan Steenekamp	**Analysed:** 26 January 2017 09:43:18 AM	
Sample bulk lot ref: CH (Sample 2)	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 14.62 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.578 %	**Result Emulation:** Off
Concentration: 0.1550 %Vol	**Span :** 1.526	**Uniformity:** 0.471	**Result units:** Volume
Specific Surface Area: 0.0822 m²/g	**Surface Weighted Mean D[3,2]:** 73.000 um	**Vol. Weighted Mean D[4,3]:** 175.070 um	

d(0.1): 61.406 um **d(0.5):** 161.504 um **d(0.9):** 307.936 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.099	0.00	11.482	0.20	120.226	8.22	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.01	13.183	0.26	138.038	9.26	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.01	15.136	0.33	158.489	9.81	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.07	17.378	0.42	181.970	9.73	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.08	19.953	0.50	208.930	9.01	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.09	22.909	0.58	239.883	7.72	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.11	26.303	0.64	275.423	6.08	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.13	30.200	0.69	316.228	4.32	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.14	34.674	0.74	363.078	2.70	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.15	39.811	0.85	416.869	1.45	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.16	45.709	1.06	478.630	0.42	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.16	52.481	1.44	549.541	0.07	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.16	60.256	2.05	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.16	69.183	2.94	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.15	79.433	4.10	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.15	91.201	5.45	954.993	0.00	10000.000	0.00
0.091	0.00	0.955	0.00	10.000	0.15	104.713	6.89	1096.478	0.00		
0.105	0.00	1.096	0.00	11.482	0.17	120.226	8.22	1258.925	0.00		

Operator notes:

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Mastersizer 2000 Ver. 5.60
Serial Number : MAL1007548

File name: 26 Jan 2017.mea
Record Number: 7
2017/01/26 09:54:45 AM

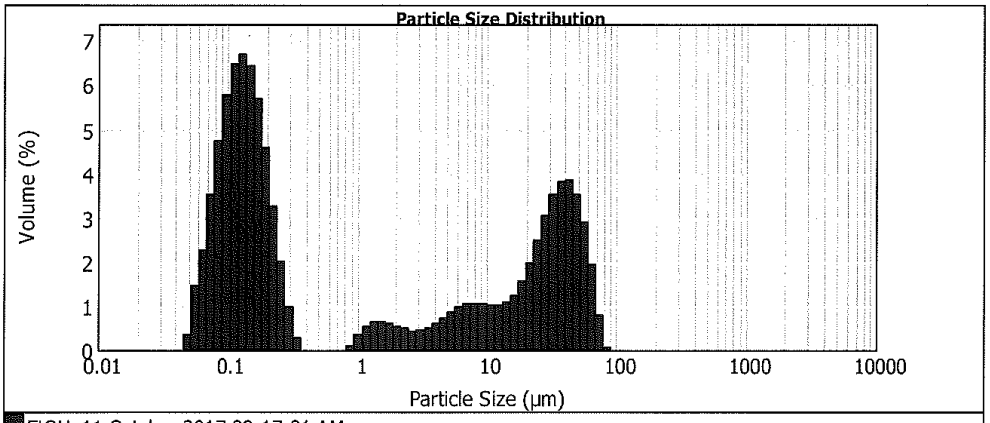
Figure C4: *Mastersizer 2000 analysis report for chitosan, B*

MASTERSIZER 2000

Result Analysis Report

Sample Name: EtOH	**SOP Name:** Mandi Erasmus Okt 17	**Measured:** 11 October 2017 09:17:01 AM	
Sample Source & type: ME	**Measured by:** Neil Barnard	**Analysed:** 11 October 2017 09:17:02 AM	
Sample bulk lot ref: EtOH (Sample 1)	**Result Source:** Measurement		
Particle Name: Yellow pigment	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.190	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 4.85 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 4.271 %	**Result Emulation:** Off
Concentration: 0.0007 %Vol	**Span :** 196.716	**Uniformity:** 56.3	**Result units:** Volume
Specific Surface Area: 28.3 m²/g	**Surface Weighted Mean D[3,2]:** 0.212 um	**Vol. Weighted Mean D[4,3]:** 12.817 um	

d(0.1): 0.086 um **d(0.5):** 0.225 um **d(0.9):** 44.408 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	6.47	1.096	0.54	11.482	1.02	120.226	0.00	1258.925	0.00
0.011	0.00	0.120	6.71	1.259	0.64	13.183	1.08	138.038	0.00	1445.440	0.00
0.013	0.00	0.138	6.45	1.445	0.66	15.136	1.25	158.489	0.00	1659.587	0.00
0.015	0.00	0.158	5.71	1.660	0.62	17.378	1.55	181.970	0.00	1905.461	0.00
0.017	0.00	0.182	4.59	1.905	0.55	19.953	1.98	208.930	0.00	2187.762	0.00
0.020	0.00	0.209	3.28	2.188	0.48	22.909	2.50	239.883	0.00	2511.886	0.00
0.023	0.00	0.240	2.00	2.512	0.44	26.303	3.04	275.423	0.00	2884.032	0.00
0.026	0.00	0.275	0.98	2.884	0.44	30.200	3.52	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.28	3.311	0.50	34.674	3.82	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.60	39.811	3.85	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.72	45.709	3.54	478.630	0.00	5011.872	0.00
0.046	0.33	0.479	0.00	5.012	0.85	52.481	2.88	549.541	0.00	5754.399	0.00
0.052	1.44	0.550	0.00	5.754	0.96	60.256	1.95	630.957	0.00	6608.934	0.00
0.060	2.27	0.631	0.00	6.607	1.03	69.183	0.80	724.436	0.00	7585.776	0.00
0.069	3.53	0.724	0.00	7.586	1.06	79.433	0.05	831.764	0.00	8709.636	0.00
0.079	4.76	0.832	0.08	8.710	1.05	91.201	0.00	954.993	0.00	10000.000	
0.091	5.79	0.955	0.34	10.000	1.02	104.713	0.00	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

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2017/10/11 12:20:32 PM

Figure C5: *Mastersizer 2000 analysis report for ethanol S. aethiopicus extract, A*

MASTERSIZER 2000

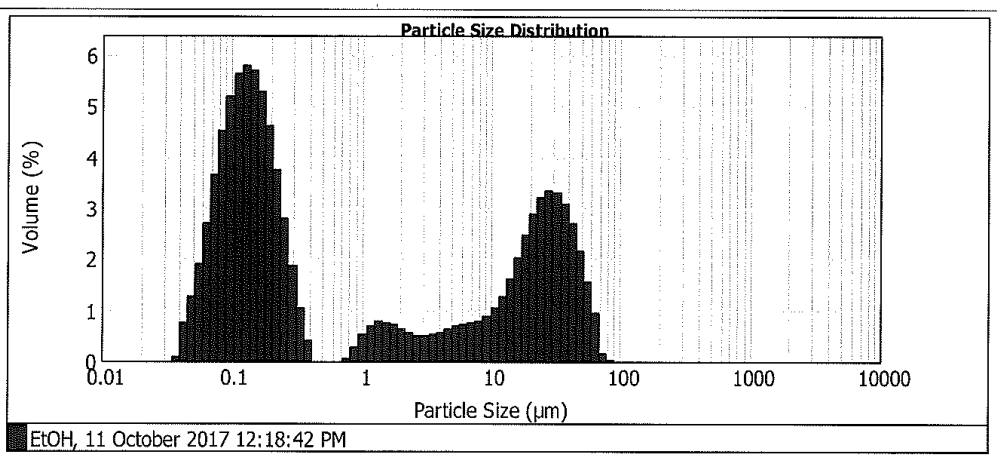
Result Analysis Report

Sample Name: EtOH	**SOP Name:** Mandi Erasmus Okt 17	**Measured:** 11 October 2017 12:18:42 PM
Sample Source & type: ME	**Measured by:** Neil Barnard	**Analysed:** 11 October 2017 12:18:43 PM
Sample bulk lot ref: EtOH (Sample 3)	**Result Source:** Measurement	

Particle Name: Yellow pigment	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.190	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 3.25 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 3.514 %	**Result Emulation:** Off

Concentration: 0.0004 %Vol	**Span :** 152.583	**Uniformity:** 43.3	**Result units:** Volume
Specific Surface Area: 31.4 m²/g	**Surface Weighted Mean D[3,2]:** 0.191 um	**Vol. Weighted Mean D[4,3]:** 10.192 um	

d(0.1): 0.079 um **d(0.5):** 0.233 um **d(0.9):** 35.579 um



Size (µm)	Volume In %
0.010	0.00
0.011	0.00
0.013	0.00
0.015	0.00
0.017	0.00
0.020	0.00
0.023	0.00
0.026	0.00
0.030	0.00
0.035	0.10
0.040	0.74
0.046	1.26
0.052	1.90
0.060	2.68
0.069	3.64
0.079	4.50
0.091	5.18
0.105	5.62
0.120	5.80
0.138	5.69
0.158	5.28
0.182	4.61
0.209	3.75
0.240	2.79
0.275	1.86
0.316	1.04
0.363	0.40
0.417	0.00
0.479	0.00
0.550	0.00
0.631	0.00
0.724	0.05
0.832	0.27
0.955	0.53
1.096	0.69
1.259	0.77
1.445	0.77
1.660	0.71
1.905	0.63
2.188	0.55
2.512	0.50
2.884	0.49
3.311	0.51
3.802	0.56
4.365	0.62
5.012	0.67
5.754	0.72
6.607	0.75
7.586	0.80
8.710	0.88
10.000	1.03
11.482	1.27
13.183	1.61
15.136	2.02
17.378	2.47
19.953	2.88
22.909	3.19
26.303	3.35
30.200	3.31
34.674	3.08
39.811	2.69
45.709	2.17
52.481	1.55
60.256	0.95
69.183	0.15
79.433	0.01
91.201	0.00
104.713	0.00
120.226	0.00
138.038	0.00
158.489	0.00
181.970	0.00
208.930	0.00
239.883	0.00
275.423	0.00
316.228	0.00
363.078	0.00
416.869	0.00
478.630	0.00
549.541	0.00
630.957	0.00
724.436	0.00
831.764	0.00
954.993	0.00
1096.478	0.00
1258.925	0.00
1445.440	0.00
1659.587	0.00
1905.461	0.00
2187.762	0.00
2511.886	0.00
2884.032	0.00
3311.311	0.00
3801.894	0.00
4365.158	0.00
5011.872	0.00
5754.399	0.00
6606.934	0.00
7585.776	0.00
8709.636	0.00
10000.000	0.00

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2017/10/11 12:20:39 PM

Figure C6: *Mastersizer 2000 analysis report for ethanol S. aethiopicus extract, B*

MASTERSIZER 2000

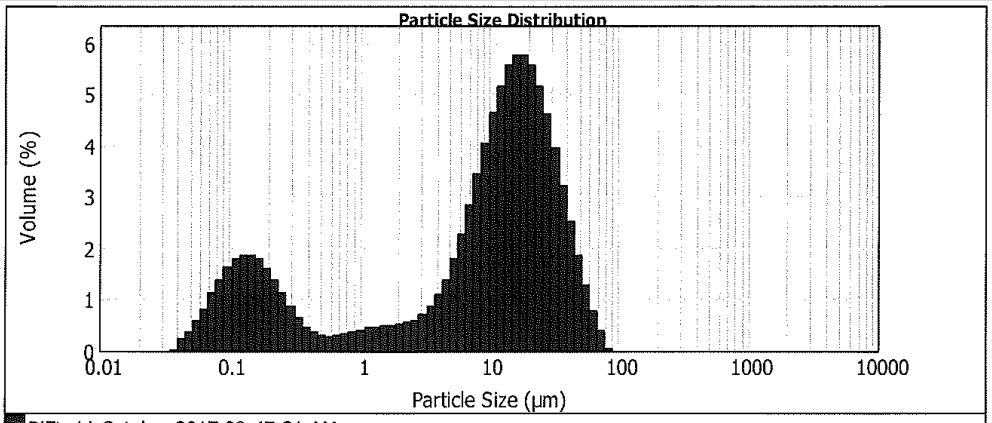
Result Analysis Report

Sample Name: DiEt	**SOP Name:** Mandi Erasmus Okt 17	**Measured:** 11 October 2017 08:47:31 AM
Sample Source & type: ME	**Measured by:** Neil Barnard	**Analysed:** 11 October 2017 08:47:32 AM
Sample bulk lot ref: DiEt (Sample 1)	**Result Source:** Measurement	

Particle Name: Yellow pigment	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.190	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 9.60 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 0.755 %	**Result Emulation:** Off

Concentration: 0.0027 %Vol	**Span :** 2.889	**Uniformity:** 0.912	**Result units:** Volume
Specific Surface Area: 10.7 m²/g	**Surface Weighted Mean D[3,2]:** 0.561 um	**Vol. Weighted Mean D[4,3]:** 15.046 um	

d(0.1): 0.140 um **d(0.5):** 11.995 um **d(0.9):** 34.790 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	1.78	1.096	0.45	11.482	5.17	120.226	0.00	1258.925	0.00
0.011	0.00	0.120	1.87	1.259	0.47	13.183	5.55	138.038	0.00	1445.440	0.00
0.013	0.00	0.138	1.87	1.445	0.48	15.136	5.76	158.489	0.00	1659.587	0.00
0.015	0.00	0.158	1.78	1.660	0.49	17.378	5.76	181.970	0.00	1905.461	0.00
0.017	0.00	0.182	1.61	1.905	0.51	19.953	5.56	208.930	0.00	2187.762	0.00
0.020	0.00	0.209	1.37	2.188	0.54	22.909	5.16	239.883	0.00	2511.886	0.00
0.023	0.00	0.240	1.11	2.512	0.60	26.303	4.60	275.423	0.00	2884.032	0.00
0.026	0.00	0.275	0.86	2.884	0.70	30.200	3.94	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.64	3.311	0.86	34.674	3.23	363.078	0.00	3801.894	0.00
0.035	0.02	0.363	0.47	3.802	1.08	39.811	2.52	416.869	0.00	4365.158	0.00
0.040	0.22	0.417	0.36	4.365	1.39	45.709	1.86	478.630	0.00	5011.872	0.00
0.046	0.38	0.479	0.30	5.012	1.78	52.481	1.28	549.541	0.00	5754.399	0.00
0.052	0.57	0.550	0.28	5.754	2.27	60.256	0.78	630.957	0.00	6606.934	0.00
0.060	0.81	0.631	0.29	6.607	2.82	69.183	0.39	724.436	0.00	7585.776	0.00
0.069	1.11	0.724	0.33	7.586	3.43	79.433	0.04	831.764	0.00	8709.636	0.00
0.079	1.39	0.832	0.37	8.710	4.05	91.201	0.00	954.993	0.00	10000.000	
0.091	1.62	0.955	0.41	10.000	4.65	104.713	0.00	1096.478	0.00		
0.105		1.096		11.482		120.228		1258.925			

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2017/10/11 09:02:02 AM

Figure C7: *Mastersizer 2000 analysis report for diethyl ether S. aethiopicus extract, A*

MASTERSIZER 2000

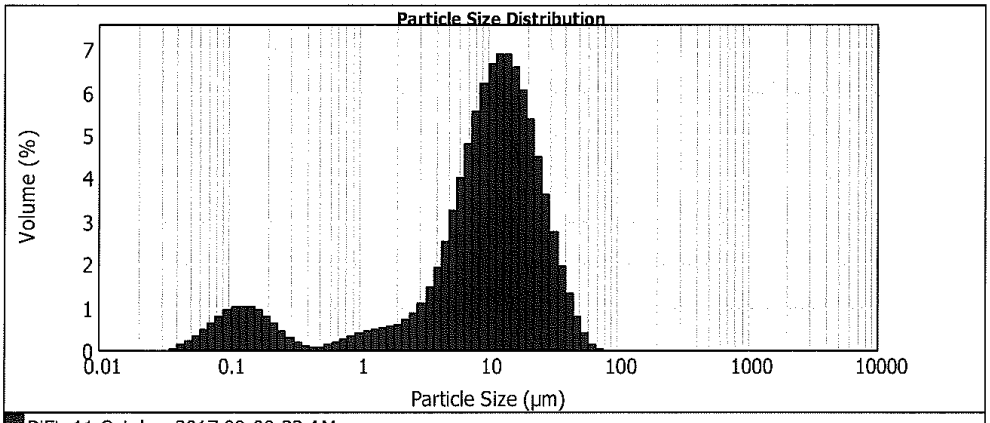
Result Analysis Report

Sample Name: DiEt	**SOP Name:** Mandi Erasmus Okt 17	**Measured:** 11 October 2017 09:00:32 AM
Sample Source & type: ME	**Measured by:** Neil Barnard	**Analysed:** 11 October 2017 09:00:33 AM
Sample bulk lot ref: DiEt (Sample 3)	**Result Source:** Measurement	

Particle Name: Yellow pigment	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.190	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 5.55 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 0.918 %	**Result Emulation:** Off

Concentration: 0.0026 %Vol	**Span :** 2.455	**Uniformity:** 0.726	**Result units:** Volume
Specific Surface Area: 6.12 m²/g	**Surface Weighted Mean D[3,2]:** 0.981 um	**Vol. Weighted Mean D[4,3]:** 13.062 um	

d(0.1): 0.584 um **d(0.5):** 10.854 um **d(0.9):** 27.225 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.99	1.096	0.45	11.482	6.89	120.226	0.00	1258.925	0.00
0.011	0.00	0.120	1.02	1.259	0.49	13.183	6.87	138.038	0.00	1445.440	0.00
0.013	0.00	0.138	1.00	1.445	0.51	15.136	6.58	158.489	0.00	1659.587	0.00
0.015	0.00	0.158	0.92	1.660	0.54	17.378	6.06	181.970	0.00	1905.461	0.00
0.017	0.00	0.182	0.79	1.905	0.59	19.953	5.35	208.930	0.00	2187.762	0.00
0.020	0.00	0.209	0.62	2.188	0.68	22.909	4.51	239.883	0.00	2511.886	0.00
0.023	0.00	0.240	0.45	2.512	0.84	26.303	3.62	275.423	0.00	2884.032	0.00
0.026	0.00	0.275	0.29	2.884	1.09	30.200	2.75	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.17	3.311	1.45	34.674	1.97	363.078	0.00	3801.894	0.00
0.035	0.01	0.363	0.09	3.802	1.92	39.811	1.30	416.869	0.00	4365.158	0.00
0.040	0.13	0.417	0.06	4.365	2.52	45.709	0.78	478.630	0.00	5011.872	0.00
0.046	0.22	0.479	0.07	5.012	3.22	52.481	0.41	549.541	0.00	5754.399	0.00
0.052	0.33	0.550	0.12	5.754	4.00	60.256	0.13	630.957	0.00	6606.934	0.00
0.060	0.47	0.631	0.18	6.607	4.79	69.183	0.02	724.436	0.00	7585.776	0.00
0.069	0.63	0.724	0.26	7.586	5.55	79.433	0.00	831.764	0.00	8709.636	0.00
0.079	0.79	0.832	0.34	8.710	6.19	91.201	0.00	954.993	0.00	10000.000	0.00
0.091	0.91	0.955	0.40	10.000	6.66	104.713	0.00	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

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2017/10/11 09:02:12 AM

Figure C8: *Mastersizer 2000 analysis report for* diethyl ether *S. aethiopicus extract, B*

ANNEXURE D

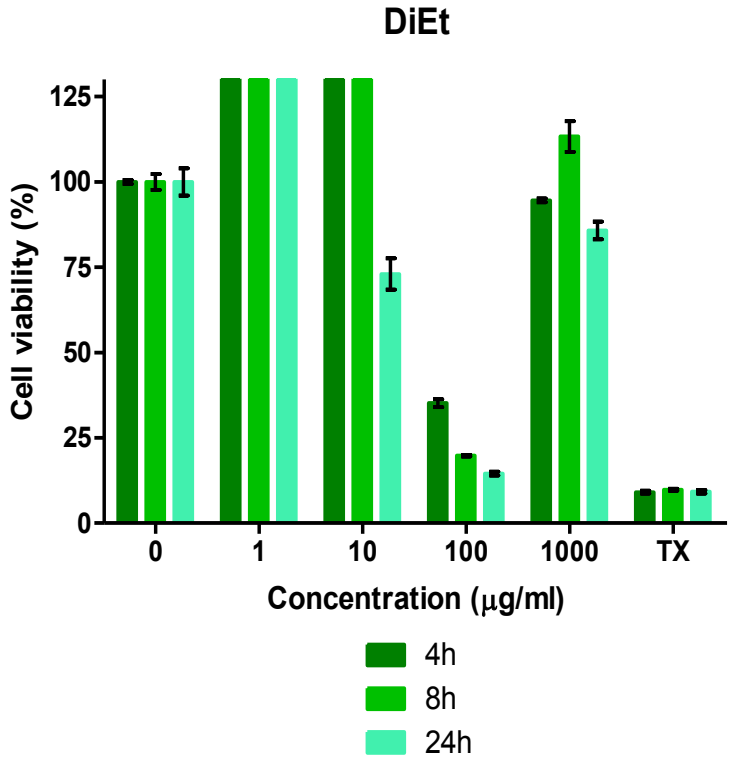


Figure D1: *Concentration range and exposure time optimisation results. HepG2 cells were exposed to diethyl ether extracts (1, 10, 100 and 1000 µg/ml) for 4, 8 and 24 h. 0 µg/ml represents vehicle controls (SFM and cells), whereas TX represents positive controls (cells exposed to 0.4% Triton X-100). Data are presented as means (n=3) ± SEM*

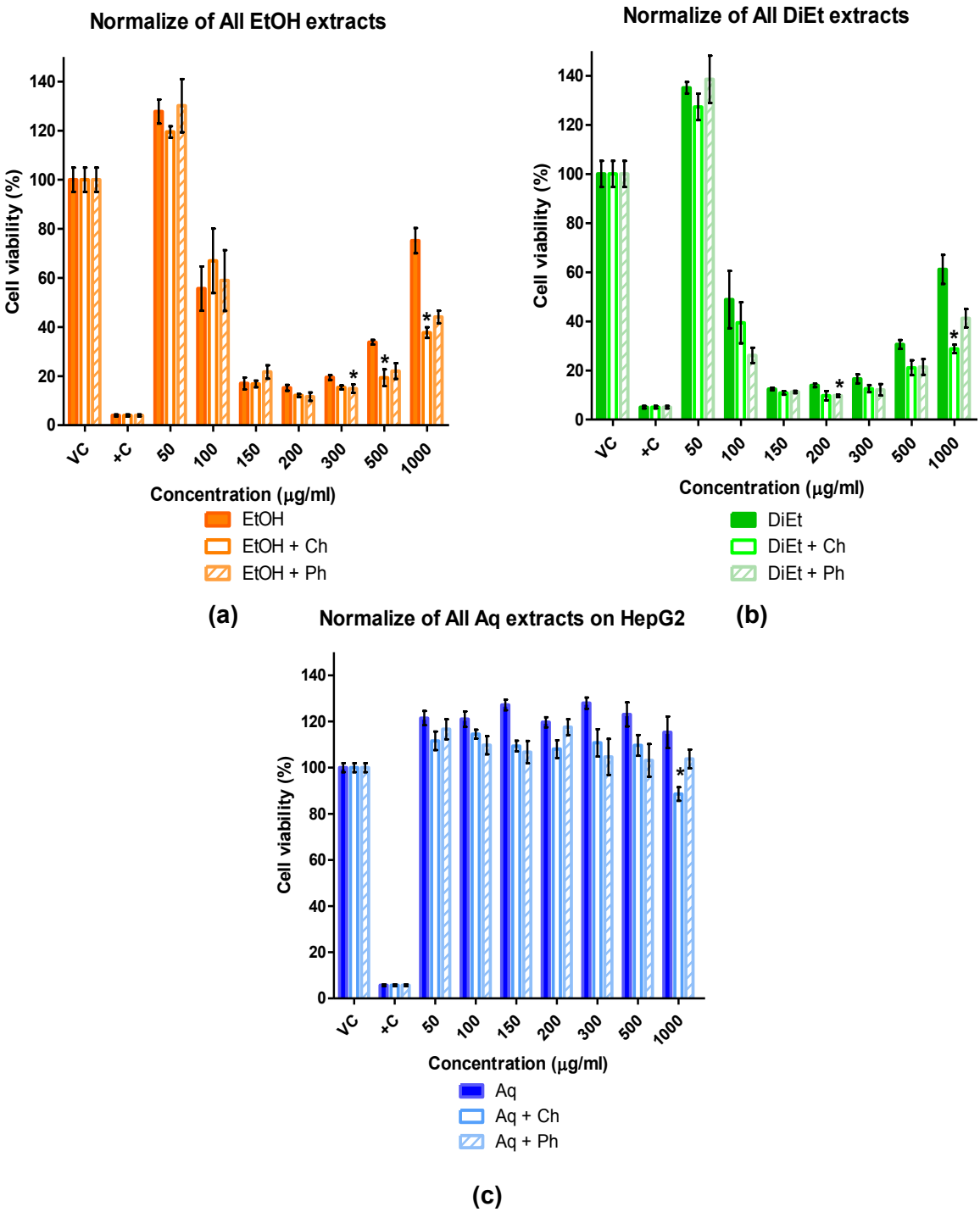


Figure D2: *Effects of* ***(a)*** *ethanol (EtOH),* ***(b)*** *diethyl ether (DiEt) and* ***(c)*** *aqueous (Aq)* *S. aethiopicus* *extracts and their various excipient combinations on the cell viability of HepG2 cells as determined by MTT assay at all concentrations. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

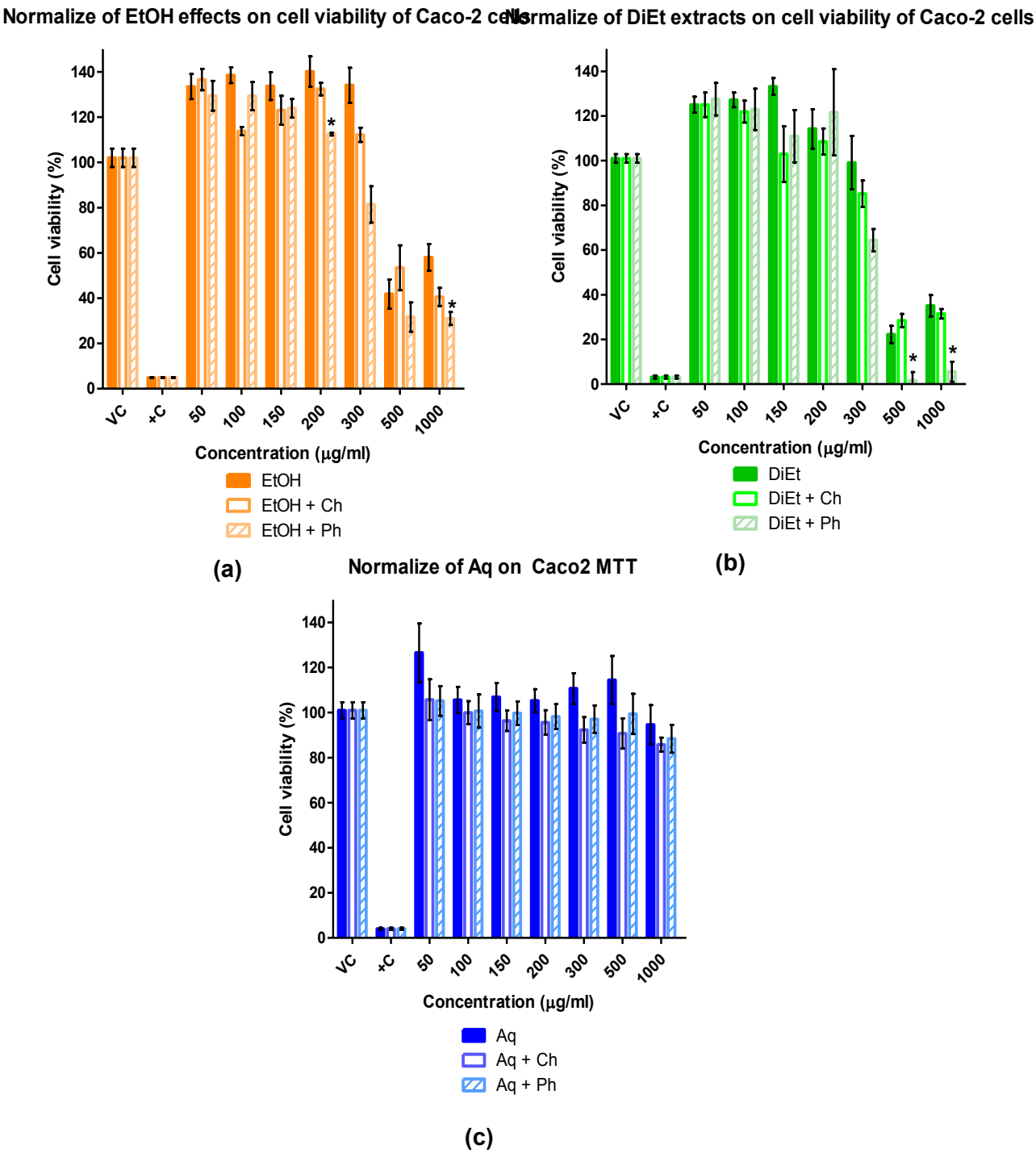


Figure D3: *Effects of* ***(a)*** *ethanol (EtOH),* ***(b)*** *diethyl ether (DiEt) and* ***(c)*** *aqueous (Aq)* S. aethiopicus *extracts and their various excipient combinations on the cell viability of Caco-2 cells as determined by MTT assay at all concentrations. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. VC represents vehicle controls (SFM and cells), whereas +C represents positive controls (cells exposed to 0.4% Triton X-100). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

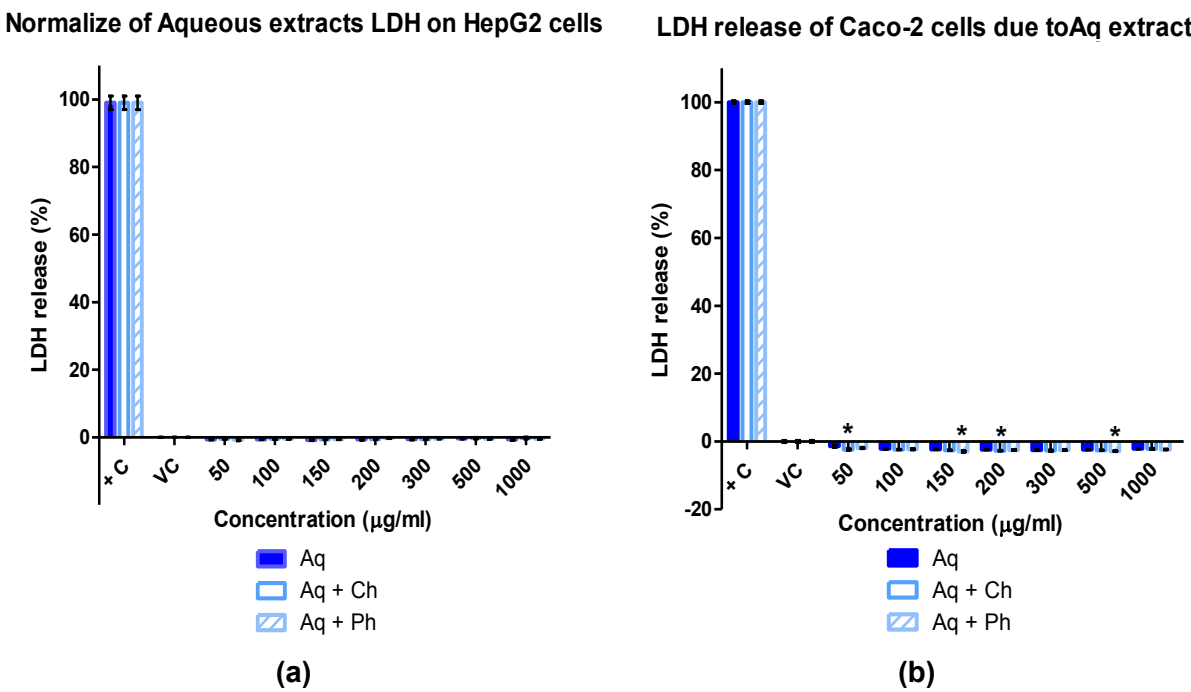


Figure D4: *Effects of aqueous (Aq) S. aethiopicus extracts and their various excipient combinations on LDH release of* ***(a)*** *HepG2 and* ***(b)*** *Caco-2 cells as determined by LDH assay at all test concentrations. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. Positive controls are indicated by +C (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

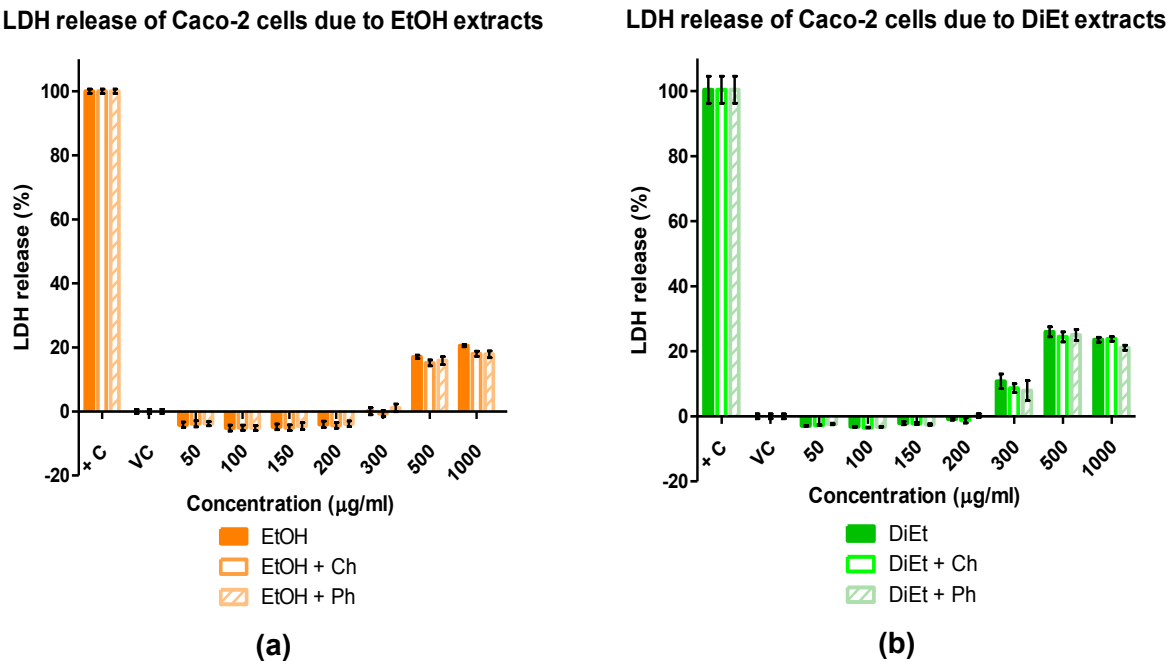


Figure D5: *Effects of **(a)** ethanol (EtOH) and **(b)** diethyl ether (DiEt) S. aethiopicus extracts and their various excipient combinations on LDH release of Caco-2 cells as determined by LDH assay at all test concentrations. Chitosan combinations are represented by + Ch, whereas Pharmacel® 101 combinations are indicated by + Ph. Positive controls are indicated by +C (cells exposed to lysis solution), whereas VC represents vehicle controls (cells exposed to SFM). Data are presented as means (n=6) ± SEM. * represents statistical significant differences with p ≤ 0.05, when extract-excipient combinations are compared to the extract only*

ANNEXURE E

Table E1: *Summary of the statistical analysis performed on the MTT results of all extracts applied to HepG2 cells. For each concentration extract-excipient combinations were selected as the independent variable, whereas the percentage cell viability was the dependant variable. Statistical significance was set at $p \leq 0.05$ for One-way ANOVA and Levene's F-test analysis and is indicated in red*

Statistical analysis summary			
Extract	**Concentration (µg/ml)**	**One-way ANOVA *P*-value**	**Levene's *F*-test *P*-value**
EtOH	50	0.306419	0.037208
	100	0.892679	0.255047
	150	0.251929	0.002301
	200	0.118608	0.247291
	300	0.079253	0.125395
	500	0.004820	0.123147
	1000	0.000004	0.292567
DiEt	50	0.346239	0.185495
	100	0.195010	0.000004
	150	0.190328	0.692965
	200	0.038332	0.001747
	300	0.226918	0.612165
	500	0.44198	0.251755
	1000	0.000227	0.092728
Aq	50	0.641493	0.413641
	100	0.474254	0.918479
	150	0.019446	0.810432
	200	0.021696	0.113317
	300	0.085823	0.024147
	500	0.070338	0.218184
	1000	0.004000	0.362816

Table E2: *Summary of the statistical analysis performed on the MTT results of all extracts applied to Caco-2 cells. For each concentration extract-excipient combinations were selected as the independent variable, whereas the percentage cell viability was the dependant variable. Statistical significance was set at $p \leq 0.05$ for One-way ANOVA and Levene's F-test analysis and is indicated in red*

Statistical analysis summary			
Extract	**Concentration (µg/ml)**	**One-way ANOVA *P*-value**	**Levene's *F*-test *P*-value**
EtOH	50	0.677986	0.650634
	100	0.269834	0.293764
	150	0.358309	0.285211
	200	0.000115	0.009034
	300	0.066604	0.136534
	500	0.174510	0.166623
	1000	0.001950	0.154058
DiEt	50	0.936322	0.197685
	100	0.827588	0.002358
	150	0.123043	0.000014
	200	0.929805	0.000357
	300	0.028438	0.003057
	500	0.000075	0.143844
	1000	0.000112	0.071646
Aq	50	0.251147	0.204776
	100	0.781663	0.318710
	150	0.387336	0.486737
	200	0.439955	0.993208
	300	0.126763	0.917036
	500	0.197011	0.102893
	1000	0.614016	0.011792

Table E3: *Summary of the statistical analysis performed on the LDH results of all extracts applied to HepG2 cells. For each concentration extract-excipient combinations were selected as the independent variable, whereas the percentage cell viability was the dependant variable. Statistical significance was set at $p \leq 0.05$ for One-way ANOVA and Levene's F-test analysis and is indicated in red*

Statistical analysis summary			
Extract	**Concentration (µg/ml)**	**One-way ANOVA *P*-value**	**Levene's *F*-test *P*-value**
EtOH	50	0.292859	0.064056
	100	0.305234	0.000000
	150	0.678234	0.609023
	200	0.316980	0.107107
	300	0.828733	0.383182
	500	0.872447	0.369294
	1000	0.446284	0.193369
DiEt	50	0.042701	0.000345
	100	0.378420	0.283508
	150	0.732011	0.480410
	200	0.295910	0.006192
	300	0.983405	0.395566
	500	0.932509	0.408573
	1000	0.664631	0.244808
Aq	50	0.737136	0.273731
	100	0.644426	0.034175
	150	0.527488	0.330425
	200	0.237108	0.233203
	300	0.508466	0.522349
	500	0.818713	0.083862
	1000	0.546253	0.011197

Table E4: *Summary of the statistical analysis performed on the LDH results of all extracts applied to Caco-2 cells. For each concentration extract-excipient combinations were selected as the independent variable, whereas the percentage cell viability was the dependant variable. Statistical significance was set at $p \leq 0.05$ for One-way ANOVA and Levene's F-test analysis and is indicated in red*

Statistical analysis summary			
Extract	**Concentration (µg/ml)**	**One-way ANOVA *P*-value**	**Levene's *F*-test *P*-value**
EtOH	50	0.947318	0.113158
	100	0.997685	0.173965
	150	0.929883	0.221982
	200	0.929829	0.696131
	300	0.542221	0.550837
	500	0.397605	0.197560
	1000	0.046926	0.004563
DiEt	50	0.060442	0.484785
	100	0.296658	0.188414
	150	0.713374	0.081858
	200	0.215870	0.164607
	300	0.666220	0.285629
	500	0.788332	0.972434
	1000	0.040612	0.816925
Aq	50	0.028381	0.269887
	100	0.489573	0.487414
	150	0.009697	0.021491
	200	0.131337	0.065669
	300	0.618959	0.399427
	500	0.008067	0.008438
	1000	0.227112	0.103411

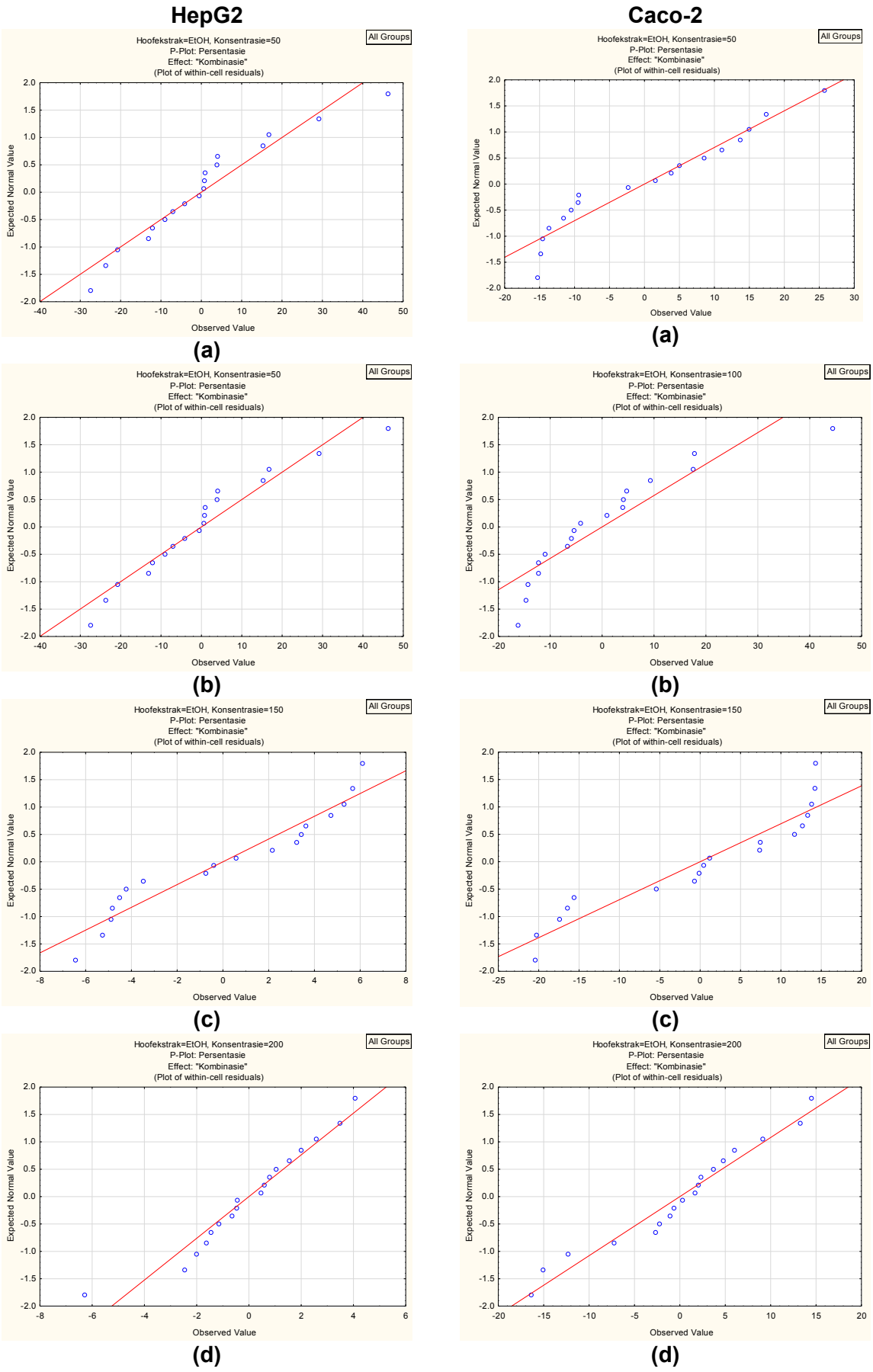


Figure E1: *P-plot distributions as a graphical indication of normality of ethanol extract MTT data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

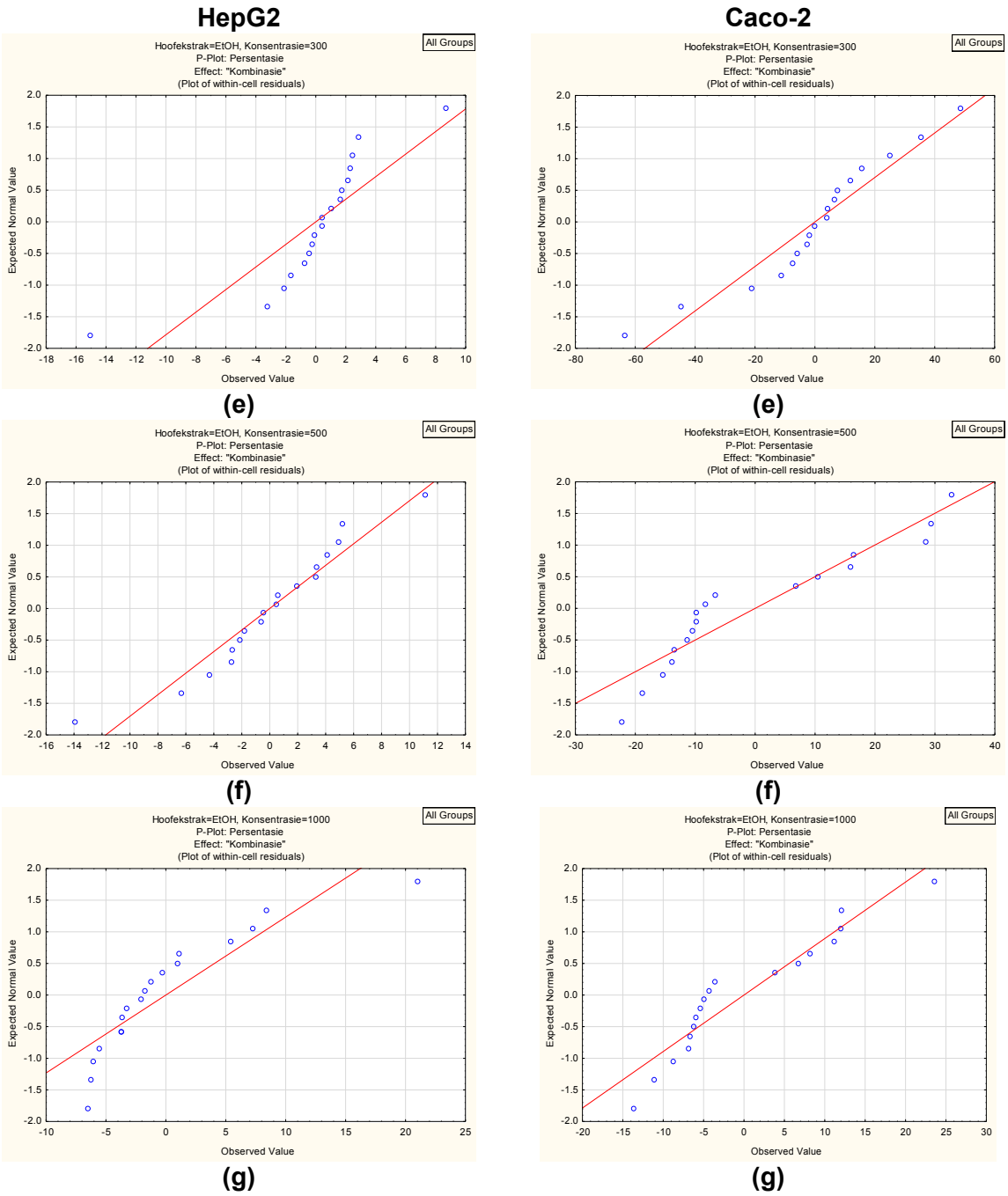


Figure E1: *P-plot distributions as a graphical indication of normality of ethanol extract MTT data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

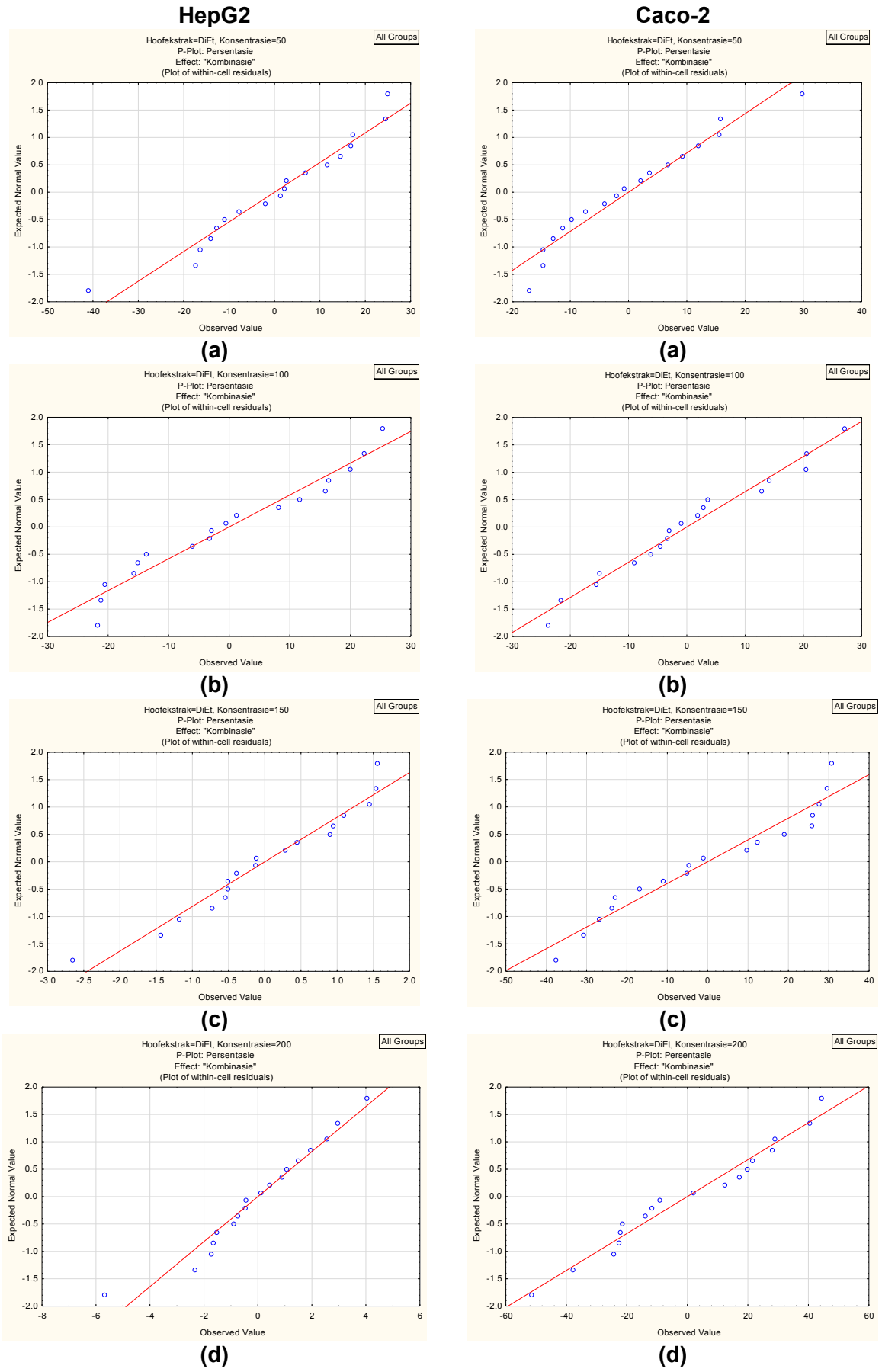


Figure E2: *P-plot distributions as a graphical indication of normality of diethyl ether extract data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

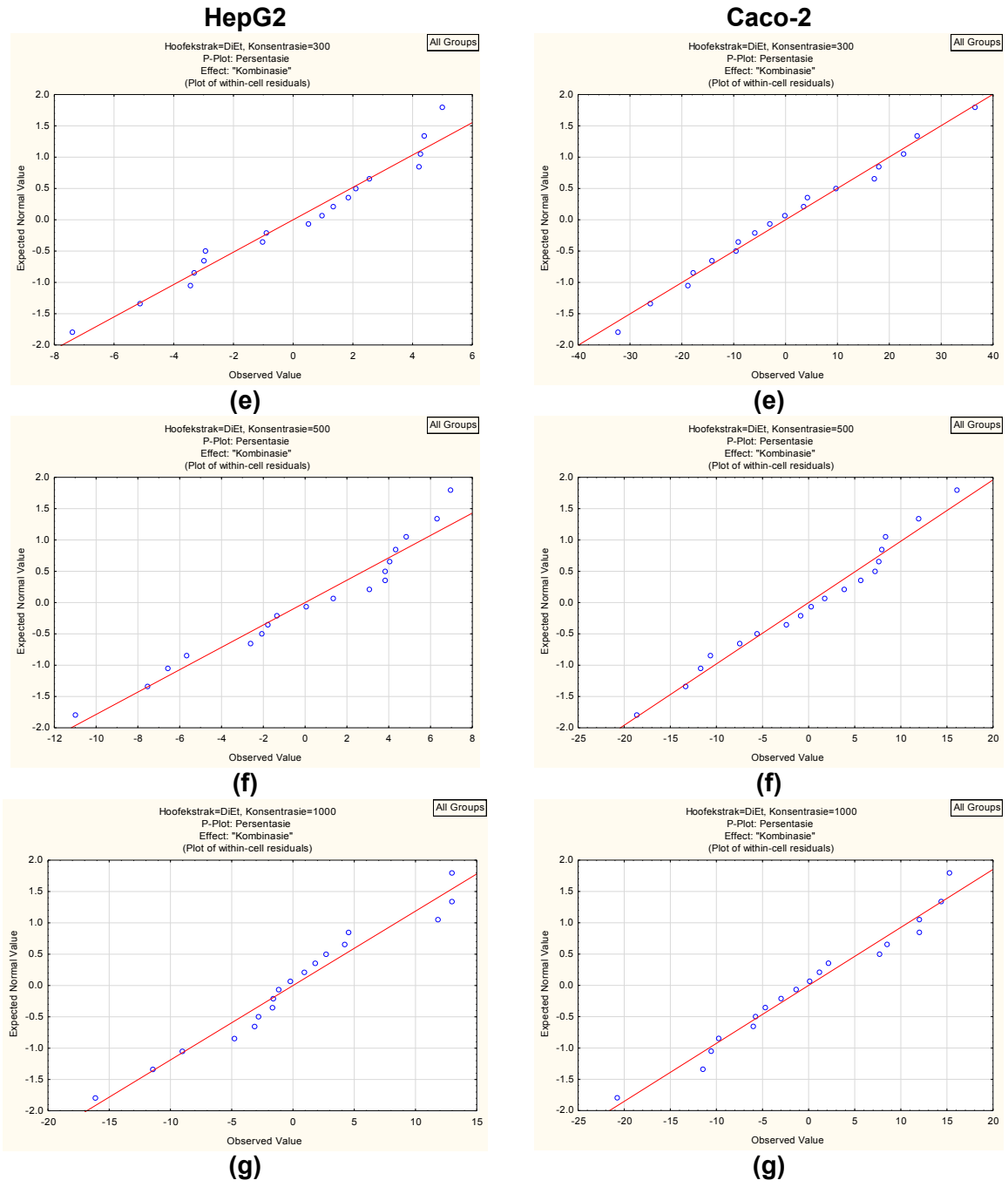
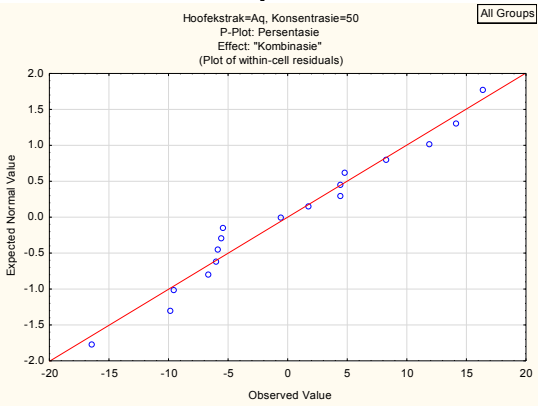
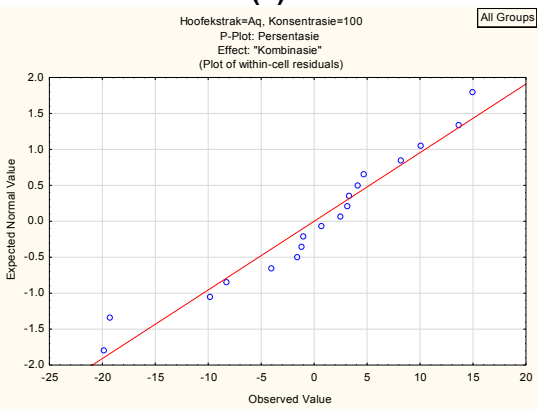


Figure E2: *P-plot distributions as a graphical indication of normality of diethyl ether extract MTT data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

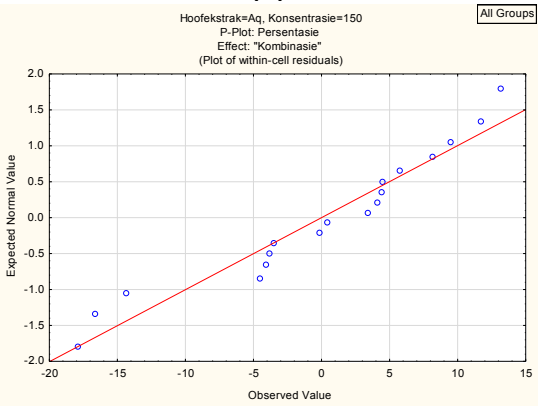
HepG2



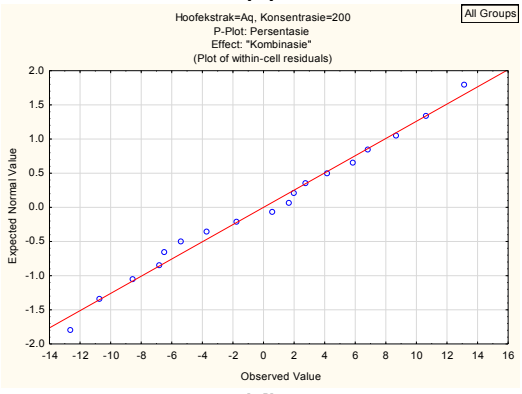
(a)



(b)

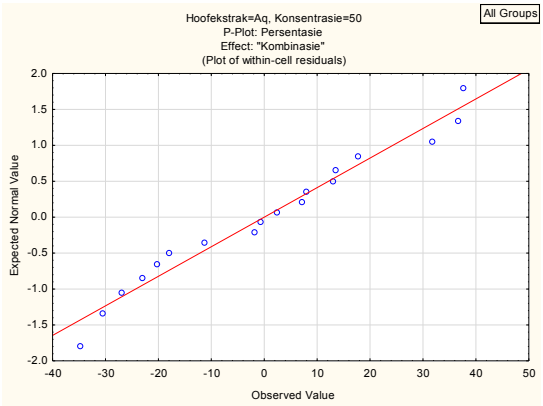


(c)

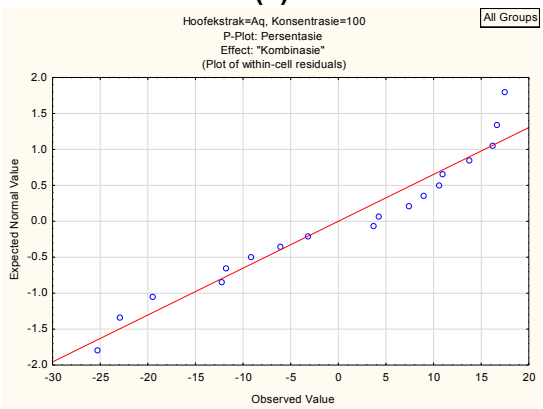


(d)

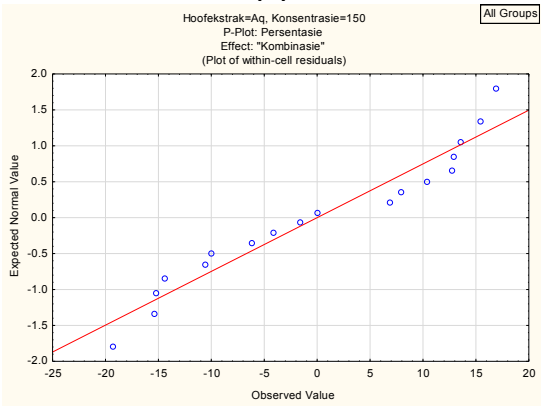
Caco-2



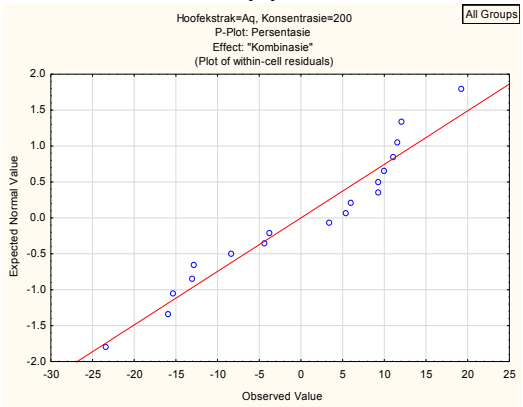
(a)



(b)



(c)



(d)

Figure E3: *P-plot distributions as a graphical indication of normality of aqueous extract MTT data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

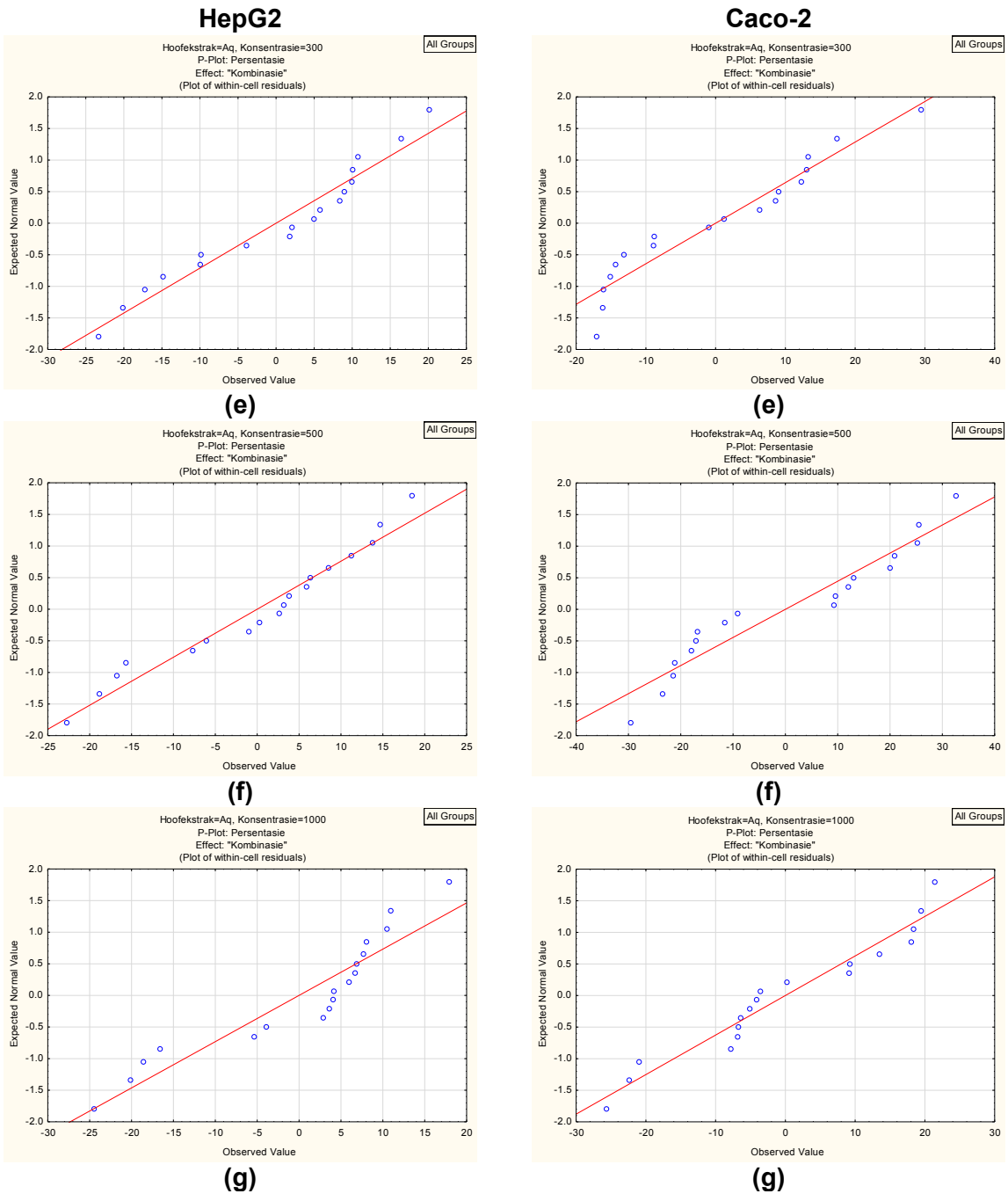


Figure E3: *P-plot distributions as a graphical indication of normality of aqueous extract MTT data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

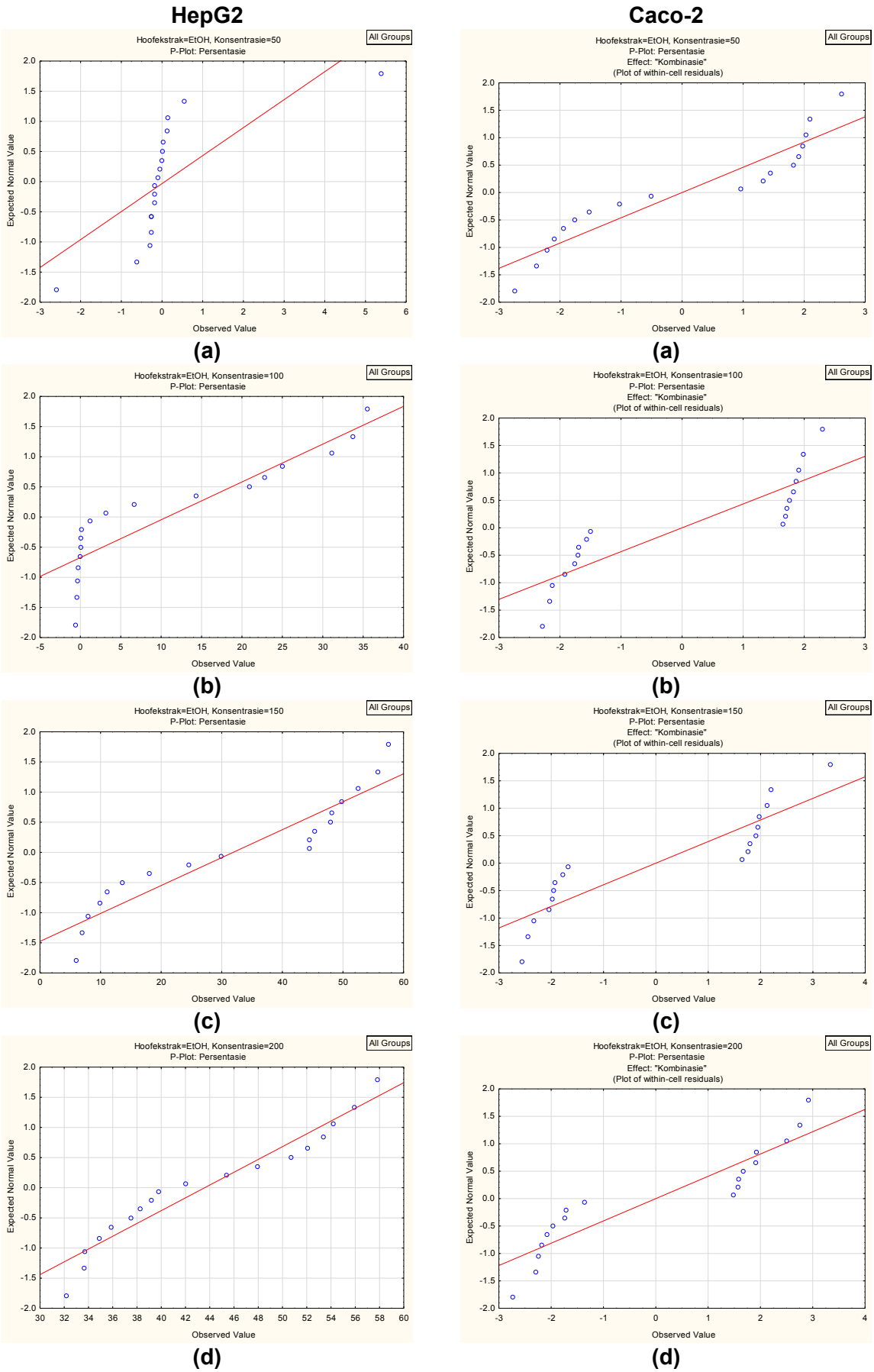


Figure E4: *P-plot distributions as a graphical indication of normality of ethanol extract LDH data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

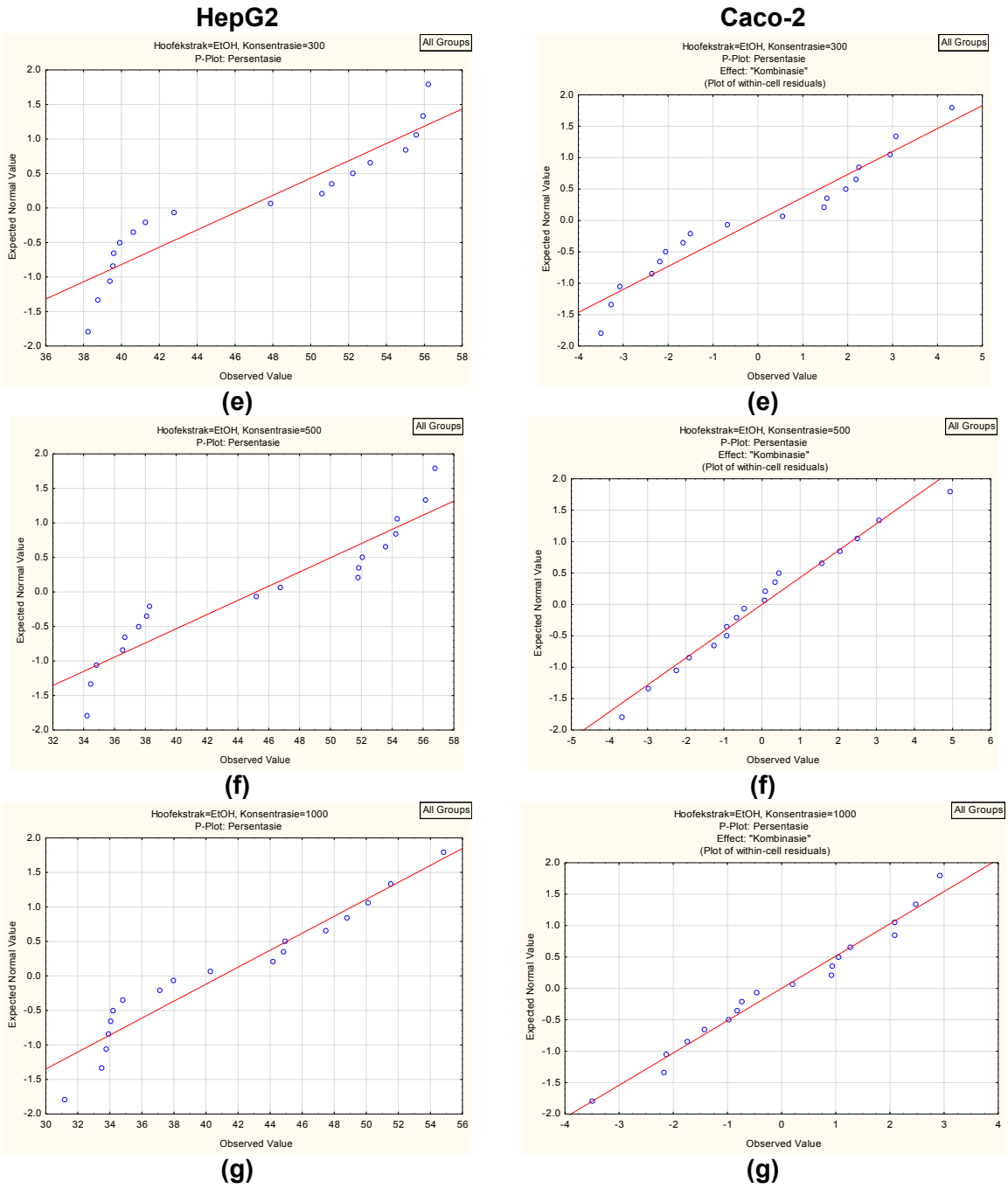


Figure E4: *P-plot distributions as a graphical indication of normality of ethanol extract LDH data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

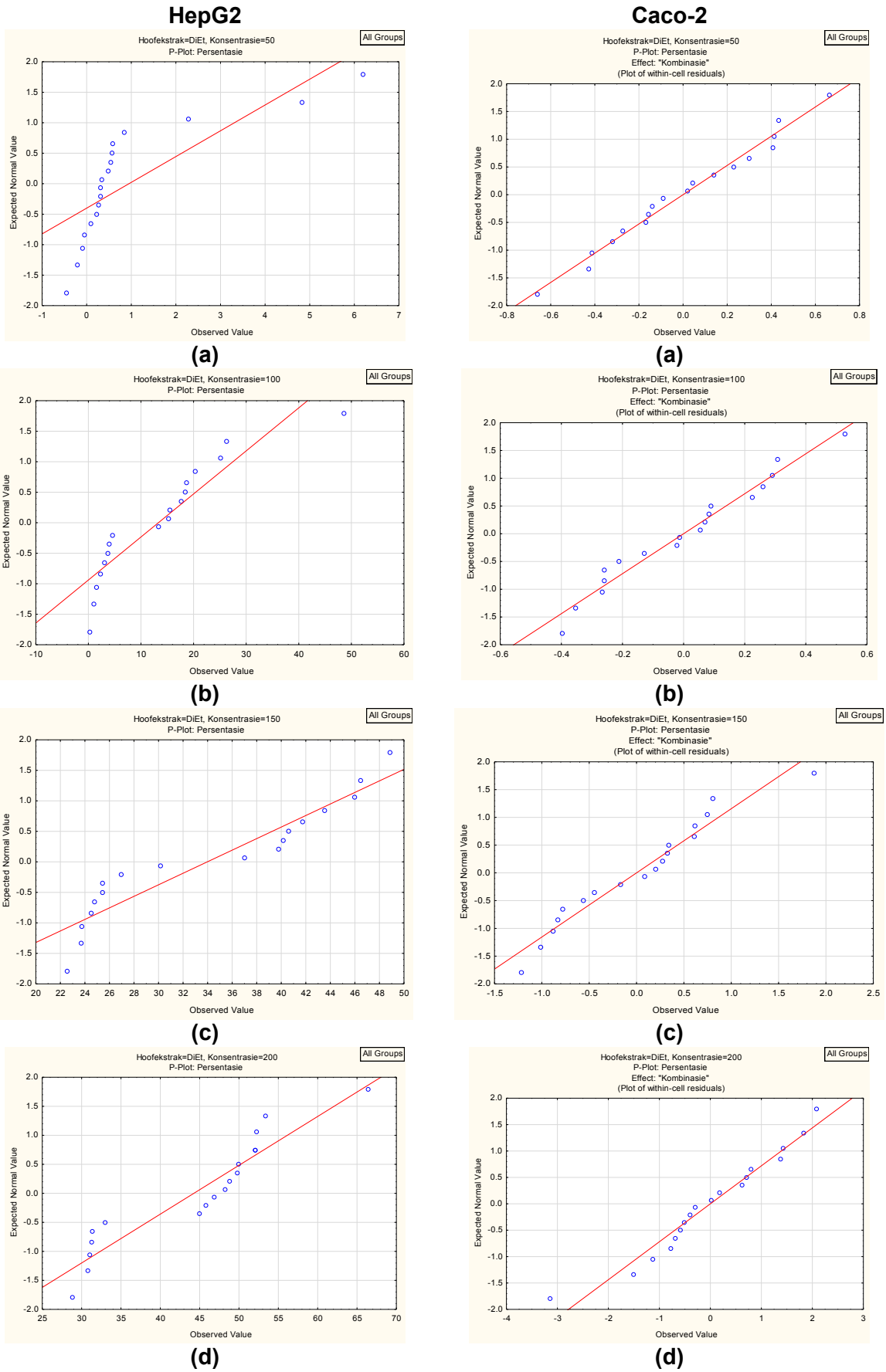


Figure E5: *P-plot distributions as a graphical indication of normality of diethyl ether extract LDH data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

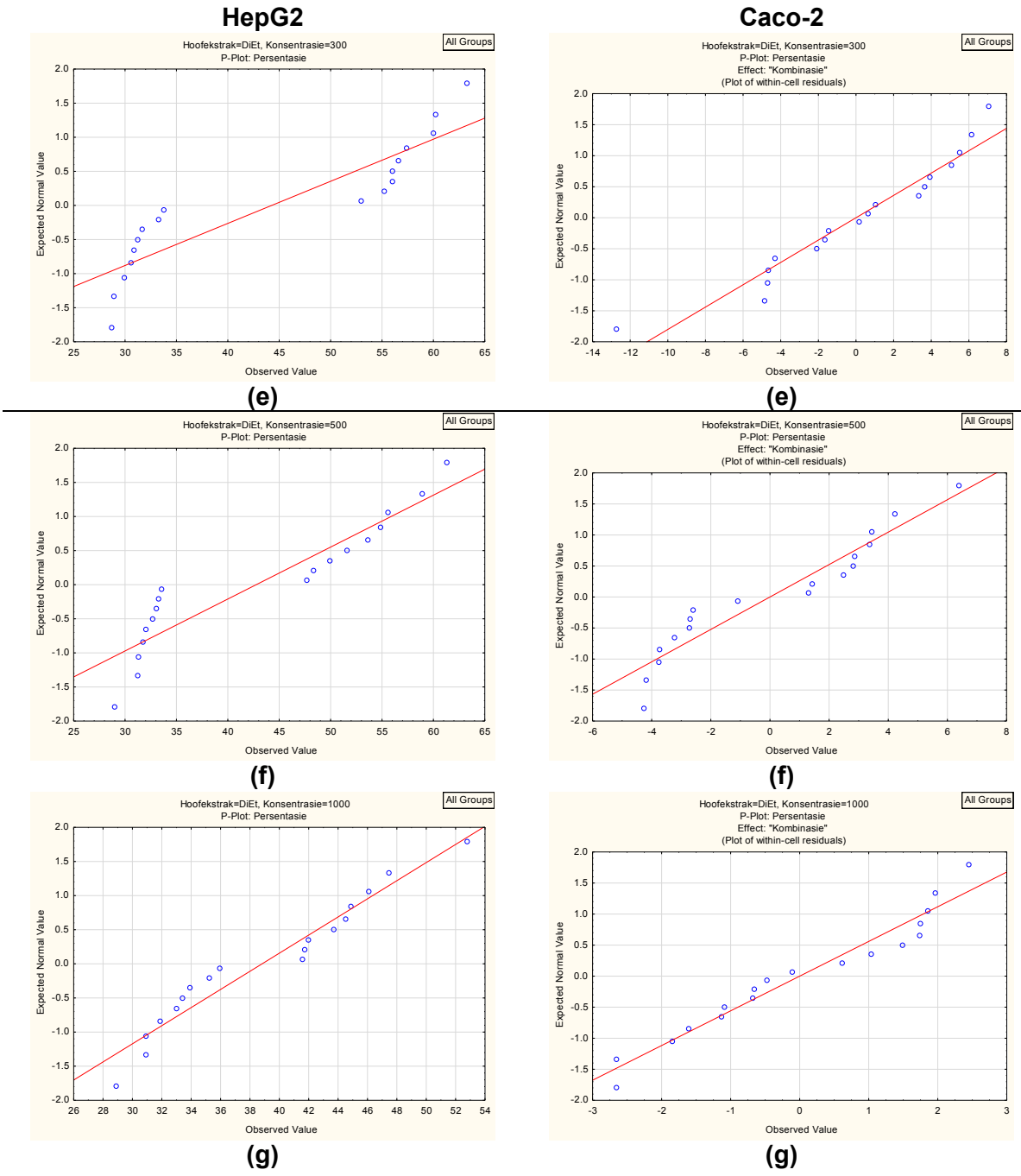


Figure E5: *P-plot distributions as a graphical indication of normality of diethyl ether extract LDH data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

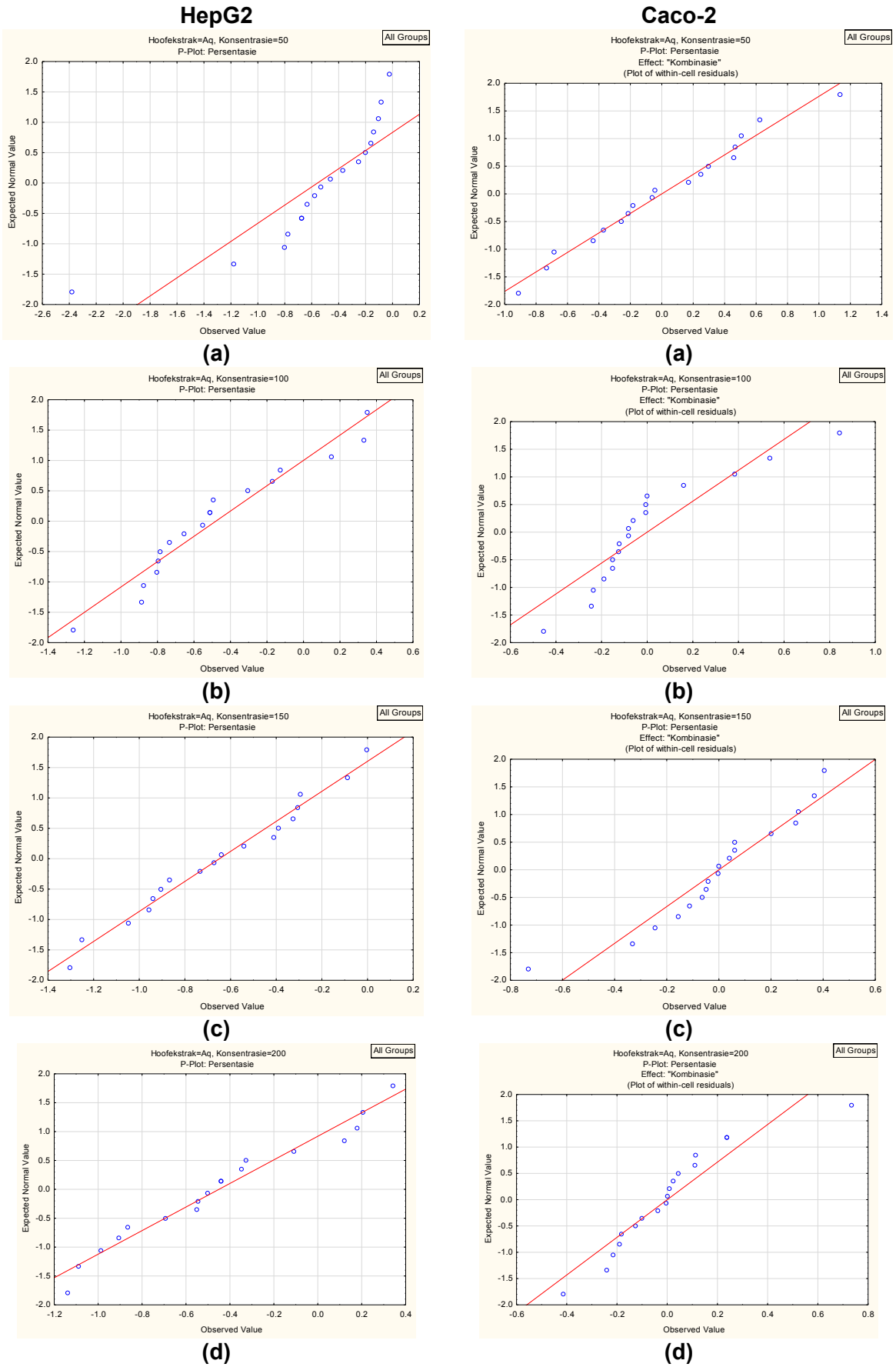


Figure E6: *P-plot distributions as a graphical indication of normality of aqueous extract LDH data on HepG2 and Caco-2 cells, individually, at (a) 50, (b) 100, (c) 150 and (d) 200 µg/ml*

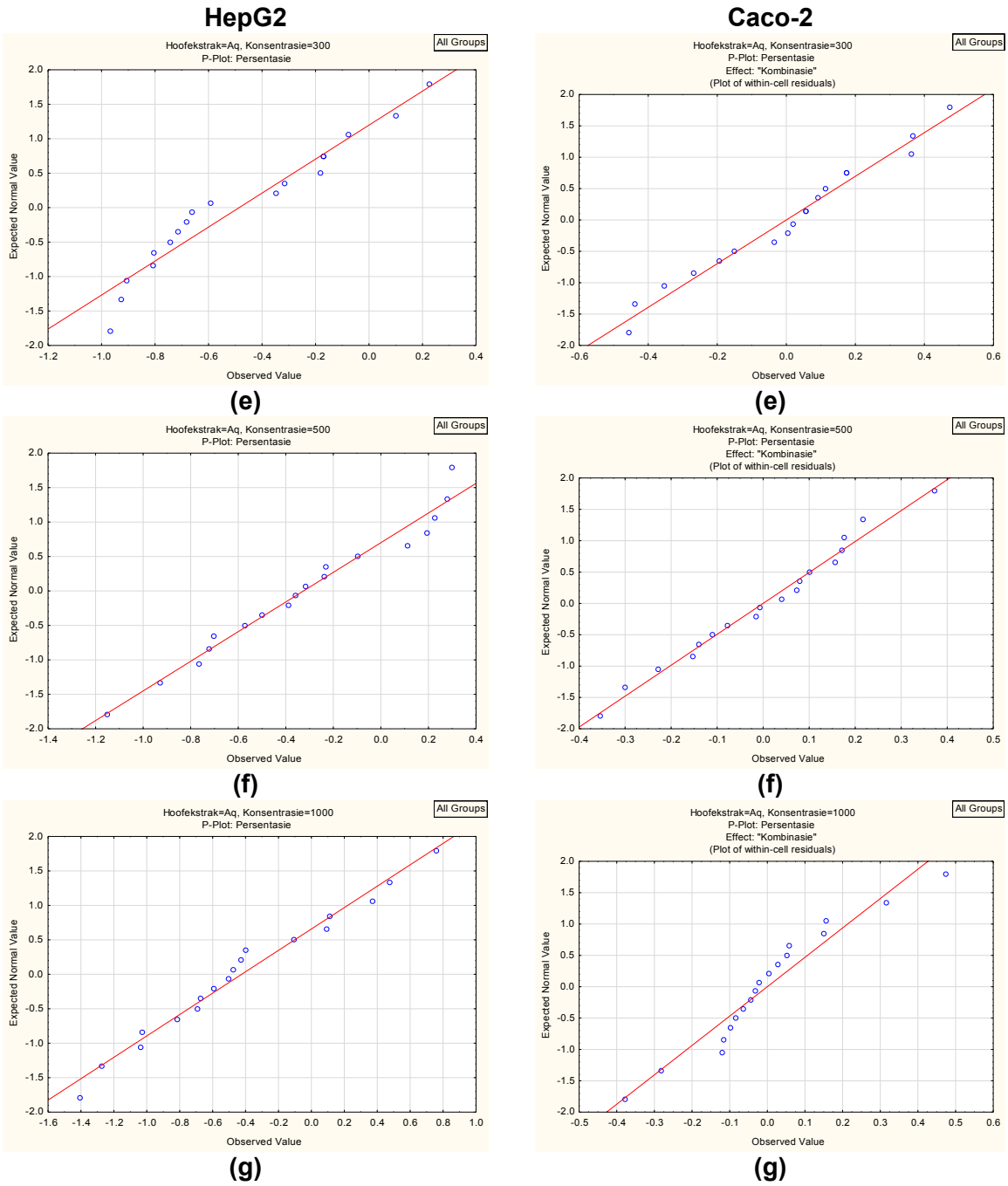


Figure E6: *P-plot distributions as a graphical indication of normality of aqueous extract LDH data on HepG2 and Caco-2 cells, individually, at (e) 300, (f) 500 and (g) 1000 µg/ml (continued)*

Table E5: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 300 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=300 Multiple Comparisons p values (2-tailed); Persentasie (Hep(Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =7.298246 p =.0260		
Depend.: Persentasie	1 R:14.167	2 R:8.1667	3 R:6.1667
1		0.154728	0.028332
2	0.154728		1.000000
3	0.028332	1.000000	

Table E6: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 500 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=500 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =8.502924 p =.0142		
Depend.: Persentasie	1 R:14.667	2 R:6.5000	3 R:7.3333
1		0.024175	0.052044
2	0.024175		1.000000
3	0.052044	1.000000	

Table E7: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 500 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=500 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =8.502924 p =.0142		
Depend.: Persentasie	1 R:14.667	2 R:6.5000	3 R:7.3333
1		0.024175	0.052044
2	0.024175		1.000000
3	0.052044	1.000000	

Table E8: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 1000 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=1000 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =13.06612 p =.0015		
Depend.: Persentasie	1 R:15.500	2 R:4.5000	3 R:8.5000
1		0.00107(	0.06942:
2	0.00107(		0.58309(
3	0.06942:	0.58309(	

Table E9: *A Kruskal-Wallis test performed on MTT data of diethyl ether (1), diethyl ether and chitosan (2) as well as diethyl ether and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 200 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=DiEt, Konsentrasie=200 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =7.064327 p =.0292		
Depend.: Persentasie	1 R:14.167	2 R:7.8333	3 R:6.5000
1		0.11969<	0.03860!
2	0.11969<		1.00000(
3	0.03860!	1.00000(	

Table E10: *A Kruskal-Wallis test performed on MTT data of diethyl ether (1), diethyl ether and chitosan (2) as well as diethyl ether and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 1000 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=DiEt, Konsentrasie=1000 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =13.13450 p =.0014		
Depend.: Persentasie	1 R:15.000	2 R:3.8333	3 R:9.6667
1		0.00087<	0.25069;
2	0.00087<		0.17524(
3	0.25069;	0.17524(	

Table E11: *A Kruskal-Wallis test performed on MTT data of aqueous (1), aqueous and chitosan (2) as well as aqueous and Pharmacel® 101 (3) extract combinations on HepG2 cells at a concentration of 1000 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=Aq, Konsentrasie=1000 Multiple Comparisons p values (2-tailed); Persentasie (He Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =8.842105 p =.0120		
Depend.: Persentasie	1 R:13.500	2 R:4.5000	3 R:10.500
1		0.01050	0.99117(
2	0.01050		0.15472
3	0.99117(	0.15472	

Table E12: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 200 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=200 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_MTT) Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =11.09942 p =.0039		
Depend.: Persentasie	1 R:14.167	2 R:10.333	3 R:4.0000
1		0.64083	0.00291
2	0.64083		0.11969
3	0.00291	0.11969	

Table E13: *A Kruskal-Wallis test performed on MTT data of ethanol (1), ethanol and chitosan (2) as well as ethanol and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 1000 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=EtOH, Konsentrasie=1000 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_MT Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =9.204678 p =.0100		
Depend.: Persentasie	1 R:14.333	2 R:9.1667	3 R:5.0000
1		0.28104	0.00738
2	0.28104		0.52927
3	0.00738	0.52927	

Table E14: *A Kruskal-Wallis test performed on MTT data of diethyl ether (1), diethyl ether and chitosan (2) as well as diethyl ether and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 500 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=DiEt, Konsentrasie=500 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_MTT) Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =11.94152 p =.0026		
Depend.: Persentasie	1 R:11.333	2 R:13.667	3 R:3.5000
1		1.00000(	0.03311(
2	1.00000(		0.00291(
3	0.03311(	0.00291(	

Table E15: *A Kruskal-Wallis test performed on MTT data of diethyl ether (1), diethyl ether and chitosan (2) as well as diethyl ether and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 1000 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=DiEt, Konsentrasie=1000 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_MTT) Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =11.36842 p =.0034		
Depend.: Persentasie	1 R:12.500	2 R:12.500	3 R:3.5000
1		1.00000(	0.01050'
2	1.00000(		0.01050'
3	0.01050'	0.01050'	

Table E16: *A Kruskal-Wallis test performed on LDH data of aqueous (1), aqueous and chitosan (2) as well as aqueous and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 50 µg/ml. Statistical significance ($p \leq 0.05$) is indicated in red*

	Hoofekstrak=Aq, Konsentrasie=50 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_LDH) Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =6.877193 p =.0321		
Depend.: Persentasie	1 R:13.167	2 R:5.1667	3 R:10.167
1		0.02833(	0.99117(
2	0.02833(		0.31427(
3	0.99117(	0.31427(	

Table E17: *A Kruskal-Wallis test performed on LDH data of aqueous (1), aqueous and chitosan (2) as well as aqueous and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 150 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=Aq, Konsentrasie=150 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_L Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =9.274854 p =.0097		
Depend.: Persentasie	1 R:14.667	2 R:8.3333	3 R:5.5000
1		0.11969	0.00881
2	0.11969		1.00000
3	0.00881	1.00000	

Table E18: *A Kruskal-Wallis test performed on LDH data of aqueous (1), aqueous and chitosan (2) as well as aqueous and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 200 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=Aq, Konsentrasie=200 Multiple Comparisons p values (2-tailed); Persentasie (Caco2_L Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =6.474518 p =.0393		
Depend.: Persentasie	1 R:13.333	2 R:5.5000	3 R:9.6667
1		0.03311	0.70258
2	0.03311		0.52927
3	0.70258	0.52927	

Table E19: *A Kruskal-Wallis test performed on LDH data of aqueous (1), aqueous and chitosan (2) as well as aqueous and Pharmacel® 101 (3) extract combinations on Caco-2 cells at a concentration of 500 µg/ml. Statistical significance (p ≤ 0.05) is indicated in red*

	Hoofekstrak=Aq, Konsentrasie=500 Multiple Comparisons p values (2-tailed); Persentasie (Cac Independent (grouping) variable: Kombinasie Kruskal-Wallis test: H (2, N= 18) =9.309942 p =.0095		
Depend.: Persentasie	1 R:14.500	2 R:8.8333	3 R:5.1667
1		0.19796	0.00738
2	0.19796		0.70258
3	0.00738	0.70258	

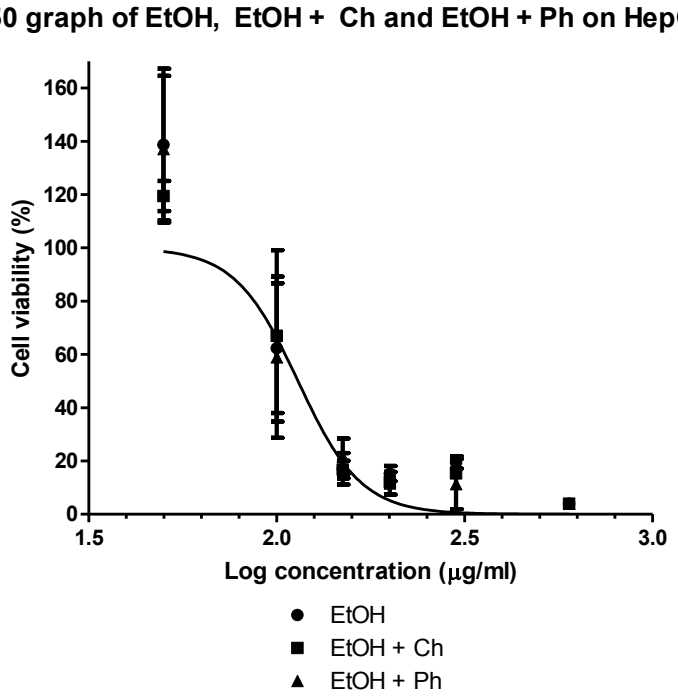


Figure E7: *Determination of IC_{50}-values of ethanol, ethanol and chitosan, as well as ethanol and Pharmacel® 101 extract-excipient combinations on HepG2 cells. By means of nonlinear regression, data sets were analysed simultaneously and the curve fitted*

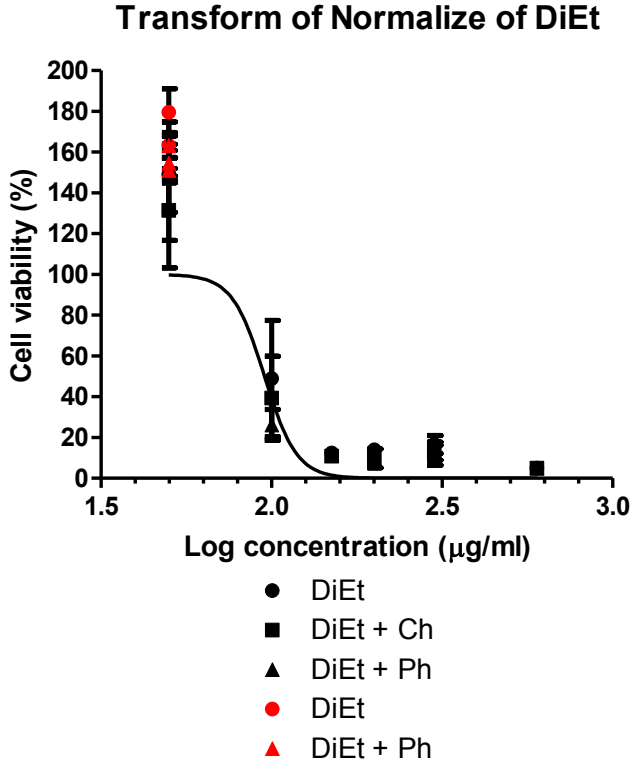


Figure E8: *Determination of IC_{50}-values of diethyl ether, diethyl ether and chitosan, as well as diethyl ether and Pharmacel® 101 extract-excipient combinations on HepG2 cells. By means of nonlinear regression, data sets were analysed simultaneously and the curve fitted. Outliers are indicated in red*

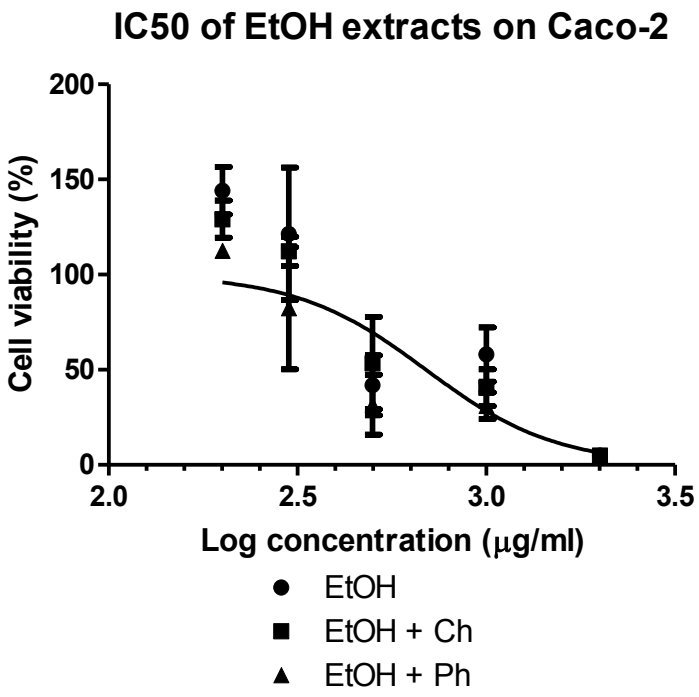


Figure E9: *Determination of IC_{50}-values of ethanol, ethanol and chitosan, as well as ethanol and Pharmacel® 101 extract-excipient combinations on Caco-2 cells. By means of nonlinear regression, data sets were analysed simultaneously and the curve fitted*

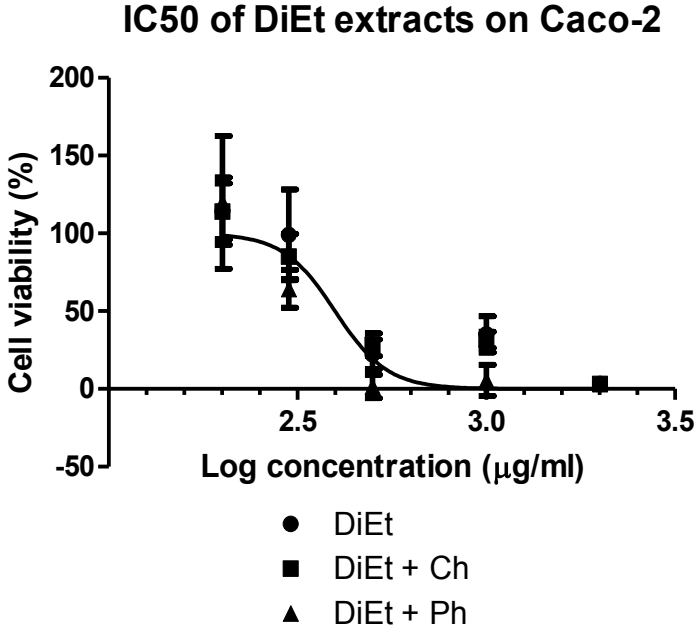


Figure E10: *Determination of IC_{50}-values of diethyl ether, diethyl ether and chitosan, as well as diethyl ether and Pharmacel® 101 extract-excipient combinations on Caco-2 cells. By means of nonlinear regression, data sets were analysed simultaneously and the curve fitted. Outliers are indicated in red*

ANNEXURE F

NORTH-WEST UNIVERSITY
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Faculty of Health Sciences
Health Sciences Ethics Office for Research, Training and Support
Health Research Ethics Committee (HREC)

Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

28 September 2016

Dr JM Viljoen
Pharmaceutics

Dear Dr Viljoen

APPROVAL OF YOUR APPLICATION BY THE HEALTH RESEARCH ETHICS COMMITTEE (HREC) OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00335-16-S1

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Study title: ***In vitro cytotoxicity of Siphonochilus aethiopicua*** **in combination with selected fillers for tableting**

Study leader/supervisor: Dr JM Viljoen

Student: M Erasmus

Application type: Single study

Risk level: Minimal

You are kindly informed that your application was reviewed *via* the expedited process of the HREC, Faculty of Health Sciences, and was approved on 28/09/2016.

The commencement date for this study is 28/09/2016 dependent on fulfilling the conditions indicated below. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC, Faculty of Health Sciences (if applicable).

The HREC, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the HREC, Faculty of Health Sciences prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form at Ethics-HRECIncident-SAE@nwu.ac.za.

A monitoring report should be submitted within one year of approval of this study (or as otherwise stipulated) and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-Monitoring@nwu.ac.za. Annually a number of studies may be randomly selected for an external audit.

Please note that the HREC, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECApply@nwu.ac.za.

Yours sincerely

Dr Wayne Towers
HREC Chairperson

Prof Minrie Greeff
Ethics Office Head

Current details: (132105/72) C:\Users\132105\72\Documents\HREC\HREC - Applications\2016 Applications\Applications 09-20 October 2016\NWU-00335-16-S1(JM Viljoen-M Erasmus)\NWU-00335-16-S1(JM Viljoen-M Erasmus)-AL\NWU-00335-16-S1(JM Viljoen-M Erasmus)-AL.docm
29 September 2016

File reference: 9.1.5.3

ANNEXURE G

DECLARATION BY THE LANGUAGE EDITOR

17 October 2017

To whom it may concern,

I, Julia Handford, herewith declare that I have partly (Chapters 1, 2 and 5) language edited the dissertation of **Mandi Erasmus** that is titled **"*In vitro* cytotoxicity of *Siphonochilus aethiopicus* in combination with selected fillers for tableting".**

Yours truly,

Signature and credentials of language editor
JULIA S HANDFORD [MBA, BCom (Acc), BSc (Hons), HED]