Pharmacokinetic interactions of *Aloe vera* gel polysaccharides with indinavir

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Vir Albert

Chan eil tuil air nach tig traoghadh...

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Appendix A

List of Abbreviations

A

A549 Folate receptor deficient cell line

AIDS Acquired immunodeficiency syndrome

ANOVA Analysis of variance

ARV's Anti-retroviral drug

ATP Adenosine tri-phosphate

AUC Area under the curve

AVG Aloe vera gel

B

BCS Biopharmaceutics Classification System

C

C_{max} Maximum plasma concentration

Caco-2 Human colonic carcinoma cell line

CAD Collision gas

CE Collision energy

CEP Collision cell entrance potential

CO_2 Carbon dioxide

CODES™ Colon targeted delivery system

CPP Crude precipitated polysaccharide

CPP Cell penetrating peptides

CSK Tyrosine-protein kinase

CUR Curtain gas

CXP Collision cell exit potential

List of Abbreviations

D

D2O Deuterium oxide

DDAVP Desmopressin acetate

DDM N-dedocyl-ß-D-maltoside

DMEM Dulbecco's modified eagle's medium

DNA Deoxyribonucleic acid

dn/dc Specific refractive increment

DP Declustering potential

E

EDTA Ethylenediaminetetraacetic acid

EP Entrance potential

ER Enhancement ratio

ESI Electronspray ionisation

F

FDA Food and drug administration

F_{rel} Relative bioavailablity

FSH Follicle-stimulating hormone

G

GAS1 Nebuliser gas

GAS2 Turbo gas

GFC Gel filtration chromatography

GPL Glucagon-like peptide-1 receptor agonists

List of Abbreviations

H

HbA1c Glycated haemoglobin A1c

hGH Human growth hormone

HIM2 Hexal insulin monoconjugate 2

HIV/HIV1 Human immunodeficiency virus

1H-NMR Proton nuclear magnetic resonance

HCl Hydrochloric acid

HDV Hepatic-directed vesicles insulin

HFS High frequency ultrasound

HIM-2 Hexyl-insulin mono-conjugate 2

HPLC High performance liquid chromatography

hPTH Human parathyroid hormone

HSV-1 Herpes simplex virus

I

IN-105 Oral prandial insulin

ISTD Indinavir-d6 internal standard

K

KB Keratin forming tumour cell line

L

LC-MS Liquid chromatography mass spectrometry

LFS Low frequency ultrasound

LS-180 Human epithelial cell line

List of Abbreviations

M

M6 Indinavir metabolite

MALLS Multi angle laser light scattering

MDR1 Multidrug resistant protein 1

M_n Weight number absolute molecular weight

MRM Multiple reaction monitoring

MS Mass spectrometer

M_w Weight absolute molecular weight

MWF Molecular weight fraction

N

NEAA Non-essential amino acids

P

PBS Phosphate buffer solution

PEG Polyethylene glycol

PEPT Peptide transporter

P-gp P-glycoprotein

PHT Phosphate transporter

PTH Parathyroid hormone

R

REAL Reversible aqueous lipidisation

RI Refractive index

%RSD Percentage relative standard deviation

List of Abbreviations

S

SC Stratum Corneum

sCT Salmon calcitonin

SKOV Sloan Kettering ovarian cancer cell line

SLN Solid lipid nanoparticle

SLS Sodium lauryl sulphate

SN38 Active metabolite of irinotecan

STD Standard

T

TD-1 11-amino acid synthetic peptide

TEER Transepithelial electrical resistance

TMC N-trimethyl chitosan chloride

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Abstract

Aloe vera (*L.*) Burm.f. (*Aloe barbadensis* Miller) gel and whole leaf materials have shown the ability to modulate the bioavailability of vitamins and to change the transport of molecules across intestinal epithelia.

In this study, precipitated polysaccharides from *A. vera* gel were separated into four different molecular weight fractions (MWF). This was done by means of membrane based centrifugal devices with specific molecular weight cut-off values (i.e. 300 kDa, 100 kDa, and 30 kDa). Chemical characterization of all the *A. vera* gel materials was done by means of nuclear magnetic resonance spectroscopy in order to quantify specific marker molecules such as aloverose, glucose and malic acid. Gel filtration chromatography linked to multi-angle laser-light scattering and refractive index detection was utilised to determine the average molecular weight of each fraction. The *A. vera* gel starting material used in this study contained all three marker molecules. Aloverose was present in different concentrations in all the aloe leaf gel materials investigated in this study except for the MWF that passed through the centrifugal device membrane with 30 kDa as molecular weight cut-off value. The aim of the study was to determine the effect of each polysaccharide fraction on drug transport and bioavailability.

The effect of each of the *A. vera* gel materials was measured on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers as well as on the metabolism of indinavir in the LS180 cell model (both from intestinal epithelial origin). The precipitated polysaccharides decreased the TEER to a higher extent than the gel material, which indicated that the molecules responsible for opening of tight junctions are more concentrated in the precipitated material than in the gel material. The effect of the different MWF of the polysaccharides on the TEER did not correlate directly to their average molecular weight values. All of the aloe gel materials showed lower metabolite (M6) to parent drug (indinavir) ratio values when compared to that of the normal control group (indinavir alone), which represents an enzyme inhibition effect (albeit not statistically significantly).

An *in vivo* bioavailability study of indinavir was done in Sprague-Dawley rats in the absence and presence of the various *A. vera* gel materials. Blood samples were analysed with a sensitive and selective liquid chromatography linked to mass spectrometry (LC-MS) method. The maximum indinavir plasma concentration (C_{max}) values were increased by *A. vera* gel,

Abstract

crude precipitated polysaccharides and two of the MWF's when compared to that of indinavir alone (control group). On the other hand, the area under the curve (AUC) values were increased by all the treatment groups. These changes represent relative bioavailability values of 1.28 for *A. vera* gel, 1.67 for crude precipitated polysaccharides, 1.84 for molecular weight fraction 1, 1.77 for molecular weight fraction 2, 1.39 for molecular weight fraction 3 and 1.95 for molecular weight fraction 4. The relatively high effect of the crude precipitated polysaccharides as well as two of the MWF's on indinavir bioavailability correlates well with their *in vitro* performances in terms of TEER reduction and metabolism inhibition. The results from this study indicate modulation of indinavir bioavailability by *A. vera* gel materials, which was higher for the precipitated polysaccharides and some of the isolated polysaccharide fractions when compared to that of the *A. vera* gel material.

Keywords: *Aloe vera*, bioavailability, Caco-2, LS180, indinavir, metabolism inhibition, transepithelial electrical resistance.

Uittreksel

Aloe vera (*L.*) Burm.f. (*Aloe barbadensis* Miller) jel en heelblaar materiale het die vermoë getoon om die biobeskikbaarheid van vitamiene te moduleer asook om die beweging van molekules oor die inestinale epiteel te verander.

In hierdie studie is gepresipiteerde polisakkariede van *A. vera* jel geskei in vier molekulêre gewigsfraksies (MGF). Dit is gedoen deur die gebruik van membraangebaseerde sentrifugale toestelle met spesifieke molekulêre gewigsafsnypunte (naamlik 300 kDa, 100 kDa, 30 kDa). Chemiese karakterisering van al die *A. vera* jel materiale is gedoen deur middel van kern magnetiese resonans spektroskopie om spesifieke merker molekules te kwantifiseer naamlik aloverose, glukose en maliensuur. Gelfiltrasie kromotografie gekoppel aan multi-hoek laserlig verspreiding en refraktiewe indeks deteksie was gebruik om die gemiddelde molekulêre gewig van elke fraksie te bepaal. Die *A. vera* jel wat as uittgangstof gebruik is, het al drie die merker molekules bevat. Aloverose was teenwoording in verskillende konsentrasie in al die aloe blaar materiale wat in hierdie studie ondersoek was, behalwe in die MGF wat deur die sentrifugale membraan toestel met die 30 kDa gewigsafsnypunt gefiltreer is. Die doel van die studie is om die effek van elke polisakkariedfraksie op geneesmiddeltransport te bepaal.

Die effek van elk van die *A. vera* jel materiale op die trans-epiteel elektriese weerstand (TEEW) van Caco-2 selmonolae asook op die metabolisme van indinavir in LS180 selle is bepaal (beide van intestinale epiteel oorsprong). Die gepresipiteerde polisakkariede het ‘n groter verlaging in die TEEW getoon as die jel materiaal, wat aandui dat die molekules verantwoordelik vir die opening van die hegte aansluitings meer gekonsentreerd voorkom in die gepresipiteerde polisakkariede as in die gel materiaal. Die effek van die MGF’s op die TEEW het egter nie direk met die gemiddelde molekulêre gewigswaarde van die fraksies gekorreleer nie. Al die aloe jel materiale het laer metaboliet (M6) tot moedergeneesmiddel (indinavir) verhoudingswaardes getoon, wat dui op ‘n ensieminhiberende effek (egter nie statisties betekenisvol nie).

‘n *In vivo* biobeskikbaarheidstudie van indinavir was in die Sprague-Dawley rotmodel uitgevoer in die teenwoordigheid en afwesigheid van die *A. vera* jel materiale. Bloedmonsters was ge-analiseer met behulp van ‘n sensitiewe en selektiewe vloeistof kromatograaf gekoppel aan ‘n massa spektometer. Die maksimum indinavir plasmakonsentrasiewaardes (C_{maks}) is verhoog deur die *A. vera* jel, die rou gepresipiteerde

polisakkariede en twee van die MGF's in vergelyking met die negatiewe kontrole groep (indinavir alleen). Aan die ander kant, die area onder die kurwe waardes was verhoog deur al die eksperimentele groepe. Hierdie resultate stel relatiewe biobeskikbaarheidswaardes van 1.28 vir die *A. vera* gel, 1.67 vir die rou presipiteerde polisakkariede, 1.84 vir molekulêre gewigsfraksie een, 1.77 vir molekulêre gewigsfraksie twee, 1.39 vir molekulêre gewigsfraksie drie en 1.95 vir molekulêre gewigsfraksie vier voor. Die relatiewe groot effek wat die gepresipiteerde polisakkariede teweeggebring het, sowel as twee van die MGF's op die biobeskikbaarhied van indinavir korreleer goed met die resultate verkry vanuit die *in vitro* studies in terme van TEEW afname en metabolisme inhibisie. Die resultate van die studie dui op modulasie van indinavir biobeskikbaarheid deur *A. vera* gel materiale, met 'n verhoogde modulasie effek deur die presipiteerde polisakkariede, sowel as sommige van die geïsoleerde polisakkaried fraksies vergeleke met die oorspronklike *A. vera* gel. Die resultate dui op die verandering van indinavir biobeskikbaarheid deur *A. vera* jel materiale, wat hoër was vir die gepresipiteerde polisakkariede en sommige van die MGF's wanneer vergelyk met die *A. vera* jel materiaal.

Sleutelwoorde: *Aloe vera*, biobeskikbaarheid, Caco-2, LS180, indinavir, metabolisme inhibisie, trans-epiteel elektriese weerstand

Foreword

This thesis is presented in the article format as prescribed by guidelines of the North-West University. It comprises introductory and conclusion chapters, one published review article (as published in the peer-reviewed journal "Protein and Peptide Letters") and one full length research article accepted for publication in the journal "Current Drug Delivery". The guides for authors for both of these journals are specified in Appendices D and E, respectively. In addition to the abovementioned chapters, detailed experimental data is given in different appendices of this thesis.

The aim of this study was to investigate the effect of *A. vera* gel, precipitated *A. vera* polysaccharides and different *A. vera* gel polysaccharide molecular weight fractions on *in vitro* transepithelial electrical resistance (TEER) using the Caco-2 cell model, the metabolism of indinavir using the LS180 cell model and the *in vivo* bioavailability of indinavir in Sprague Dawley rats. Chemical characterization of the *A. vera* materials was also performed by means of nuclear magnetic resonance spectroscopy (^{1}H-NMR), gel filtration chromatography (GFC) linked to multi-angle laser-light scattering (MALLS) and refractive index (RI) detection.

Chapter 1

Introduction and problem statement

1. INTRODUCTION

1.1 Herb-drug pharmacokinetic interactions

Herbal remedies or traditional medicines are being considered at a growing rate as an alternative to conventional medicines and treatments all over the world. It has been estimated that in the United States of America alone, approximately 15 million patients are looking to herbal remedies as an alternative means of treatment (Tachjian *et al*., 2010:515; Cheng *et al*., 2015:370). In Sub-Saharan Africa, more patients are now considering traditional medicines as an alternative when it comes to the management of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) (Nagata *et al*., 2011:501). Because of potential pharmacokinetic and pharmacodynamic interactions between the traditional or herbal medicines and anti-retroviral drugs (ARV's), there are serious concerns amongst health practitioners for the safety of these patients (Nagata *et al*., 2011:502). Decreased or increased drug bioavailability is one of the unwanted pharmacokinetic interactions that may occur with co-administration of herbs and allopathic medicines. Relatively low increases or decreases in drug plasma levels may be potentially harmful in the case of drugs with narrow therapeutic indices (Tarirai *et al*., 2010). St. John's wort (*Hypericum perforatum*) and garlic (*Allium sativum*) supplements have, for example, been proven to reduce the plasma concentrations of the anti-retroviral drugs such as indinavir and saquinavir, which could lead to possible drug resistance and therefore reduced efficacy of the drug (Pal & Mitra, 2006:2134). Many herbal agents are substrates of the same cytochrome P450 enzyme (CYP3A4), which is responsible for the metabolism of xenobiotics. Co-administration of herbal products that interfere with an allopathic medicine's metabolism may lead to modified plasma levels of the drug (Pal & Mitra, 2006:2136).

Pharmacokinetic interactions that may occur between herbs and drugs include modulation of active efflux transporters and/or changes in the metabolism of the co-administered drug (Varma *et al*., 2003). P-glycoprotein (P-gp) efflux has been found to have a significant impact on the absorption, distribution, metabolism, elimination and toxicity of drug molecules (Balimane *et al*., 2006:2). In the gastrointestinal tract, P-gp is located on the apical exterior surface of epithelial cells and acts as a biological barrier by exporting xenobiotics,

which are substrates for this active transporter, out of the epithelial cells (Deferme *et al*., 2008:187). This attribute enables P-gp to limit or prevent the absorption of certain compounds into the systemic circulation (Chan *et al*., 2004:34). It has previously been shown that the bioavailability of some orally administered drugs can be extensively enhanced by inhibiting intestinal P-gp efflux (Evans, 2003:539). P-gp is susceptible to inhibition, activation or induction by herbal constituents. The modulation of P-gp activity and/or expression may result in a change in the absorption and bioavailability of drugs that are P-gp substrates such as methotrexate, protease inhibitors and steroids. Alterations in plasma drug concentrations may, to a large extent, be attributable to a change in the activity of drug transporters such as P-gp and/or a change in the activity of cytochrome P450 enzymes (Huisman *et al*., 2002:2296).

On the other hand, controlled herb-drug interactions such as efflux or metabolism inhibition and opening of tight junctions potentially presents an opportunity for enhancing the permeability of orally administered drugs with poor bioavailabilities.

1.2 *Aloe vera* leaf composition

Aloe vera (L.) Burm.f. (*Aloe barbadensis* Miller) is one of the most commercialised aloe species worldwide and although it is currently cultivated globally, it originated most probably from North Africa or Arabia (O'Brien, 2005:31-52). The innermost part or pulp of the leaves of the *A. vera* plant contains a clear and viscous mucilage or gel. This gel is composed of polysaccharides, amino acids, vitamins, minerals, enzymes, lipids, phenolic compounds and organic acids. The polysaccharides found in aloe leaf gel (e.g. acetylated polymannose or acemannan, mannan, galactan, arabinan, arabinorhamnogalactan) are made up of monomers such as mannose, glucose, xylose, arabinose, galactose, fucose, hexose, rhamnose (Grace *et al.,* 2013: 79). Aloe leaf pulp also contains other chemical compounds such as pectin substance, xylan and cellulose (Ni *et al*., 2004:1745-1755). Aloe gel materials has been used commercially in food as a preservative, in cosmetic products as gelling agent and in the treatment of minor ailments such as burns, skin irritations, constipation, ulcers and coughs (Bozzi *et al*., 2007:1).

Chapter 1

1.3 Biological and pharmacological activities of *A. vera* gel materials

Claims pertaining to the pharmacological activities of the polysaccharides in *A. vera* gel include immunostimulation, promotion of radiation damage repair, anti-inflammatory, anti-bacterial, anti-viral, anti-fungal, anti-diabetic, anti-neoplastic and anti-oxidant effects (Ni *et al.*, 2004:1746; Reynolds & Dweck, 1999:3). *In vitro* studies indicated that acetylated polymannose (acemannan or aloverose) has potential synergistic effects when used in combination with certain nucleoside reverse-transcriptase inhibitors in human U1 cells infected with herpes simplex virus (HSV-1) and HIV-1. Lower doses of anti-retroviral (ARV) drugs were found to be effective when given in conjunction with acemannan, although the plant material on its own had no effect on CD4 cell decline (Stargrove *et al*., 2008:4). In another study, acemannan was used to treat human immunodeficiency virus (HIV) infected patients that developed acquired immunodeficiency syndrome (AIDS) and a noticeable reduction in symptoms was recorded mainly due to the stimulation of the immune system. Acemannan increased cell viability and reduced the viral load in human lymphocyte cultures infected with HIV-1. A possible explanation for this anti-viral effect could be the inhibition of glycosylation of the viral proteins (Reynolds & Dweck, 1999:19).

A. vera juice or health drink has been shown to promote the expression of various enzymes including cytochrome P450 1A2 (CYP1A2) and cytochrome P450 3A4 (CYP3A4) as well as active transporters such as multidrug resistant protein 1 (MDR1). Although no significant effect on digoxin's P-glycoprotein related efflux in *in vitro* studies was found for *A. vera* gel (Djuv & Nilson, 2008:1623; Cordier & Steenkamp., 2011:58), *in vitro* inhibition of efflux of cimetidine was observed for a complex mixture of precipitated polysaccharides from *A. vera* gel (Beneke *et al*., 2013:S44).

1.4 Effect of *A. vera* gel materials on drug pharmacokinetics

Aloe vera gel and whole leaf extract have been reported to increase the bioavailability of both vitamins C and E in humans (Vinson, Al Kharrat & Andreoli, 2005:760). This is caused by phytochemicals in the aloe leaf material, which amongst other mechanisms also protected ascorbic acid from degradation in the intestinal tract (Vinson, Al Kharrat & Andreoli, 2005:763). *In vitro* studies showed that *A. vera* gel and whole leaf extracts have the ability to increase the transport of drugs across Caco-2 cell monolayers as well as excised rat intestinal

tissue due to the opening of tight junctions (Chen *et al*., 2009:587; Beneke *et al*., 2012:475; Lebitsa *et al*., 2012:297). In recent *in vivo* studies, *A. vera* leaf pulp was found to decrease fasting blood glucose levels, as well as improve the levels of the anti-oxidant enzyme in diabetic rats (Ezuruike & Prieto, 2014:862). In another study inalloxan-induced diabetic rabbits, *A. vera* whole leaf gel extract was found to prevent the commencement of hyperglycaemia (Akinmoladun & Akinlove, 2007:1028).

2 RESEARCH PROBLEM

Many herbal medicines exhibit pharmacokinetic or pharmacodynamic interactions with allopathic medicines when administered simultaneously, which may lead to potential side effects or decreased efficacy. However, some pharmacokinetic interactions may be used to the benefit of the patient, for example, to enhance drug absorption and thereby the dose to be administered can be reduced. *A. vera* gel materials have shown potential to enhance *in vitro* drug transport across intestinal epithelial cell monolayers and tissues by means of opening of tight junctions between adjacent epithelial cells. Furthermore, the precipitated polysaccharides from *A. vera* gel material showed the ability to inhibit drug efflux across the intestinal epithelium.

It is not yet known if the complex mixture of polysaccharides in *A. vera* gel or specific polysaccharides are responsible for pharmacokinetic interactions with co-administered anti-retroviral drugs. Furthermore, the *in vivo* effect of *A. vera* gel materials on drug pharmacokinetics has not yet been investigated. Clinical significance of herb-drug interactions can only be effectively determined by means of *in vivo* studies. The knowledge generated by this *in vivo* study can be used to potentially prevent treatment failure or adverse effects in HIV/AIDS patients that ingest *A. vera* juice or leaf extracts simultaneously with indinavir treatment. The data will indicate which part of the leaf material is responsible for herb-drug pharmacokinetic interactions, i.e. the gel material, precipitated polysaccharides or isolated polysaccharide fractions. It will further reveal if *A. vera* gel materials are suitable permeation enhancers *in vivo* for the development of advanced drug delivery systems for potentially increased bioavailability of drugs.

3 AIM AND OBJECTIVES

The aim of this study was to isolate and characterise different polysaccharide fractions from *A. vera* gel material based on molecular weight and to identify pharmacokinetic interactions between these *A. vera* gel polysaccharide fractions and indinavir by means of *in vitro* and *in vivo* studies.

The objectives of the study were:

- To conduct a literature review on *A. vera* gel as drug absorption enhancer, as well as interactions with the pharmacokinetics of drugs in general.
- To fractionate polysaccharides precipitated from *A. vera* gel into four different molecular weight fractions using membrane based centrifugal devices and characterise these fractions by means of nuclear magnetic resonance spectroscopy (^{1}H-NMR), gel filtration chromatography (GFC) linked to multi-angle laser-light scattering (MALLS) and refractive index (RI) detection;
- To evaluate the effect of the *A. vera* gel, precipitated polysaccharides as well as different polysaccharide molecular weight fractions on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers;
- To evaluate the effect of the *A. vera* gel, precipitated polysaccharides as well as different polysaccharide molecular weight fractions on the metabolism of indinavir in LS180 cells;
- To evaluate the effect of the *A. vera* gel, precipitated polysaccharides as well as different polysaccharide molecular weight fractions on the *in vivo* bioavailability of indinavir in Sprague-Dawley rats.

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Chapter 2

Article published in Protein and Peptide Letters

Chapter 2 is presented in the form of a review article that was published in the journal "Protein and Peptide Letters" in 2014 (Volume 21, number 11 p.1087-1101). The student contributed the bulk of the contents of this review article, as part of the literature review on the topic of drug absorption enhancement, since *A. vera* gel has shown the ability to enhance drug absorption.

The complete guide for authors, for publishing in this journal, is given in Appendix D. These guidelines state that the manuscript should be written in 10 pt Times New Roman font, according to the Microsoft Word template file.

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Novel Non-Invasive Protein and Peptide Drug Delivery Approaches

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Abstract: Protein and peptide based therapeutics are typically administered by injection due to their poor uptake when administered via enteral routes of drug administration. Unfortunately, chronic administration of these drugs through multiple injections presents certain patient related problems and it is difficult to mimic the normal physiological release patterns via this mode of drug administration. A need therefore exists to non-invasively deliver these drugs by means of alternative ways such as via the oral, pulmonary, nasal, transdermal and buccal administration routes. Although some attempts of needle free peptide and protein drug delivery have progressed to the clinical stage, relatively limited success has been achieved in terms of commercially available products. Despite the low frequency of clinical breakthroughs with non-invasive protein drug delivery this far, it remains an active research area with renewed interest not only due to its improved therapeutic potential, but also due to the attractive commercial outcomes it offers. It is the aim of this review article to reflect on the main strategies investigated to overcome the barriers against effective systemic protein drug delivery in different routes of drug administration. Approaches based on chemical modifications and pharmaceutical technologies are discussed with reference to examples of drugs and devices that have shown potential, while attempts that have failed are also briefly outlined.

Keywords: Absorption enhancers, enzymatic and physical barriers, needle-free, non-invasive, peptide, protein.

1. INTRODUCTION

Advances in biotechnology have led to an increased number of therapeutic proteins and peptides that have become commercially available on a large scale at an affordable price and the number of these therapeutics is expected to accelerate further in the future [1]. A relatively quick rise in the development of biotechnology based therapeutics due to advanced technologies (e.g., recombinant DNA technology) has unfortunately not been matched by the same rate in the development of effective delivery systems for these types of drugs. The challenges associated with effective systemic delivery of these types of drugs can be related to their unfavourable physicochemical properties [2]. Protein-based drugs usually fall within class III of the Biopharmaceutics Classification System (BCS) and the main reasons for their low oral bioavailability include susceptibility to enzymatic degradation, hydrophilic properties, large molecular size and in some cases low solubility [3,4]. Despite the enormous challenges associated with the effective delivery of biotechnology based drugs via non-invasive routes of drug administration, investigations with respect to this concept remain active due to its large clinical and commercial potential and the ever-increasing role that these drugs can play in the effective treatment of diseases [5,6].

Although there is room for parenteral delivery of protein drugs, a need exists for less invasive delivery of therapeutic proteins that are administered chronically (e.g., more than 60 000 insulin injections need to be administered during a patient's lifetime to effectively control blood glucose levels) [2,6]. Other shortcomings associated with peptide drug delivery by injection besides the pain, discomfort and potential for infections include a lack of replication of the physiological release pattern. Examples of this problem include insulin with its basal and post-prandial release patterns that are difficult to mimic with a series of injections and growth hormone with several secretion events spaced unevenly throughout the day [7,8]. Furthermore, fear of needles and resistance to self-injection are considered to be some of the main reasons for limiting insulin therapy and reduced compliance in diabetic patients [9]. The clinical advantages of non-invasive protein and peptide drug delivery include enhanced patient compliance and potentially improved efficacy by certain routes of drug administration due to a better resemblance of the physiological secretion patterns [10]. Therefore, non-invasive protein and peptide drug delivery is still a relevant area of investigation by formulation scientists [11].

This review aims to reflect on different strategies employed for non-invasive protein and peptide drug delivery via different routes of drug administration as demonstrated in Figure 1.

2. CHEMICAL APPROACHES

Chemical modification of peptide and protein drugs to improve bioavailability has received considerable attention. This interest is primarily due to enhanced enzymatic stability, intestinal permeability and immunogenicity [12,13]. Promising chemical modifications that have been employed to improve the unfavourable properties of peptide and protein drugs include amino acid substitution (analogue forma-

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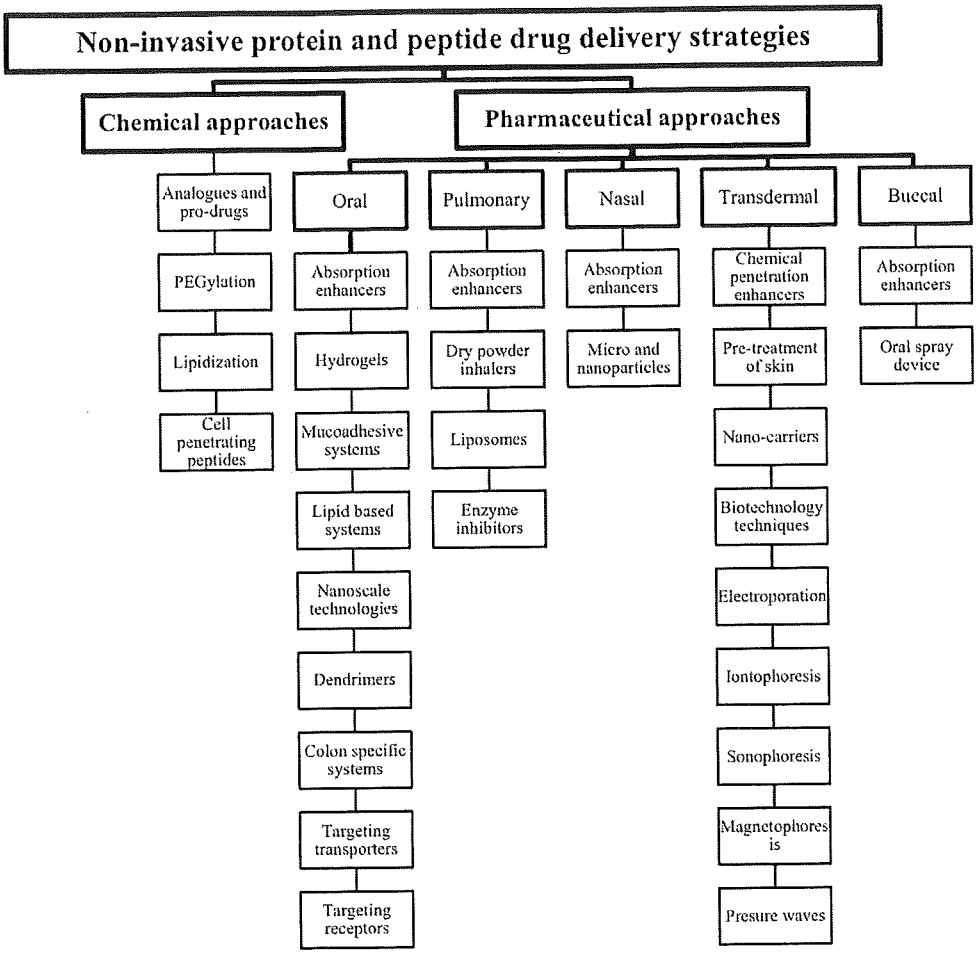

Figure 1. Summary of strategies for non-invasive protein and peptide drug delivery via different routes of drug administration.

tion), synthesis of pro-drugs, conjugation with fatty acids (lipidisation) and conjugation with polyethylene glycol (PEGylation) [12,14,15].

2.1. Analogues and Pro-Drugs

Substituting a specific amino acid in the structure of a peptide or protein with a different amino acid is known as analogue formation. Modifications based on amino acid substitution can be done by substituting an L-amino acid with a D-amino acid or by replacing a particular amino acid with a different amino acid [13]. Desmopressin acetate (DDAVP), a synthetic cyclic analogue of 8-arginine vasopressin (a 9-amino acid peptide), is an example of analogue formation which is currently the treatment of choice for diabetes insipidus and is marketed by Aventis Pharmaceuticals. In contrast to vasopressin, DDAVP is available as injection, intranasal solution and tablets for oral administration. Although the oral bioavailability of DDAVP is <1%, this produces a sufficient antidiuretic effect. Although analogue formation for non-invasive delivery of peptides has been successful for DDAVP, it seems that the application of this modification technique will be limited to small peptides [16].

2.2. PEGylation

PEGylation is a technique that has attracted considerable research attention in terms of structural modifications of peptides and proteins and is defined as the conjugation of poly(ethylene glycol) (PEG) with peptide or protein drugs [17,18]. In research done by Hinds and Kim [19], PEG was successfully conjugated with insulin via an amide bond. This conjugation did not alter insulin's secondary/tertiary structure or potency *in vivo*. However, the conjugate was almost completely devoid of immunogenicity, allergenicity and antigenicity. The conjugated insulin also remained in the systemic circulation for longer periods of time compared to unmodified insulin after subcutaneous administration. In terms of the non-invasive delivery of insulin, perhaps one of the most promising insulin formulations to date for oral administration into the gastrointestinal tract is hexal-insulin-monoconjugate-2 (HIM2). HIM2 was developed by Nobex

Corporation in partnership with GlaxoSmithKline. This conjugate has a small polyethylene glycol 7-hexyl group attached to the B29 amino acid (i.e. lysine) of recombinant human insulin. Clinical trials suggested an oral bioavailability of approximately 5%, which is thought to result in acceptable glucose lowering. HIM2 was as effective as a subcutaneous injection of 8 units of regular insulin to control postprandial glycemia with respect to parameters such as 2 h postprandial glucose concentration, maximum glucose concentration, and glucose $AUC_{0\text{-}240}$ [20,21,22]. However, Biocon acquired the technology from Nobex and conducted clinical studies. One of the longer-term studies in type 2 diabetes mellitus patients did not meet the endpoint of a reduction of HbA1c of greater than 0.7% compared to the placebo. The reduction in HbA1c in the placebo group was, however, larger than expected, thereby decreasing the difference between the two treatment groups. Unfortunately, the statistics of the trial are not freely available [8]. Currently, Biocon in co-operation with Bristol-Myers Squibb are pursuing further clinical development of this conjugated molecule [23].

2.3. Lipidisation

Another conjugation technique that has been used successfully to improve the lipophilicity and thereby the bioavailability of peptide drugs is lipidisation. This technique usually involves the conjugation of a fatty acid to a peptide or protein [21,24]. Due to difficulties associated with the conjugation of fatty acids to peptides and proteins, a modified technique to overcome the drawbacks of conventional lipidisation, was developed. This method is referred to as "reversible aqueous lipidisation" (REAL) and can be carried out in an aqueous solution, while the original peptide can be regenerated in the blood or tissues. With regard to oral peptide delivery, Wang and co-workers [25] have shown that REAL-technology improved the oral bioavailability of salmon calcitonin, however, the exact mechanism of improved absorption from the gastrointestinal tract was not clear. The authors concluded that REAL-technology can be used in future for designing polypeptide drugs for oral delivery. In another study, a non-reversible aqueous-soluble lipid conjugate of calcitonin was prepared [26]. This derivative of salmon calcitonin produced hypocalcaemic activity comparable to salmon calcitonin after subcutaneous injection, however, activity after oral administration was inconclusive due to within group variation in the rat model. With regard to calcitonin, Nobex Corporation and Elan Corporation initiated Phase I clinical trials with Oratonin™, an oral calcitonin derivative for the treatment of osteoporosis. This derivative of calcitonin encompasses the attachment of a polymer to calcitonin, however, the nature of this polymer is not revealed, only that it involves proprietary Nobex technology [27]. Currently Oratonin™ has completed Phase I clinical trials and is ready for Phase II clinical trials (Seachaid Pharmaceuticals). Publicly available information on Oratonin™ is limited.

2.4. Cell-Penetrating Peptides

Cell-penetrating peptides (CPPs) received considerable interest during the last two decades for the non-invasive delivery of peptides and proteins. CPPs can cross the cell membrane with limited toxicity and can act as vector for protein and peptide drugs. However, the exact mechanism by which CPP-drug conjugates cross the cell membrane and/or how they get internalised is still unclear [28,29,30]. It is postulated that the inter-molecular interactions between the CPP and the drug are important for enhanced absorption [13]. Improved absorption of insulin was obtained after nasal administration of insulin in the presence of penetratin, a CPP. This study also showed that penetratin did not cause any mucosal damage over the concentration range used [31]. However, a great deal of research is still needed before CPP-mediated delivery will be clinically utilised [29].

3. PHARMACEUTICAL APPROACHES

3.1. Oral Delivery

Oral drug delivery remains the most popular and patient compliant route of drug administration [31], which explains the numerous efforts of pharmaceutical scientists to overcome challenges such as low stability and poor permeability of protein and peptide drugs in the gastrointestinal tract. Although limited success has been achieved in terms of the clinical translation of protein based drugs into registered oral products [10], it is important to highlight formulation approaches that have shown potential for effective oral delivery of these drugs.

3.1.1. Polymeric Hydrogels

Hydrogels are three-dimensional water-absorbing networks consisting of cross-linked hydrophilic polymers that can be used in novel drug delivery systems [32]. Different types of hydrogel drug delivery systems have been investigated for improved protein and peptide drug delivery such as microparticles consisting of poly(methacrylic acid grafted with poly(ethylene glycol)) that protected insulin from degradation in the gastrointestinal tract with improved bioavailability[33,34].

An example of a polymeric hydrogel shuttle system which consisted of superporous hydrogel composite for site-specific controlled release of *N*-α-benzoyl-L-arginine ethylester. This system showed a promising time-controlled release profile with the potential to bring the drug in close contact with the intestinal epithelium at a specific site in the gastrointestinal tract and in addition also partly inhibited the activity of trypsin together with opening of the tight junctions. The system was designed to produce a double phase release profile to provide the enzyme inhibitors and absorption enhancers first before releasing the active ingredient as illustrated in Figure **2** [35].

3.1.2. Mucoadhesive Systems

Intestinal transit time can be prolonged by the use of mucoadhesive delivery systems that slows down the movement of a delivery system through the gastrointestinal tract by adhering to the mucosal surface. Furthermore, it improves the contact between the drug and the mucosa resulting in an increased drug concentration gradient preventing dilution or degradation of the drug in the luminal fluid to a certain extent [36].

In a previous study, a gastrointestinal mucoadhesive patch system was designed in the form of a four layered en-

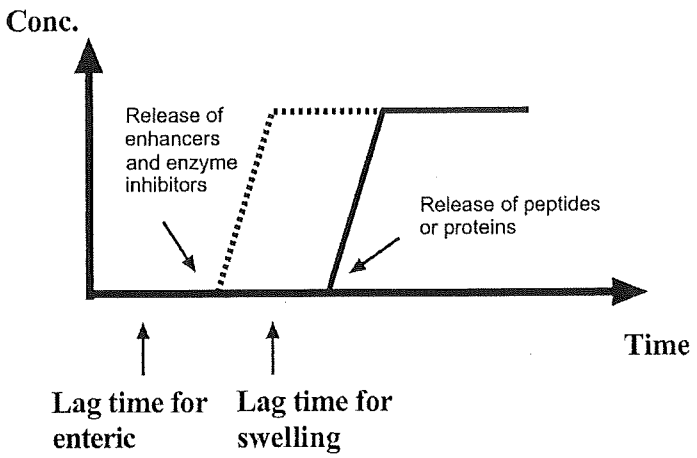

Figure 2. Graph illustrating the double phase time controlled release profile as intended to be obtained from the polymeric hydrogel shuttle system [Reprinted from 35 with permission from Elsevier].

teric coated capsule for the oral delivery of recombinant human granulocyte colony-stimulating factor. This patch system protected the drug from proteolytic enzymes and depended on environmental pH for drug release [37]. Thiolated polymers (thiomers, e.g., thiolated chitosan) contain thiol-bearing ligands that are bound to the polymer backbone and have been shown to enhance intestinal macromolecular drug absorption by means of mechanisms such as mucoadhesion and opening of tight junctions. A third generation of pre-activated thiomers have recently been introduced that enhanced the stability of the polymer, improved its mucoadhesive properties and further increased macromolecular drug delivery [38,39,40].

3.1.3. Lipid-Based Formulations

Solid lipid nanoparticles (SLNs) is an example of a lipid-based drug delivery system formulated to contain part of the drug dose in the internal core and part in the external coat of solid lipid delivery systems [41]. A significant hypoglycaemic effect was observed in diabetic rats after the oral administration of insulin loaded SLNs. It was found that glucose levels of rats were lower when given insulin-loaded SLNs due to the ability of the matrix of the SLNs to protect insulin against chemical degradation [42]. Self-dispersing or self-emulsifying lipid formulations consist of a drug that is solubilised in oil, surfactant and co-surfactant, which form an emulsion when in contact with the gastrointestinal fluid. Another type of lipid based formulations is colloidal lipid carriers that are capable of solubilising drugs in the locus of the bilayer lipid domain to enhance the dissolution of drugs. Both self-dispersing lipid formulations and colloidal lipid carriers are recent examples of lipid-based formulations investigated for the oral delivery of protein drugs [41].

3.1.4. Nanoscale Technologies

The development of nanoparticles has been considered to be a revolutionary step in the delivery of drugs with the potential of not only improving drug bioavailability, but also targeting drugs towards certain tissues in the body. However, despite the high potential of nanoparticles to provide new treatment opportunities only a few nanoparticle-based systems have reached the market due to challenges such as low encapsulation efficiencies, too rapid drug release profiles and toxicological issues [43,44].

Vitamin B_{12} conjugated dextran nanoparticles demonstrated increased bioavailability of insulin due to particle receptor mediated endocytosis and the protection of insulin from proteolytic degradation [45]. N-trimethyl chitosan chloride (TMC) insulin loaded nanoparticles were modified with a targeting peptide (i.e. CSKSSDYQC or CSK) and compared with unmodified nanoparticles. In comparison to the unmodified nanoparticles, the CSK modification facilitated the uptake of the nanoparticles in the villi, enhanced permeation of the insulin across the epithelium and induced higher internalisation of insulin via endocytosis on goblet cell-like HT29-MTX cells. The modified CSK nanoparticles also showed enhanced transport across Caco-2/HT29-MTX co-cultured cell monolayers and produced improved hypoglycemic effects with a 1.5-fold higher relative bioavailability compared to unmodified nanoparticles [46].

In another study, the anti-diabetic effect of an oral insulin nanoparticulate system consisting of an alginate-dextran sulfate core and a chitosan-polyethylene glycol-albumin shell was evaluated. The nanospheres were loaded with insulin (25, 50, 100 IU/kg) and when administered to diabetic rats, blood glucose reduced in a dose-dependent manner with maximal effect after 14h and a continued effect for up to 24h. The success of these nanospheres was attributed to the reason that albumin prevents protease attack on the insulin and PEG stabilises the nanosphere [47].

3.1.5. Dendrimers

Dendrimers are defined as spherical, macromolecules in the nanosize range with a specific architectural structure consisting of branches stretching from the core containing terminal functional groups [48]. Peptide and protein dendrimers that are synthesised without a core using lysine and

consisting of cationic and aromatic groups in the structure attracted attention due to their potential applications as protein mimetics or drug delivery vehicles [49].

Conjugation of SN38, the active metabolite of irinotecan to poly(amido amine)dendrimers was studied by determining their *in vitro* transport profiles using Caco-2 cell monolayers. The conjugated dendrimers resulted in an increase in the transepithelial transport with decreased toxicity [50].

3.1.6. Colon-Specific Delivery Systems

Site-specific delivery in the colon is considered an attractive approach for oral delivery of proteins and peptides due to decreased proteolytic activity, increased residence time and potentially improved absorption [38]. Approaches for colon-specific delivery include pressure-induced drug delivery, micro-activated systems, pH and time dependent delivery systems and particulate drug delivery systems [51].

Cross-linked bovine serum albumin nanospheres layered with fatty acids presented little drug release in the gastric area and were capable to control the release of vancomycin in the colon area responding to stimuli such as pH, mucin interactions and enzyme absorption [52]. CODES™ (Colon-targeted delivery system), developed by Katsuma and co-workers achieved colon-specific delivery in humans and dogs by releasing the drug content when the lactulose is degraded by enterobacteria in the colon [53].

3.1.7. Targeting Active Transporters

Drug molecules can be transported against a concentration gradient across intestinal epithelium and oral mucosa by means of active transport. This is an energy dependent, carrier-mediated transport of small or macromolecules by transmembrane proteins [13,54]. Transporter proteins exist for amino acids, monosaccharides, monocarboxylic acids, nucleosides, dipeptides, organic cations, phosphates and water-soluble vitamins. These active transporters are driven by Na^+- or H^+-ion electrochemical gradients, or by adenosine tri-phosphate (ATP) hydrolysis [56]. Absorption of di- and tri-peptides across intestinal epithelium has been shown to be facilitated by carrier-mediated oligopeptide transporters [13,55].

A strategy to enhance intestinal absorption of protein and peptide drugs involves its covalent conjugation to carrier molecules, creating targeted pro-drugs, which facilitates membrane transport by enabling recognition by the endogenous membrane transporters [13,56].

Of these endogenous systems, peptide transporters have several advantages as a target system including broad substrate specificity [57]. Although a number of peptide transporters have been identified, including PHT1, PHT2 and PepT2, the proton-coupled PepT1 has been described extensively and is known to be widely distributed in the small intestine and in Caco-2 cells [55,57]. It has been shown that a variety of derivatised dipeptides may be targeted to PepT1, thereby possibly improving the compound's oral bioavailability including dipeptidyl derivatives of α–methyl-Dopa. Although studies indicate that di/tripeptidomimetic and dipeptidyl pro-drugs targeting PepT1/2 showed promise for effective delivery of small drug molecules, its use for larger peptides or macromolecules appears to be limited [58,59].

3.1.8. Targeting Receptors

Receptors, unlike active transporters, transport drug molecules across epithelial and enterocytic cells via receptor-mediated transcytosis and are not limited by their ability to transport molecules based on their size [13,60]. Therefore, the conjugation of protein or peptide drugs to receptor ligands (e.g., vitamin B_{12}, biotin, transferrin, etc.) can enable site specific absorption without affecting the cellular membrane integrity [13,60,61]. This has made receptor-mediated endocytosis an important target for drug delivery research. One study showed that oligomeric transferrin has the potential to act as a carrier of protein and peptide drugs such as insulin for oral delivery with sustained release. They cross-linked insulin with transferrin, which resulted in increased intracellular retention in enterocyte-like Caco-2 cells and CF-1 mice. Insulin conjugated to the tranferrin oligomer was also more effective after oral administration in streptozotocin-induced diabetic rats [61].

The folate receptor has been identified as an ideal marker to target drug delivery to various tumours, including ovarian carcinomas and, in various studies, folate was attached directly to drugs or chemically bound to polymer-drug or polymer/lipid-drug conjugates [59,62]. Kim and co-workers proposed a poly(L-lysine)-poly(ethylene glycol)-folate conjugate (a di-block copolymer conjugate) for therapeutic proteins, since physical PEGylation might prolong circulation in the blood [62]. Folate has also been conjugated to trimethyl chitosan chloride (TMC) to protect it from enzymatic degradation, but to decrease the toxicity of TMC and to increase binding of folate to its receptor, a folate-poly(ethylene glycol)-grafted-trimethylchitosan complex was prepared [63]. Both conjugated molecules demonstrated enhanced intracellular delivery of fluorescein isothiocyanate conjugated bovine serum albumin as a model protein in a folate receptor over-expressing cell line (KB cells or SKOV3 cells) through folate receptor-mediated endocytosis when compared with A549 cells (a folate receptor deficient cell line). It is possible that this intracellular protein delivery system can be suitable to enhance delivery of other negatively charged therapeutic proteins. However, Kim and co-workers noted that intracellular delivery of the model protein was decreased when serum proteins were present, and suggested that for *in vivo* applications the protein complexes should have enhanced stability [62]. Bi-functional fusion proteins, including a human growth hormone-transferrin fusion protein for oral delivery, have been genetically engineered to link protein and peptide drugs with carrier antibodies which target transferrin receptors, and this system has been shown not to be limited to the rat [64,65].

3.2. Pulmonary Delivery

A promising non-invasive delivery route for protein and peptide drugs is via the airway or pulmonary system, in the form of dry powder inhalants and nanoparticles [66]. Macromolecules are promptly absorbed from the alveolar airspace mainly because of the large alveolar surface area, decreased enzymatic activity as well as the thin epithelial air-blood barrier [67]. As with many other delivery routes, the pulmonary route of administration also has its drawbacks such as the thick mucus layer covering the upper and central

respiratory tract, which prevents adequate absorption of proteins [68]. This mucus layer along with the closely joined tight junctions in the respiratory epithelium act as a significant physical barrier against the penetration of macromolecules [69], which needs to be overcome before therapeutic levels of the drug could be reached.

Macrophage targeting carrier systems, mucoadhesive systems as well as compounds that could reversibly open tight junctions is some novel approaches that have been explored to optimise pulmonary delivery of macromolecules [69,70]. Optimal deep lung delivery of drug molecules is ideally obtained by particles with a diameter of between 1 and 5 μm. Polymeric micro and nanoparticles that comprise of degradable and biocompatible materials such as polyethylene glycol and poly(lactide-co-glycolide) are retained well in the pulmonary tissues and are able to evade macrophages [71,72]. Besides these novel dosage form designs, various devices such as metered dose inhalers, jet and ultrasonic nebulisers and dry-powder inhalers have been used in the pulmonary drug delivery [73].

3.2.1. Absorption Enhancers

The use of absorption enhancers is one of the common methods employed to improve absorption of molecules across different mucosal surfaces. Several mechanisms of action are known for absorption enhancers employed in pulmonary drug delivery, which include reduction of the mucus viscosity, reversible opening of tight junctions, increase in membrane fluidity and momentarily disruption of the structural integrity of epithelial membranes [13]. Various absorption enhancers have been investigated for the improvement of protein and peptide absorption through the pulmonary epithelium such as surfactants, chelating agents, fatty acids, bile salts, anionic and cationic polymers [13,74]. Studies over recent years have, however, proved that only few absorption enhancers are regarded as safe for use in humans. Some of these include: chitosan, alkylsaccharides and polyethylene glycol (PEG) [75,76].

Chitosan and its derivatives have long been used in improving uptake of proteins across epithelial tissue mainly because of its muco-adhesive and absorption enhancing properties, but also because of its low toxicity [69,70]. Microencapsulation of dry insulin powder in chitosan nanoparticles significantly increased the distribution of the drug to the deep lung after intra-tracheal administration thereof. The inhaled nanoparticles induced a more pronounced and prolonged hypoglycaemic effect in rats compared to the control group [71].

3.2.2. Dry Powder Inhalers

Exubera® is a spray-dried inhalation product that contains mannitol, glycine and sodium citrate in addition to recombinant human insulin and proved to enhance the pulmonary absorption of insulin when compared to regular subcutaneous administration [77,78]. The manufacturing of this product, marketed by Pfizer, was discontinued in 2007 due to several reasons such as its high cost, relative difficulty to use the device, poor acceptance by patients and clinicians, considerations of damage to lungs and a potential increased risk in bronchial carcinoma of smokers [77,79].

Technosphere® technology (Afrezza®, Mannkind Corp) is a system developed for insulin delivery to the pulmonary area by an inhaler containing single dose cartridges [78]. This technology uses fumaryl diketopiperazine as a particle substrate, which enables improved absorption of insulin in the deep lung because it encapsulates the drug, which can be lyophilised into dry powder. The surface charge of each particle is slightly negative, which causes a charge difference between the particle and the pulmonary tissue, thereby enhancing the adsorption of the particles to the tissue. The dry powder is inhaled and the particles disintegrate in the neutral pH of the lung, where encapsulated peptide drugs are then released to be absorbed into the systemic circulation [80]. This technology has been used in delivering not only insulin, but also parathyroid hormone (PTH) and salmon calcitonin (sCT) in healthy human volunteers, as well as felbamate in mice [81]. Aspirair® is a new device, which makes use of compressed air and a vortex separation chamber in order to enhance fine particle fraction lung delivery by de-agglomeration [82].

3.2.3. Liposomes

Liposomes are spherical lipid bilayer vesicles capable of encapsulating drugs and are formulated from cholesterol and phospholipids. Insulin was encapsulated in muco-adhesive liposomes, which caused a reduced plasma glucose level in the blood of rats over a period of 12 h [70]. In another *in vivo* study that was carried out in rats, elactonin, a peptide drug used in the treatment of osteoporosis, had increased absorption in pulmonary tissue when administered in surface-modified liposomes [78].

3.2.4. Enzyme Inhibitors

Enzyme inhibitors, for example N-lauryl-ß-D-maltopyranoside, have shown the ability to enhance the pulmonary absorption of peptide drugs, such as insulin, when *in vivo* testing on rats was carried out [74]. However, limited information on the use of enzyme inhibitors for pulmonary peptide delivery is available in literature which can probably be explained by the problems experienced with long term enzyme inhibition.

3.3. Nasal Delivery

Although the nasal route of drug administration was mainly utilised for the treatment of local diseases of the upper respiratory tract, researchers later acknowledged the value of this route for the systemic delivery of peptide and protein based drugs [83,84].

The structure and composition of the nasal mucosa provides a challenging barrier, especially for the systemic delivery of large molecules (i.e. above 1.0 kDa). Nasal delivery of hydrophilic proteins and peptides usually exhibit bioavailability values of less than 1%. The sub therapeutic bioavailability of these drugs can predominantly be attributed to weak mucosal membrane permeability and degradation due to proteolytic enzyme activity in the nasal mucosa. The rapid mucocilliary clearance of drugs from the nasal mucosa also has a negative effect on bioavailability. It is therefore common practice to make use of nasal absorption enhancers to achieve therapeutic systemic drug levels [75,85].

The following approaches have been pursued to improve the nasal absorption of drugs with a peptide and/or protein nature: 1) chemical structure modification to improve metabolic stability and/or permeability [86]; 2) the addition of enzyme inhibitors to protect the drug from mucosal enzymatic degradation [87]; 3) the addition of absorption enhancers to aid the passage of drug molecules through the nasal mucosa [88]; 4) development of novel drug carrier systems such as liposomes, niosomes, nano and microparticles [89,90,91]. Recent trends showed that the focus is primarily on the use of absorption enhancers as well as nano and microparticulate systems and these strategies will therefore be discussed in more detail.

3.3.1. Absorption Enhancers

As mentioned before, chemical absorption enhancers are co-administered with drugs to improve their absorption across mucosal surfaces via different mechanisms of action. Cyclopenta decalactone is derived from the plant *Angelica archangelica* and is listed on the Food and Drug Administration (FDA) approved inactive ingredient list for use in drug delivery applications. This excipient acts as an absorption enhancer; however, thespecific mechanism of its drug absorption enhancement property in the nose is only speculative at this stage. The surfactant nature of the molecule suggests that interaction with the nasal membrane may increase fluidity, which in turn may result in improved transcellular absorption of co-administered substances. Cyclopenta decalactone has proved effective in enhancing the nasal delivery of insulin (e.g., Nasulin®) in phase I and phase II clinical trials [92].

Alkylsaccharides (e.g., Intravail®) consist of a polar sugar head (e.g., sucrose, maltose), which is esterified with non-polar alkyl chains of varying lengths. Tetradecyl maltoside, with an alkyl chain length of C_{14}, has been shown to be a very effective nasal absorption enhancer for various drug molecules [93,94]. The proposed mechanism of drug absorption enhancement is attributed to a stimulation of the rate of endocytosis and an increase in fluidity of the mucosal membrane. Studies also showed a significant reduction in transepithelial electrical resistance (TEER) after treatment of human tracheal/bronchial cell cultures, which suggested that paracellular transport enhancement may also contribute to improved absorption [95]. Tetradecyl maltoside was tested in an anaesthetised rat model in concentrations ranging from 0.06 - 0.50%. The study showed that tetradecyl maltoside had improved the nasal absorption of insulin (5.7 kDa), leptin (16.0 kDa), somatropin (22.1 kDa) and epoetin-α (30.4 kDa) to varying degrees of success when administered within the specified concentration range [96]. Another study showed that the alkylsaccharide, N-dodecyl-β-D-maltoside (DDM), enhanced the nasal delivery of a PTH analogue in rodent and primate models when used at a concentration of 0.18%. A relative bioavailability of 35 - 40% was achieved in a primate model when comparing the bioavailability of the DDM-PTH combination to that of a subcutaneous injection [97].

Hydroxy fatty acid esters of polyethylene glycol (e.g., CriticalSorb™ containing macrogol 15 hydroxy stearate) are well known as effective mucosal absorption enhancers. A relatively recent study in a conscious rat model has shown that the nasal absorption of insulin can be dramatically enhanced when combined with fatty acid esters. In a previous study, a solution of insulin (4 IU/kg) and macrogol 15 hydroxy stearate was nasally administered that resulted in a relative bioavailability of 100% when compared to a subcutaneous injection based on an $AUC_{0\text{-}1h}$ [98]. Macrogol 15 hydroxy stearate was also used in a similar study to enhance the nasal absorption of human growth hormone (hGH). A 5 mg/kg dose of hGH was administered nasally to conscious rats in a simple solution of macrogol 15 hydroxy stearate. The results showed a relative bioavailability of 49.9% as compared to a control group (i.e. hGH solution) with a bioavailability of 0.7% based on an $AUC_{0\text{-}2h}$. The optimal ratio of Macrogol 15 hydroxy stearate to hGH, when used as a solution for nasal administration, was reported to be 4:1 on a weight:weight basis [99,100]. The mechanism of action of Macrogol 15 hydroxy stearate was reported to be related to improved permeation in conjunction with a 60% reduction in TEER values which were observed in Caco-2 cell monolayers after a 2 h exposure period [101].

3.3.2. Micro- and Nanoparticles

Micro and nanoparticulate systems for nasal drug delivery usually consist of mucoadhesive polymers, which improve the retention time of the drug on the nasal mucosa that lead to enhanced drug absorption. These particulate systems not only protect the incorporated peptide and protein drugs against enzymatic degradation, but also establish a tight contact between the drug and nasal mucosa [102].

Chitosan is a positively charged polymer with pronounced mucoadhesive properties and is regularly used to aid in the nasal delivery of macromolecules. Chitosan improves drug penetration primarily by transiently opening the tight junctions in the nasal mucosa and by improving bioadhesive properties [103,104]. Chitosan glutamate or hydrochloride salts with a degree of deacetylation of approximately 83% and molecular weight of approximately 200 kDa (e.g., ChiSys®) are mostly used. A study in a sheep model showed that chitosan microspheres were capable of improving the nasal absorption of goserelin. The relative bioavailability of the microsphere formulation as compared to a subcutaneous injection was 36.6% [105,106].

In another study, a thiomer microparticulate system containing polycarbophil-cysteine and gluthatione (as permeation enhancer) was used to sucessfully administer hGH via the nasal cavity. The polycarbophil-cysteine/gluthatione combination microparticulate system achieved a 3-fold increase in bioavailability when compared to a polycarbophil-cysteine formulation which did not contain gluthatione [107].

3.4. Transdermal Delivery

The main diffusional barrier against effective transdermal drug delivery remains the stratum corneum (SC). The SC particularly excludes the transit of polar, hydrophilic molecules such as proteins and peptides. Several strategies have been followed to increase the number of compounds that can be administered transdermally without permanently compromising the SC. These strategies can be divided into two

categories namely passive and active approaches [108,109, 110,111].

3.4.1. Passive Approaches

Passive approaches to improve transdermal drug delivery include chemical penetration enhancers, pre-treatment of skin, nano-carriers and biotechnologies such as peptide cell-penetrating enhancers and peptide chaperones [38,112,113].

3.4.1.1. Chemical Penetration Enhancers

Chemical penetration enhancers facilitate transdermal delivery of drugs by different mechanisms such as enhancing solubility, increasing partitioning into the SC, fluidising the crystalline structure of the SC and/or disrupting ordered skin lipids. Examples of transdermal penetration enhancers include fatty acids/esters, solvents, surfactants, terpenes, ureas, essential oils and sulfoxides [114,115,110,111,116]. Only a relatively small number of chemical enhancers have been able to induce substantial therapeutic enhancement of drug transport across the skin without causing toxic effects and even less depicted enhanced delivery of peptides and proteins [117,118]. Fatty acids were used as penetration enhancers for arginine vasopressin and insulin delivery [119,120] and urea for leuprolide delivery through the skin [121].

3.4.1.2. Pre-Treatment of Skin

The skin contains sulfhydryl groups that may react with certain peptide and protein molecules and thereby presenting a potential biochemical barrier. Neutralisation of this barrier by means of topically applying oxidising agents could increase transdermal delivery of disulphide containing peptides such as insulin, calcitonin, somatostatin lanreotide, octreotide and oxytocin/vasopressin analogues. Sintov and Wormser showed that skin pre-treatment with iodine followed by dermal application of insulin resulted in significantly reduced glucose levels and elevated insulin concentrations in the plasma presumably through inactivation of endogenous sulfhydryl groups [122]. Another study reported that pre-treatment with menthol modifies the barrier properties of the SC by distributing into the intercellular spaces and possibly causes reversible disruption of lipid domains [114]. Skin pre-treatment with ethanol usually increases the permeation of hydrophilic drugs, whereas it decreases diffusion of hydrophobic drugs [38]. Ethanol skin pre-treatment has been investigated for transdermal delivery of leoprolide [123], while dimethyl sulfoxide was used for amphotericin B delivery [124].

3.4.1.3. Nanocarriers

Lipososmes (including niosomes and ethosomes), transfersomes, dendrimers, nanoparticles and nanoemulsions are nanocarriers most commonly utilised for transdermal drug delivery. Although liposomes are considered as nanocarriers in this discussion, it is important to note that they may also fall within the micro size range [112,115,125].

Niosomes, analogues of liposomes, are non-phospholipid vesicles created by the self-assembly of non-ionic surfactants in aqueous dispersions. Liposomes with high alcohol content are known as ethosomes. The surfactants in all of these particles aid in local fluidisation of the lipids, allowing for the particles to sit in the upper layers of the SC where they are able to form a depot for prolonged effects [112,115,125]. The encapsulation of peptides in liposomes to increase transdermal peptide delivery has been investigated for insulin [126], melatonin [127,128] and tetanus toxoid [129,130].

Transfersomes consist of a deformable structure, making them more elastic in nature and allowing them to squeeze through the pores on the surface of skin into the deeper layers [131]. Drug-loaded transfersomes can transport relatively large amounts of drug per unit time across skin (up to 100 $\mu g.cm^{-2}.h^{-1}$). Even when transfersomes are loaded with peptide-like molecules of high molecular weight, a relatively high rate of transdermal delivery remains reproducible [125,132,133]. Examples of peptide-like drugs that have been investigated for transdermal delivery with transfersomes include calcitonin, alpha-interferon, gamma-interferon, insulin and serum albumin [132,133,134].

Nanoparticles have been employed to encapsulate protein drugs for transdermal delivery. The interaction between the nanoparticles and the skin barrier leads to increased skin permeability, which was shown for example with insulin, cyclosporin A and interferon alfa-2a [135,136,137,138,139, 140,141].

Nanoemulsions can incorporate hydrophobic and hydrophilic drugs because it is possible to make both water-in-oil or oil-in-water nanoemulsions [125,142,143,144]. Examples of peptide drugs that have been incorporated in nanoemulsions for transdermal delivery include insulin [145] and bovine serum albumin [146], however, the use of this technique has become less popular due to inherent stability problems with the nanoemulsions.

3.4.1.4. Biotechnology Techniques

Increased passive delivery of insulin via the transfollicular route was achieved when a short 11-amino acid synthetic peptide (TD-1) was co-administered [147]. Enhanced transdermal delivery was also attempted for human epidermal growth factor and human growth hormone by utilising TD-1 [148,149]. A natural pore-forming peptide, magainin, was employed to increase skin permeability by a mechanism proposed to target bilayer disruption in SC lipids and not in deeper tissue [150]. Magainin was, however, only effective when utilised in combination with a surfactant.

Lipophilic derivatives of peptides were reported to have increased skin permeability, for example, covalent binding of cyclosporine to a polyarginine-heptamer cell-penetrating peptide led to improved skin absorption. However, it is important to note that alteration of the chemical structure of peptides might also affect their pharmacological activity [126,151,152,153].

3.4.2. Active Approaches

Active approaches to improve transdermal drug delivery involve the use of an energy source to overcome skin barrier properties. The device and application factors can be adjusted to accommodate the skin properties of different patients. Active transdermal delivery approaches can be subdivided into invasive (e.g., intravenous or intramuscular injection), minimally invasive (e.g., photopolymerisation, microdermabration, liquid jet injectors, powder injectors, mi-

croneedles, thermal ablation, radiofrequency ablation, laser ablation, tape stripping) and non-invasive methods (e.g., electroporation, iontophoresis, sonophoresis, magnetophoresis and pressure waves) [111,126,154]. Although some of the invasive and minimally invasive techniques have been successfully used to deliver protein based drugs across the skin, only non-invasive methods will be discussed here as delimited by the scope of this review article.

3.4.2.1. Electroporation

Electroporation is a technique that causes a temporary structural perturbation of the lipid bilayers of the SC in order to create transient pores through application of short (microsecond or millisecond), high voltage (50-1000 V) pulses to the skin. Transport of charged macromolecular drugs (up to 40 kDa) occurs mainly due to enhanced diffusion, electrophoresis and to a small extent also electro-osmosis. Electroporation has effectively been used to enhance permeability of peptides and proteins across the skin such as insulin [155], calcium regulating hormones [156] and sCT [157].

Genetronics, Inc (San Diego, California) has developed a prototype electroporation device in order to attain gene delivery and to improve drug delivery. However, safety in the clinical application of this technique is still questionable due to mild skin reactions [158,159,160].

3.4.2.2. Iontophoresis

Iontophoresis is a procedure that employs a small electrical potential difference (0.5 mA/cm^2) to enhance delivery of charged and neutral molecules with relatively low molecular weight through the skin. It offers controlled or pulsatile drug delivery, since the amount of drug delivered is directly proportional to the potential difference applied [161,162,163, 164]. Peptides that remain charged throughout transdermal delivery are ideal for iontophoretic delivery [160]. Some examples of biotechnology based drugs that have been investigated for transdermal delivery with iontophoresis include ribonuclease A [164], calcitonin [156,157,165], serum human parathyroid hormone [166,167], vasopressin [168] and luteinizing-hormone-releasing hormone [169,170].

Various companies have developed iontophoresis devices which are currently commercially available, e.g., Ionto-Patch® (Travanti Pharma Inc.) which includes the corporation's wearable electronic disposable drug delivery stand (WEDD®) that is based on a thin, elastic, low-cost battery technology within a single-use, disposable, iontophoretic patch [171,172].

3.4.2.3. Sonophoresis

Sonophoresis is the movement of compounds through intact skin into soft tissue under the influence of ultrasonic agitation. Either low-frequency ultrasound (LFS; 20-100 kHz) or high frequency ultrasound (HFS; 0.7-16 MHz) can be employed [158,173]. It was shown that LFS is significantly more effective than HFS in delivering drugs across the skin. Collapse cavitation (i.e. the generation and oscillation of gas bubbles in the skin) was identified as the mechanism of action for improved transdermal drug delivery by sonophoresis [174,175,176]. The SonoPrep® from Sontra Medical Corporation and Prelude™ SkinPrep from Echo Therapeutics are examples of skin permeation devices which employ ultrasound to increase permeability through the skin [108,160].

3.4.2.4. Magnetophoresis

Magnetophoresis is the application of a magnetic field on the skin that acts as a peripheral driving force to improve transdermal drug delivery. Alteration in the structure of skin is induced, which most likely contributes to an increase in permeability [142,177,178]. Magnetophoresis is mostly used in cancer therapy, but it has also been investigated for transdermal delivery of lipofectamine and certain genetic materials [179,180].

3.4.2.5. Pressure Waves

Pressure waves are generated by intense laser radiation and are only applied for a very short time (i.e. microseconds to milliseconds). It is hypothesised that the pressure waves form a continuous or hydrophilic pathway across skin due to the development of lacunae domains in the SC. Adequate permeation of the SC is possible with only a single pressure wave, which allows the transport of peptides and proteins into the dermis and epidermis. Additionally, drugs can also pass into the vasculature and yield a systemic effect [142,177,181]. Insulin delivered across the skin by means of pressure waves reduced blood glucose concentrations over several hours [142,181].

3.5. Buccal Delivery

The mucosa of the oral cavity offers an opportunity for systemic drug delivery since it is highly vascularized with relatively low enzyme activity and avoids first pass metabolism. However, the limited absorption surface area combined with the barrier functions of the mucosa constitutes challenges against effective drug delivery. Permeation of protein and peptide drugs is specifically limited by their high molecular weight and hydrophilic nature [54,182].

The term "buccal" refers to the lining of the cheek and the lips, which only represents about one third of the total surface area of the oral cavity. The lining of the buccal mucosa consists of non-keratinised, multilayered squamous epithelium supported by the underlying lamina propria [183].

Different approaches have been used to improve drug permeation across the buccal mucosa such as incorporation of chemical permeation enhancers, enzyme inhibitors and mucoadhesive polymers to retain the drug in close contact with the site of delivery for a prolonged period.

3.5.1. Absorption Enhancers

Buccal delivery of several protein and peptide drugs with different types of permeation enhancers have been investigated in different *in vitro* and *in vivo* models, which include insulin, octreotide acetate, recombinant human interferon alpha B/D hybrid, gonadotropin releasing hormone, buserelin, luteinising hormone-releasing hormone and glucagon-like-peptide I [184].

When transforming growth-factor β was incorporated into a 2% w/v chitosan gel, a marked increase in the amount that moved across the different strata of the buccal mucosa was observed in comparison with when the drug was applied alone. The improved permeability of the drug caused by the

Table 1. Examples of non-invasive peptide drug delivery products under clinical investigation or available on the market.

Name	Company	Active ingredient	Status	Reference
Oral route of administration				
Peptelligence®	This platform is the proprietary of Unigene Laboratories, licenced to other companies for product development	Salmon calcitonin	Phase III clinical trial completed	[187, 188]
Eligen®	This platform is the proprietary of Emisphere, licenced to other companies for product development	Semaglutide (GPL-1 agonist), insulin, calcitonin	Under clinical investigation	[187, 189]
IN-105	Biocon (taken over from Nobex)	Insulin analogue based on HIM2	Under clinical investigation	[190]
Capsulin™	Diabetology	Insulin	Under clinical investigation	[190]
HDV-I	Diasome	Insulin linked to a hepatic targeting molecule	Under clinical investigation	[190]
Pulmonary route of administration				
Exubera®	Pfizer	Insulin	Discontinued in 2007	[78]
Technosphere®	This platform is the proprietary of MannKind Corp, licenced to other companies for product development	Insulin (AFREZZA™)	Comparative trials	[191]
Nasal route of administration				
µCO™ system in Fitlizer™ devices	SNBL Ltd	Insulin, Parathyroid hormone, calcitonin	Pre-clinical studies in non-human primates	[192]
Miacalcin®	Novartis	Salmon calcitonin	Marketed	[78, 192]
Minirin®	Ferring Pharmaceuticals	Desmopressin	Marketed	[78, 192]
Synarel®	Pharmacia	Nafarelin	Marketed	[78, 192]
Transedrmal route of administration				
PassPort™	Proprietary of Altea Therapeutics Corporation licenced to other companies for product development	e.g., KAI Pharmaceuticals Proprietary peptides, insulin	Under clinical investigation	[193]
ViaDor™	TransPharma Medical Ltd	hPTH, GLP-1, calcitonin	Under clinical investigation (Phase Ib completed for GLP-1)	[193]
P.L.E.A.S.E. (precise laser epidermal system)	Pantec Biosolutions	Therapeutic proteins, e.g., FSH	Under clinical investigation	[193]
Buccal route of administration				
Oral-lyn™	Generex	Insulin	Marketed	[194]

chitosan gel was explained by a potential interference of the polymer with the lipid organisation in the superficial layers of the buccal epithelium combined with a mucoadhesive interaction with the tissue [185]. This permeation enhancing effect of chitosan across the buccal mucosal epithelium was confirmed with statistically significantly increased permeation of hydrophilic and macromolecular model compounds across an *in vitro* buccal cell culture model [182].

3.5.2. Oral Spray Device

Oral-lyn® is a product that delivers insulin systemically through the buccal mucosa and is administered with a spraying device (i.e. Rapidmist®). One spray from the Rapidmist® device contains 10 insulin units and with an absorption of 10% of the total dose, one insulin unit is therefore delivered to the systemic circulation with each spray. The insulin reaches the blood within 5 min, peaks at 30 min and returns back to baseline after 2 h. A clinical trial in Type 1 diabetes mellitus patients showed a very high degree of reproducible insulin delivery in the blood when administered weekly over a three week period. A two year safety study in dogs with four applications daily indicated no adverse effects or pathological changes in the buccal epithelium [186].

4. CONCLUSION

In recent years, there has been a renewed interest in the quest for the non-invasive delivery of peptide and protein drugs. Different routes of drug administration have been investigated for this purpose, including the oral, pulmonary, nasal, transdermal and buccal routes. Although the number of successes in terms of commercialised products does not correlate with the research efforts so far, promising technologies and devices have been developed to serve as platforms for further investigations. Certain approaches and technologies have resulted in products that are in different stages of clinical testing. However, it is important to note that factors such as production cost, difficulty of use, acceptance by clinicians as well as patients, and safety need to be carefully taken into consideration to ensure the marketing potential of a product. Despite all the obstacles, recent progress has highlighted the possibility of non-invasive delivery of biotechnology based drugs.

A summary of some products that are under clinical investigation or commercially available for non-invasive protein and peptide drug delivery via different routes of administration is given in Table 1.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Chapter 3

Article submitted for publication in Current Drug Delivery

Chapter 3 is presented in the form of a research article and was accepted for publication in the journal "Current Drug Delivery" in October 2015. The student was responsible for conducting the research and writing the first draft of the article.

The complete guide for authors, for publishing in this journal, is given in Appendix E. These guidelines state that the manuscript should be written in 10 pt Times New Roman font, according to the Microsoft Word template file.

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Evaluation of Isolated Fractions of *Aloe vera* Gel Materials on Indinavir Pharmacokinetics: *In vitro* and *in vivo* Studies

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Abstract: *Aloe vera* is a plant with a long history of traditional medicinal use and is consumed in different products, sometimes in conjunction with prescribed medicines. *A. vera* gel has shown the ability to modulate drug absorption *in vitro*. The aim of this study was to fractionate the precipitated polysaccharide component of *A. vera* gel based on molecular weight and to compare their interactions with indinavir pharmacokinetics. Crude polysaccharides were precipitated from a solution of *A. vera* gel and was fractionated by means of centrifugal filtration through membranes with different molecular weight cut-off values (i.e. 300 KDa, 100 KDa and 30 KDa). Marker molecules were quantified in the aloe leaf materials by means of nuclear magnetic resonance spectroscopy and the average molecular weight was determined by means of gel filtration chromatography linked to multi-angle-laser-light scattering and refractive index detection. The effect of the aloe leaf materials on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers as well as indinavir metabolism in LS180 cells was measured. The bioavailability of indinavir in the presence and absence of the aloe leaf materials was determined in Sprague-Dawley rats. All the aloe leaf materials investigated in this study reduced the TEER of Caco-2 cell monolayers, inhibited indinavir metabolism in LS 180 cells to different extents and changed the bioavailability parameters of indinavir in rats compared to that of indinavir alone. These indinavir pharmacokinetic modulation effects were not dependent on the presence of aloverose and also not on the average molecular weight of the isolated fractions.

Keywords: *Aloe vera*, area under the curve, indinavir, metabolism, pharmacokinetic interaction, transepithelial electrical resistance.

1. INTRODUCTION

1.1. Herb-Drug Pharmacokinetic Interactions

Herbal medicines are being used worldwide at a growing rate as an alternative to conventional medicines. Traditional medicines or supplements are sometimes used in conjunction with prescribed medications. This is particularly important in developing countries where Governments provide free medicines for life threatening diseases such as anti-retroviral drugs for patients with acquired immunodeficiency syndrome. A relatively large portion of these patients commonly use herbal medicines to complement the efficacy of highly active anti-retroviral therapy. Unfortunately, simultaneous use of herbal remedies with allopathic medicines may cause herb-drug pharmacokinetic or pharmacodynamic interactions that may result in treatment failure or drug resistance due to plasma concentration reduction or in toxic effects due to plasma concentration augmentation [1-3].

Herbal remedies can cause drug pharmacokinetic interactions via a number of mechanisms, which include activation or inhibition of enzymatic metabolism and/or active transporters. Inhibition of enzymes will cause higher drug plasma concentrations, while enzyme induction will cause reduction in drug plasma levels. Drug transport can be modulated by different mechanisms such as interference with active transporters (e.g. inhibition of efflux transporters) and modification of permeation of the mucosal epithelium (e.g. modulation of tight junctions). Such effects are more likely to occur in the gastro-intestinal tract where high concentrations of phytochemicals are achieved [4].

1.2. Aloe Vera Gel

Aloe vera (L.) Burm.f. *(Aloe barbadensis Miller)* leaf gel consists of different phytochemical substances, which

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include minerals, enzymes, polysaccharides, water- and fat soluble vitamins, organic acids and phenolic compounds [5]. Some of the therapeutic properties of *A. vera* gel such as promotion of radiation damage repair, anti-bacterial, anti-viral, anti-fungal, anti-diabetic, anti-neoplastic, immuno-stimulating, anti-inflammatory and anti-oxidant effects have been attributed to the polysaccharides found in the gel [6,7].

The processed leaf pulp or gel of *A. vera* is used globally in many products such as functional foods, cosmetic products, household toiletry products as well as health drinks and beverages [8]. When *A. vera* containing products are taken simultaneously with prescribed drugs, it may cause pharmacokinetic interactions because a clinical trial has shown that *A. vera* gel and whole leaf liquid preparations significantly increased the overall extend of absorption of both vitamins C and E in humans after oral administration [9]. Furthermore, *in vitro* pharmacokinetic studies have shown that *A. vera* gel and whole leaf materials have the capability of increasing the transport of insulin across human intestinal epithelial cell culture monolayers [10] as well as other drugs such as cimetidine across excised animal intestinal tissues [11-13].

2. MATERIALS AND METHODS

2.1. Fractionation of Polysaccharides Precipitated from *A. vera* Gel

Crude polysaccharides were precipitated from aqueous solutions (50 g in 500 ml and passed through a cheese cloth) of *A. vera* gel extract powder 200:1 (Ecuadorian Rainforest LLC, Belleville, New Jersey, USA) after adjusting the pH of the filtrate to a value of 3.2 with the addition of 6 M hydrochloric acid (HCl). Absolute ethanol was added in a ratio of 4:1 to this *A. vera* gel solution and left for 24 h [14,15] to allow the precipitate to settle under gravitation. The precipitated polysaccharides were further separated centrifugally at 4000 rpm for 10 min and then washed twice with 80% v/v ethanol. The precipitate was freeze dried and dissolved in water before it was fractionated with Macrosep® advance centrifugal devices (PALL Corporation, Ann Arbor, Michigan, USA) containing Omega membranes (modified polyethersulfone) with molecular weight cut-off values of 30 KDa, 100 kDa and 300 kDa. Each molecular weight fraction (MWF) produced by the centrifugal filtration of the precipitated polysaccharides was freeze dried and kept in air tight containers until chemically characterised and used in the *in vitro* as well as *in vivo* experiments.

2.2.1. Quantitative Proton Nuclear Magnetic Resonance Spectrometry (^{1}H-NMR)

^{1}H-NMR spectrometry was used to identify and quantify specified marker molecules (i.e. acetylated polymannose or aloverose, glucose and malic acid) in the different aloe leaf gel materials (i.e. *A. vera* gel, crude precipitated polysaccharides and the four molecular weight fractions of the polysaccharides). To record ^{1}H-NMR spectra of the materials, approximately 30 mg of each material were dissolved in 1 mL D_2O and then measured with an Avance III HD 500 MHz NMR spectrometer (Bruker, Germany) equipped with auto-sample changer and BBO Prodigy cryo probe. The internal standard was nicotinic acid amide (NSA; Fluka Chemie AG, Buchs, Switzerland), while the same reference standards for glucose, malic acid and acetylated polysaccharides were used as published before. The quantitative analysis of the marker molecules was performed according to a validated ^{1}H-NMR method for quality assurance of *A. vera* products as published before [16].

2.2.2. Gel Filtration Chromatography Linked to Multi-Angle Laser Light Scattering and Refractive Index Detection (GFC-MALLS-RI)

Gel filtration chromatographic (GFC) separation of the polysaccharide species in the different aloe materials was done with an HP 1100 series chromatograph (Hewlett Packard, Waldbronn, Germany) under isocratic conditions. The chromatograph was interfaced first with a multi-angle laser light scattering (MALLS) photometer (DAWN DSP, Wyatt Technology Corp., Santa Barbara, USA) and then a refractive index (RI) detector (Hewlett Packard, Waldbronn, Germany). Samples were prepared at a concentration of approximately 30 mg/ml and dissolved for 12 h. Triplicate samples were prepared from each fraction and aliquots (100 µl) were injected twice for each analysis that was each filtered through 0.22 µm syringe filters. Two columns (7.8 mm internal diameter and 300 mm length) were connected in series (i.e. a TSK gel $GMPW_{XL}$ column with a molecular weight range of 1 000 to 8 000 000 g/mol and a TSK gel $G5000PW_{XL}$ with molecular weight range of 4 000 to 800 000 g/mol, TOSOH Corp., Japan) to perform the size-exclusion separation of the polysaccharides. Phosphate buffered saline (PBS, pH 7.4) was used as elution liquid and solvent for the test samples. The flow rate was maintained at 1 ml/min and the column thermostat compartment and RI detector flow cell at 35 °C. The specific refractive increment (dn/dc) of the samples was determined online [17] using a pullulan standard (Polymer Laboratories, Amherst, MA, USA). The dn/dc of pullulan was measured as 0.1471 ± 0.0001, which corresponds to the reported value of 0.148 measured at 632.8 nm [18]. MALLS and RI signals were recorded and processed using ASTRA™ 4.73 software (Wyatt Technology Corp., Santa Barbara, CA). The Zimm formalism was used to fit the MALLS detector data utilizing detection angles between 52 and 132. Equation (1) describes the extrapolation of scattered light intensities at various angles to the molecular weight [19]:

$$\frac{R_\Theta}{K_c} = MP(\Theta) - 2A_2cM^2P^2(\Theta) + \ldots \qquad \text{Eq. (1)}$$

Where M is the molecular weight of a defined slice in the chromatogram, R_Θ is the excess Rayleigh scattering ratio at the angle (Θ). P(Θ) is the wavelength and angle-dependent particle scattering factor. A_2 is the second virial coefficient, which accounts for the solvent-excluded effect, here taken as unity and *c* is the concentration of the sample. K is a constant that overlays the concentration (RI) signal with the MALLS signal to calculate a weight number absolute molecular weight (M_n) and a weight average absolute molecular weight (M_w) for the sample and is given by Equation (2):

$$K = \frac{4\ ^2n_0^2}{\lambda_0^2 N_A}\left(\frac{dn}{dc}\right)^2 \qquad \text{Eq. (2)}$$

Where n_0 is the refractive index of the solvent in vacuum at the wavelength of incidence, λ_0 of 632.8 nm. N_A is the Avogadro number, and the specific refractive increment of the sample is dn/dc.

2.2. Transepithelial Electrical Resistance (TEER) Studies

2.2.1. Culturing of Caco-2 Cells

The human colorectal carcinoma cell line (Caco-2, European Collection of Cell Cultures or ECACC) was used in the transepithelial electrical resistance studies. Caco-2 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Johannesburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% penicillin/streptomycin (Separations, Johannesburg, South Africa), 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% of amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa). The cells were cultured at 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The growth medium was exchanged every second day. Prior to exchange of the growth medium, the cells were inspected by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments Tokyo, Japan) to estimate the percentage confluence as well as ensuring the absence of any contamination. Once the cells reached a confluency of 50-60%, subculturing took place by means of trypsinisation.

2.2.2. Seeding of Caco-2 Cells onto Transwell® Filter Membranes

Caco-2 cells were seeded onto Transwell® 24-well filter membranes (Corning Costar® Corporation, Tewksbury, USA) with a pore diameter of 0.4 µm and surface area of 0.33 cm^2. A cell suspension was obtained by means of trypsinisation of the cells with Trypsin-Versene (EDTA) mixture (Whitehead scientific, Cape Town, South Africa). The cells in the suspension were counted by means of Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) staining and a haemocytometer. The cell suspension was hereafter diluted to obtain a concentration of 20 000 cells per ml. A volume of 0.2 ml of the final cell suspension was pipetted into each apical chamber of the wells in order to seed cells onto the filter membranes. The growth medium was replaced every second day and cultured until confluent monolayers were obtained (21-24 days).

2.2.3. Measurement of Transepithelial Electrical Resistance (TEER)

A TEER value of more than 250 Ω (which is equal to 1167.5 Ω/cm^2) was required for each Caco-2 cell monolayer before commencement of the experiment. Solutions (0.2 ml) of each aloe gel material in serum-free DMEM at 1.0 % w/v were added to the apical chambers of the Transwell® 24-well plates with confluent Caco-2 cell monolayers. Sodium lauryl sulphate (0.2% w/v) was used as the positive control group. TEER readings were recorded at time intervals of 20 min over a total period of 2 h, but it was started 40 min prior to addition of the test solutions to the apical chamber. The TEER was measured using a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) connected to chopstick electrodes. All the experiments were done in triplicate at 37 ºC in a humidified atmosphere of 95 % air and 5 % CO_2. The percentage TEER was plotted as a function of time.

2.3. Metabolism Studies

2.3.1. Culturing of LS180 Cells

The human epithelial cell line (LS180, European Collection of Cell Cultures) was cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Randburg, South Africa), 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa) and 1% penicillin/streptomycin (Separations, Thermo Scientific). The cells were cultured at 37°C and 95% humidified air with 5% carbon dioxide, in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The growth medium was exchanged every second day. Prior to sub-culturing, the cells were inspected by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) to estimate the percentage confluence as well as ensuring the absence of any contamination. Sub-culturing was only conducted when the LS180 cells reached a confluency of 50% or more. The LS180 cells were detached from the cell culture flask by means of scraping and not by trypsinisation.

2.3.2. Seeding of LS180 Cells into 6-well Plates

The LS180 cells were seeded into 6-well plates (Corning Costar® Corporation, Tewksbury, USA) 24 h prior to the addition of the test solutions. After scraping the cells from the culturing flask, a cell suspension was obtained and the cells were counted by means of Trypan blue staining in a haemocytometer. The cell suspension was diluted to 250 000 cells/ml and a volume of 2 ml of the final cell suspension was pipetted into each well. The cells were grown under sterile conditions for a period of 24 h.

2.3.3. Metabolism Inhibition Study

After the 24 h growth period, the growth medium was removed from the cells and the different test and control solutions were applied. The test solutions consisted of each aloe gel material in serum-free DMEM at 1.0 % w/v together with indinavir (40 µM). Indinavir (40 µM) alone was used as the negative control group, while the positive control group consisted of indinavir (40 µM) together with ketoconazole (40 µM) in serum-free DMEM. After addition of the test and control solutions, the plates were incubated for 4 h at 5% CO_2 and 95% humidity. The test solutions were removed and the cells immediately washed with 1 ml pre-warmed phosphate buffered saline (PBS) (Separations, Randburg, South Africa). The cells were gently scraped in in 1 ml PBS in order to detach and harvest the cells. The harvested cells were transferred into clearly labelled 2.5 ml micro-centrifuge tubes and immediately placed on ice. The samples were centrifuged (Wirsam Scientific, Johannesburg, South Africa) for

5 min at 300 x g after which the supernatant was decanted and the cell pellets re-suspended in 200 µl ice-cold methanol to lyse the cell membranes. The samples were vortex mixed for 40 s, centrifuged over a period of 10 min at 10 000 x *g* and the supernatant was then transferred to 2.5 ml micro centrifuge tubes. The supernatant of each sample was evaporated under nitrogen gas and kept at -80°C until analysis by liquid chromatography linked to mass spectroscopy as described below.

2.3.4. Liquid Chromatography Linked Mass Spectrometry Metabolism Sample Analysis (LC-MS)

A volume of 200 µl of ice cold 0.1% v/v formic acid in methanol containing 20 ng/ml indinavir-d6 internal standard (ISTD) was added to a 20 µl sample and vortexed for 60 s. This was followed by ultra-sonication for 5 min and centrifugation at 10000 rpm for 10 min. A volume of 180 µl of the supernatant was transferred to clean tubes and evaporated under nitrogen gas at 40 ^{o}C. The residue of the ISTD, as well as that of the metabolism samples, was reconstituted with 100 µl of 0.1% formic acid in water, vortex mixed for 60 s and transferred into a 96-wellplate for liquid chromatography linked to mass spectrometry (LC-MS/MS) analysis. A volume of 10 µl of each of the reconstituted solutions was injected onto the column.

Chromatography was performed on an Eclipse plus C18 (2.1 x 50 mm, 3.5 µm) analytical column using an Agilent 1100 series HPLC. Detection of indinavir, M6 (indinavir metabolite) and the internal standard (indinavir-d6) was performed on an AB Sciex API 3200 mass spectrometer (electrospray ionisation [ESI] in the positive ion mode) and the settings on the apparatus are summarised in Tables **1** and **2**, respectively.

Table 1. Ionization source settings.

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	50
Turbo gas (Gas 2) (arbitrary unit)	40
Curtain gas (CUR) (arbitrary unit)	20
Collision gas (CAD) (arbitrary unit)	3
Source temperature (TEM) (°C)	500
Ion Spray Voltage (IS) (V)	3500

The mobile phase consisted of a mixture of A and B at 65:35 v/v; where A was 0.1 % formic acid in water and B was 0.1 % formic acid in a mixture of acetonitrile and isopropanol (90:10; v/v). The mobile phase was delivered at a constant flow rate of a 380 µl/min. The column was kept in a column compartment at 40 °C. The injection needle was rinsed with mobile phase before each injection for 30 s using the flush port wash station. The samples were cooled to 4 °C while awaiting injection. The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 614.4, m/z 523.4 and m/z 620.5 to the product ions at m/z 421.3, m/z 273.1 and m/z 421.2 for indinavir, M6 and the internal standard, respectively.

Table 2. Mass spectrometer detector settings.

MS/MS Settings	Indinavir	M6	ISTD
Protonated molecular mass (*m/z*)	614	523	620
Product ion molecular mass (*m/z*)	421	273	421
Dwell time (ms)	200	200	200
Declustering potential (DP) (V)	45	40	40
Entrance potential (EP) (V)	12	9	12
Collision cell entrance potential (CEP) (V)	35	40	25
Collision energy (CE) (eV)	50	45	45
Collision cell exit potential (CXP) (V)	11	11	8
Scan Type	MRM	MRM	
Polarity	Pos	Pos	
Pause time	5 ms	5 ms	

2.3.5. Metabolite to Drug Ratio

The chromatographic peak area was used to determine the relative amount of the metabolite (M6) in relation to the relative amount residual drug (indinavir) in each sample as previously described [20]. This equation enables normalization between samples.

$$\text{Metabolite to drug ratio} = \frac{\text{Peak area Metabolite}}{\text{Peak area Drug}} \qquad \text{Eq. (3)}$$

2.4. *In vivo* Pharmacokinetic Study Design

A total of 48 male Sprague Dawley rats weighing 250-300 g were randomly selected and divided into seven different groups with six animals per group. The solutions were administered by means of an oral gavage at a volume of 500 µl per animal. The experimental solutions consisted of 5% w/v of each of the aloe materials (i.e. *A. vera* gel, crude precipitated polysaccharides and each of the four molecular weight fractions) together with indinavir (40 mg/kg) in distilled water. Indinavir (40 mg/kg) was administered alone as a negative control. Blood samples (200 µl) were collected from the tail veins of the animals at 10, 20, 30, 60, 120, 180 and 240 min after oral administration of each experimental and control solution. The blood samples were centrifuged for 20 min at 5000 rpm, after which the plasma was recovered from each blood sample. The plasma samples were kept at -80° C until analysis for indinavir.

2.4.1. Liquid Chromatography Linked Mass Spectrometry Blood Plasma Analysis (LC-MS)

A stock solution of indinavir was prepared in DMSO at a concentration of 1 mg/ml. Blank Sprague Dawley plasma was spiked with the stock solution to obtain standard 1 (STD 1) at a concentration of 2000 ng/ml. Dilution with blank plasma resulted in STD 2 (500 ng/ml), STD 3 (125 ng/ml), STD 4 (32 ng/ml), STD 5 (8 ng/ml) and STD 6 (2 ng/ml). Quality control samples were also prepared in the same pool of rat plasma at 1600 ng/ml, 400 ng/ml, 50 ng/ml, 10 ng/ml and 2 ng/ml. The calibration standards and quality control samples were briefly vortexed, aliquotted into labelled polypropylene tubes, and also stored at -80 °C. The plasma concentration of indinavir was measured with the LC-MS method as described above for the metabolism study. Samples above the upper limit of quantification were diluted 4 times with blank plasma and reanalyzed in a repeat batch.

2.4.2. Pharmacokinetic Data Processing

Bioavailability profiles were constructed using WinNonlin software (Pharsight Corporation, California USA) to obtain the relevant pharmacokinetic parameters (i.e. peak plasma concentration (C_{max}) and area under the curve (AUC)). The relative bioavailability (F_{rel}) of indinavir was calculated by the following equation:

$$F_{rel} = \frac{[AUC]_A}{[AUC]_B} \quad \text{Eq. (4)}$$

Where $[AUC]_A$ is the area under the curve for indinavir in the presence of the experimental material and $[AUC]_B$ is the area under the curve for indinavir alone.

2.5. Statistical Data Analysis

Data analyses were performed with STATISTICA Ver 12. ANOVA's with Tukey's Honest significant post-hoc tests were performed and statistically significant differences were accepted when $p < 0.05$. All results were verified with non-parametric Kruskall-Wallis and Dunn's post-hoc tests.

3. RESULTS AND DISCUSSIONS

3.1. Quantitative ^{1}H-NMR

The ^{1}H-NMR spectrum for *A. vera* gel (starting material) is shown in Fig. (**1**), while the quantities of the marker molecules in the aloe materials are summarised in Table **3**.

From Table **3** it is clear that the *A. vera* gel starting material contained all three marker molecules expected to be present in fresh *A. vera* leaf gel. Aloverose (or acetylated polymannose) was present in different concentrations in all the aloe leaf gel materials except for molecular weight fraction 4 (i.e. the fraction that passed through the centrifugal device membrane with 30 kDa as molecular weight cut-off).

3.2. GFC-MALLS-RI

Successive filtering of the precipitated polysaccharides through the membranes of the centrifugal devices resulted in fractions with molecular weight values as shown in Table **4**.

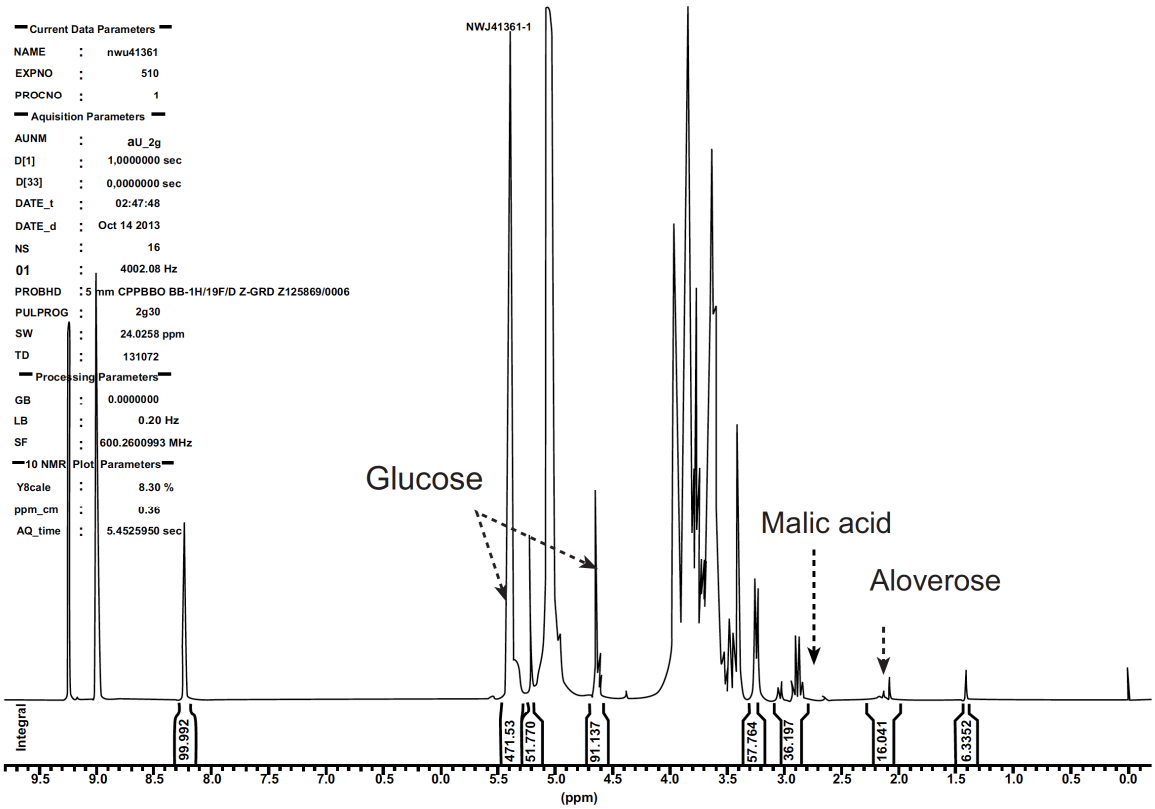

Fig. (1). ^{1}H-NMR spectrum of *Aloe vera* gel (starting material).

Table 3. Quantities (% w/w dry mass) of marker molecules in the *A. vera* leaf gel materials.

Aloe material	Aloverose (% w/w)	Glucose (% w/w)	Malic acid (% w/w)
A. vera gel	1.0	19.3	1.7
CPP	0.4	4.9	ND
MWF 1	1.1	1.4	ND
MWF 2	1.7	1.1	ND
MWF 3	detected	detected	ND
MWF 4	ND	2.7	ND

CPP = crude precipitated polysaccharides, MWF = molecular weight fraction, ND = not detected.

Table 4. Molecular weight values of the different *A. vera* leaf gel materials.

Aloe material	M_n (g/mol) ($\times 10^3$)	M_w (g/mol) ($\times 10^4$)	M_w/M_n	*dn*/*dc* (mL/g)
AVG	60.0 ± 20[1]	13.1 ± 18	2.20	0.030 ± 1.3
CPP	22.1 ± 4.7	3.81 ± 2.4	1.74	0.125 ± 2.5
MWF 1	14.2 ± 4.2	3.32 ± 1.5	2.35	0.109 ± 3.7
MWF 2	12.9 ± 4.5	2.27 ± 2.7	1.77	0.131 ± 0.7
MWF 3	11.4 ± 2.1	1.76 ± 1.5	1.51	0.125 ± 1.8
MWF 4	7.03 ± 7.2	1.30 ± 6.1	1.75	0.119 ± 4.1

[1]Error values indicate % coefficient of variance for $n = 6$, AVG = *Aloe vera* gel, CPP = crude precipitated polysaccharide, MWF = molecular weight fraction, M_w = weight absolute molecular weight, M_n = weight number absolute molecular weight.

Considering the large size exclusion ranges of the Macrosep® advance centrifugal devices that were used for separation of the precipitated polysaccharide fraction of the *A. vera* gel, it was surprising to find molecular weights that were considerably lower than expected. However, traces of a very high molecular weight material resided in the unfractionated starting material. It was estimated from the areas of the refractive index (RI) signal in the chromatogram that ~0.22% of this 10^6 g/mol material was present in the starting material, which is in the same order as the 200:1 (~0.5%) ratio indicated by the manufacturer. Furthermore, with successive filtering, the traces of the high weight polymer virtually disappeared as seen in Fig. (**2**).

The relatively large molecule (10^6 g/mol) appeared only during the first steps of centrifugal filtration followed by the lower molecular weight fractions. As centrifugal filtration proceeded, only a single fraction could be observed for all polymers, which demonstrated a molecular weight in the order of 10^4 g/mol. The fairly large elution volumes also illustrate the polydispersity of the polysaccharide fractions.

The filtered fractions showed M_w values on the lower order of 10^4 g/mol, suggesting that polymeric material was present in the fractionated samples. In addition, the molecular weight was in the same range as found for acemannan (4.5×10^4 g/mol) extracted from fresh samples of *A. vera* using water as extractant [21]. Qualitatively, the dn/dc values of the filtered fractions analyzed in this study would suggest the presence of a polysaccharide since the reported dn/dc value for glucomannan is 0.140 [22] and for trimethylated chitosan is 0.130 [23].

3.3. TEER Studies

The TEER values of the Caco-2 cell monolayers exposed to the *A. vera* gel materials (1.0% w/v) and sodium lauryl sulphate (positive control group) plotted as a function of time are shown in Fig. (**3**). A decrease in the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers is associated with opening of the tight junctions between adjacent epithelial cells [24] and gives an indication of the potential to enhance paracellular drug transport across the intestinal epithelium.

From Fig (**3**) it is clear that all the aloe materials decreased the TEER of the Caco-2 cell monolayers to different extents, which is in line with results from previous *in vitro* TEER studies involving *A. vera* gel and whole leaf materials [10]. The precipitated polysaccharides decreased the TEER to a higher extent than the aloe gel material, which indicates that the molecules responsible for opening of tight junctions are more concentrated the precipitated material than in the gel material. Interestingly, MWF 3 consisting of the second lowest average molecular weight polysaccharides showed

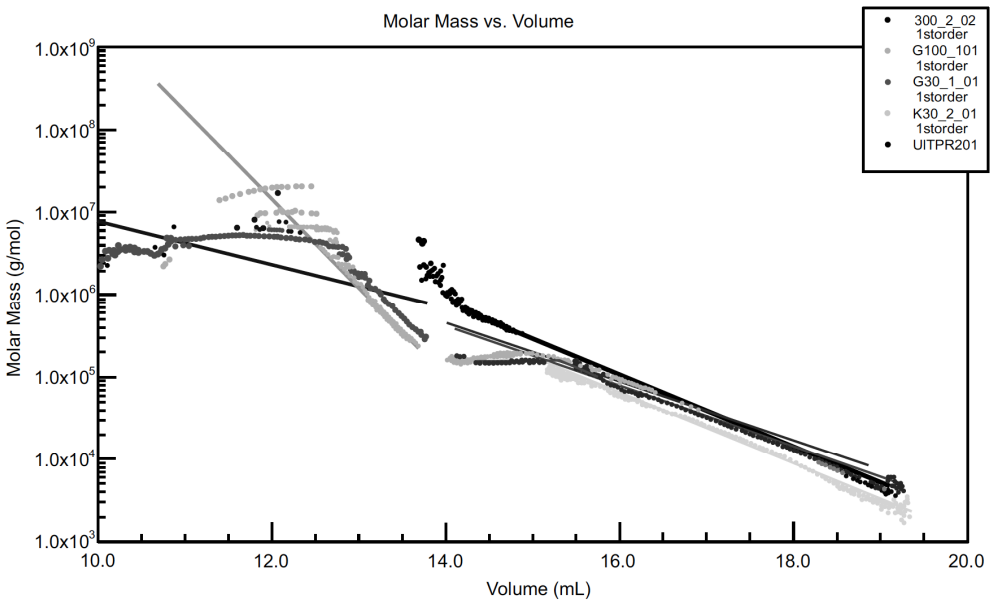

Fig. (2). Molar mass as a function of volume of the polymers in the *A. vera* gel materials.

the highest effect on TEER reduction. The effect of the different molecular weight fractions of the polysaccharides on the TEER did not correlate directly to their average molecular weight values as determined by GFC-MALLS-RI.

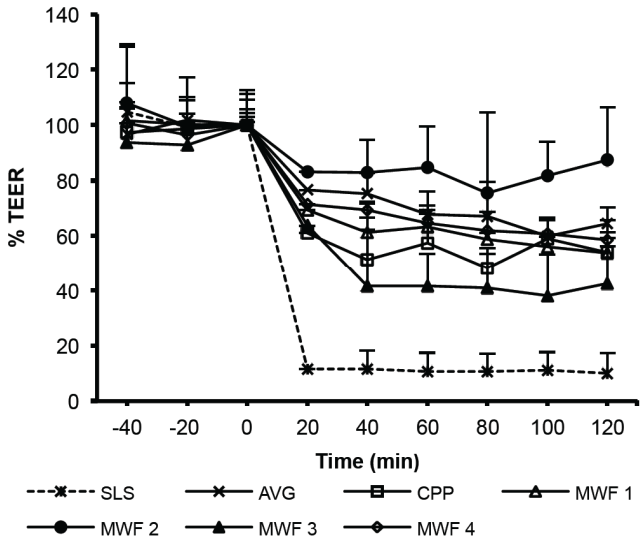

Fig. (3). Percentage transepithelial electrical resistance (TEER) of Caco-2 cell monolayers plotted as a function of time when exposed to 1.0% w/v of the *A. vera* gel materials. AVG = *Aloe vera* gel, CPP = crude precipitated polysaccharide, MWF = molecular weight fraction, SLS = Sodium Lauryl Sulphate (positive control).

3.4. Metabolism Study

Fig. (**4**) provides the metabolite (i.e. M6) to parent drug (i.e. indinavir) ratio values obtained from the *in vitro* metabolism study for all the experimental groups as well as normal control (indinavir alone) and positive control (indinavir with ketokonazole) groups. The positive control group (indinavir with ketoconazole) decreased the M6/indinavir ratio value when compared to that of the normal control group (indinavir alone), which indicated the suitability of the LS180 cell model for investigation of indinavir metabolism.

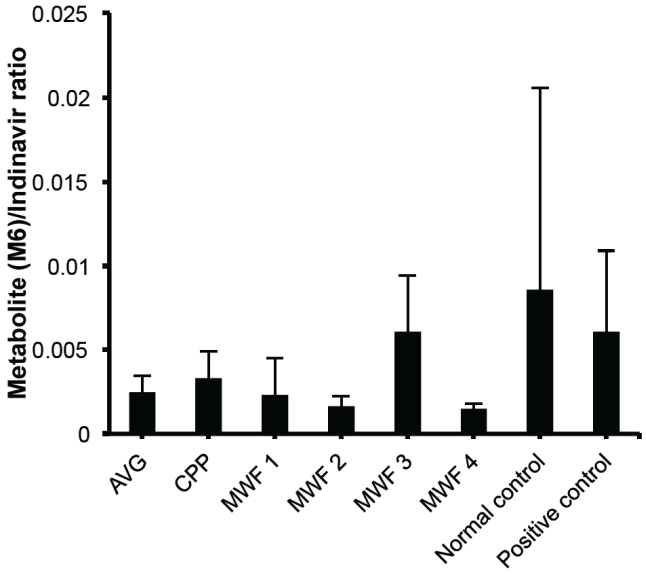

Fig. (4). Metabolite (M6) to indinavir ratio values in the presence of different aloe leaf gel materials as well as ketokonazole (positive control) and indinavir alone (normal control). AVG = *A. vera* gel, CPP = crude precipitated polysaccharides, MWF = molecular weight fraction.

From Fig. (**4**), it is clear that all the aloe gel materials showed lower metabolite (M6) to parent drug (indinavir) ratio values as compared to that of the normal control group (indinavir alone), which represent an enzyme inhibition

effect. The M6/indinavir ratio values were, however, not statistically significantly ($p > 0.05$) different from that of either of the control groups. Nonetheless, this enzyme inhibition effect may contribute to pharmacokinetic interactions and specifically enhance the bioavailability of drugs that are substrates for the same enzymes when administered together with aloe leaf materials.

3.5. *In vivo* Pharmacokinetic Study

The plasma concentration time curves of indinavir administered to rats with and without the aloe gel materials are shown in Fig. (**5**), while the bioavailability parameters namely area under the curve (AUC) and maximum plasma concentration (C_{max}) values are listed in Table **5**.

In the MWF1 and MWF 2 groups, some of the C_{max} values were obtained at different time points within the individual rats, which resulted in double peak curves with apparent lower C_{max} values (distributed over 2 time points). However, the average C_{max} values calculated from the highest plasma concentrations obtained in each rat (irrespective of time) resulted in higher C_{max} values as indicated in Table **5**.

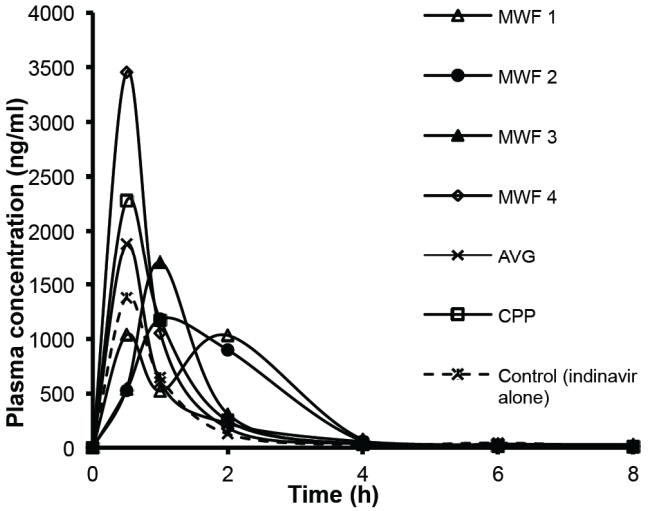

Fig. (5). Plasma concentration (ng/ml) time curves of indinavir in rats administered with and without the different aloe gel materials (error bars omitted for reasons of clarity, please refer to Table 3 for standard deviations).

From the results of the *in vivo* study, it is clear that the maximum indinavir plasma concentration (C_{max}) values were increased when compared to that of indinavir alone (control group) by *A. vera* gel, crude precipitated polysaccharides and MWFs 3 and 4. On the other hand, the areas under the curve (AUC) values were increased by all the treatment groups when compared to that of indinavir alone (Table **5**). These changes represent relative bioavailability values of 1.28 for *A. vera* gel, 1.67 for crude precipitated polysaccharides, 1.84 for MWF 1, 1.77 for MWF 2, 1.39 for MWF 3 and 1.95 for MWF 4. However, these changes were not statistically significantly ($p > 0.05$) different from that of the control group (indinavir alone). The relatively high effect of the crude precipitated polysaccharides as well as MWFs 3 and 4 on indinavir bioavailability correlates well with their performance *in vitro* in terms of TEER reduction and metabolism inhibition.

Table 5. Area under the curve ($AUC_{0-\infty}$) and maximum plasma concentration (C_{max}) values for indinavir administered to rats with and without the aloe leaf gel materials

Group	$AUC_{0-\infty}$ (min.µmol/L)	C_{max} (ng/ml)
Indinavir alone (control group)	148.8 ± 55.5	1396.4 ± 823.6
Indinavir with AVG	190.0 ± 96.4	1879.1 ± 1100.2
Indinavir with CPP	248.0 ± 167.5	2276.7 ± 1095.4
Indinavir with MWF 1	274.0 ± 234.1	1712.5 ± 1400.2
Indinavir with MWF 2	263.0 ± 173.9	1859.1 ± 1068.1
Indinavir with MWF 3	207.0 ± 46.4	1782.0 ± 337.4
Indinavir with MWF 4	290.0 ± 152.5	3456.7 ± 2146.7

The increased relative bioavailability values of indinavir by the aloe materials may be explained by a combination of mechanisms as indicated in the *in vitro* studies namely opening of tight junctions (as indicated by the decrease in TEER of Caco-2 cell monolayers) as well as inhibition of indinavir metabolism (as indicated by the decrease in M6/indinavir ratio values in LS180 cells). However, additional mechanisms for the precipitated and isolated polysaccharide fractions such as modulation of P-gp related efflux of indinavir cannot be excluded as previously shown by *in vitro* studies [12].

The results from this study indicate modulation of indinavir bioavailability by *A. vera* gel materials, which was higher for the precipitated polysaccharides and the isolated polysaccharide fractions when compared to that of the *A. vera* gel material (Table **5**). This indicates that the biologically active components in terms of drug pharmacokinetic modulation and absorption enhancement most probably lie within the polysaccharide part of *A. vera* gel, but it cannot be directly correlated to the molecular size of these molecules. Although aloverose was not detected in MWF 4 (Table **1**), this fraction had a relatively large effect on indinavir bioavailability. Aloverose may therefore contribute to drug pharmacokinetic interactions, but is not required to be present to give such an effect.

The results suggest potential overdosing of indinavir in patients that take *A. vera* leaf materials simultaneously, but further research is necessary to determine if this effect will result in adverse effects. One area of concern is the relatively high standard deviations observed for the *in vitro* results as well as the relatively high intra-subject variations for the *in vivo* indinavir bioavailability parameters. This may complicate the use of *A. vera* gel materials as drug absorption enhancers because large intra-subject variation has been highlighted as one of the major problems experienced with

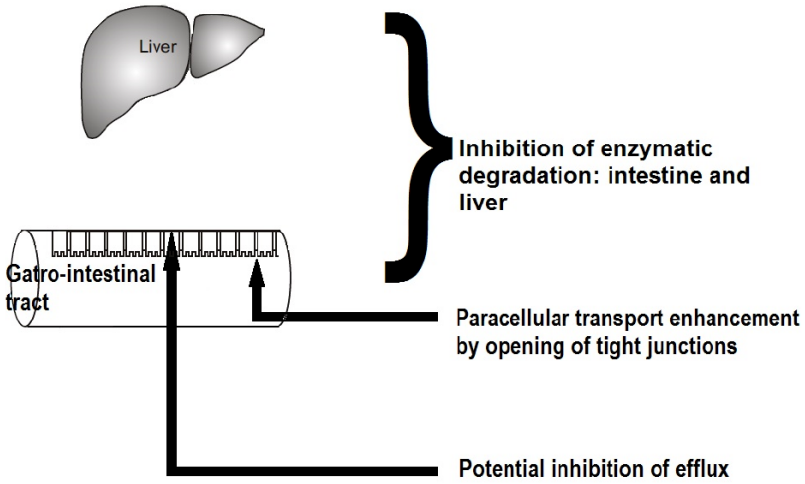

Fig. (6). Schematic illustration of the potential mechanisms of interactions between *A. vera* and indinavir.

chemical drug absorption enhancing agents that prevented further clinical development [25].

4. STANDARD PROTOCOL ON APPROVALS, REGISTRATIONS, PATIENT CONSENTS & ANIMAL PROTECTION

The *in vivo* study in Sprague Dawley rats was approved by the Faculty of Health Sciences Animal Ethics Committee of the University of Cape Town (reference number: 014/0140, date of approval: 06/11/2014). All animal experiments were conducted according to international and national standards for humane handling of animals.

CONCLUSION

The *in vitro* and *in vivo* results from this study indicate that *A. vera* gel materials have the potential to modulate drug pharmacokinetics by opening tight junctions and inhibiting metabolism, but inhibition of efflux transporters cannot be excluded (Fig. (**6**)). The effects observed on cell cultures and plasma levels of indinavir in rats were higher for the precipitated polysaccharides and for some of the isolated molecular weight fractions when compared to that of the *A. vera* gel material. This indicates that the biological active molecules in terms of modulating drug pharmacokinetics are amongst the polysaccharide portion of the aloe leaf gel material. The drug pharmacokinetic modulation effect by the aloe leaf materials was not dependent on the presence of aloverose (acetylated polymannose).

CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors have no other relevant financial involvement other than mentioned in the acknowledgements section below.

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PATIENT CONSENT

Declared none.

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Chapter 4

Final conclusions and future prospects

4.1 Final conclusions

Aloe vera (*L.*) Burm.f. (*Aloe barbadensis* Miller) has long been implemented for its medicinal purposes, in various cultures, ranging from the pre-modern Grecian empire to the innovative Egyptian of old (Surjushe e*t al.,* 2008:163). Some of the biological uses for this succulent include the treatment of skin conditions, viral- and parasitic infections and as an anti-inflammatory agent (Amoo e*t al.,* 2014:19). The anti-microbial, anti-plasmodial, anti-oxidant, anti-cancer and immune-modulatory properties of *A. vera* have also been widely documented (Ni e*t al.,* 2004:1746; Kang e*t al.,* 2014:366). Most of these biological activities of the plant have been contributed to polysaccharides found within the leaf gel. It is due to these biological properties that the use of this plant, specifically in the management of HIV/AIDS, by traditional healers in Southern Africa has drastically increased over the past few years (Chinsembu *et al.,* 2015:35). In previous studies, *A. vera* has been found to have an *in vitro* synergistic effect, when used in combination with specific nucleoside reverse-transcriptase inhibitors, in cells infected with both the HIV-1 and herpes simplex virus (HSV-1) (Stargrove e*t al.,* 2008:4). This anti-viral mechanism of action is attributed to the inhibition of glycosylation of the viral proteins (Reynolds & Dweck, 1999:19). Because of the opening of tight junctions, associated with the paracellular pathway, *A. vera* is also regarded as an excellent absorption enhancer, allowing macromolecular drugs to permeate biological membranes and therefore reach the site of pharmacological action (Renukuntla *et al.,* 2013:76).

During this study, precipitated polysaccharides from *A. vera* gel were separated into different molecular weight fractions (MWFs) ranging between 30 and 300 kDa. These fractions, along with *A. vera* leaf gel and precipitated polysaccharides from the *A. vera* gel, were chemically characterised by means of nuclear magnetic resonance spectroscopy, gel filtration chromatography linked to multi-angle laser-light scattering and a refractive index detector. The average molecular weight of each of the aloe leaf materials investigated in this study as well as the presence and quantities of specific marker molecules in each of the fractions was determined. All of the *A. vera* marker molecules (including aloverose, glucose and malic acid) were present in the starting *A. vera* gel material, whereas aloverose was present in all of

the aloe leaf gel materials at different concentrations except for the MWF that passed through the centrifugal device membrane with 30 kDa as molecular weight cut-off value.

The Caco-2 cell model was implemented in order to determine the effect of each of the *A. vera* gel materials on the transepithelial electrical resistance (TEER) of the cell monolayers. The precipitated polysaccharides decreased the TEER to a higher extent than the *A. vera* gel material. This indicated that the molecules responsible for opening of the tight junctions were more concentrated in the precipitated material, than in the gel material. The MWFs decreased the TEER of the Caco-2 cell monolayers to different extents. The average molecular weight value of each of the MWFs, however, did not correlate directly to the effect that each MWF had on the TEER of the Caco-2 cell monolayers. With regard to the metabolism of indinavir, all of the aloe gel materials displayed lower metabolite (M6) to parent drug (indinavir) ratio values, when compared to the values exhibited by the control group (indinavir alone), which signifies an enzyme inhibition effect.

During the *in vivo* bioavailability study of indinavir done in the Sprague-Dawley rat model, the effect of the *A. vera* gel materials was determined on the blood plasma levels of indinavir. Blood samples obtained at pre-determined time intervals were analysed with a sensitive and selective liquid chromatography linked to mass spectrometry (LC-MS) method. The maximum indinavir plasma concentration (C_{max}) values were increased by *A. vera* gel, the crude precipitated polysaccharides and two of the MWF's, when compared to that of indinavir alone (control group). The area under the curve (AUC) values were, however, increased by all of the treatment groups. This data represented relative bioavailability values between 1.28 (*A. vera* gel) and 1.95 (MWF 4). The effect of the *A. vera* materials on the indinavir bioavailability, correlated well with the data procured for each of the materials during the *in vitro* studies, in terms of TEER reduction and metabolism inhibition. This indicated modulation of indinavir bioavailability by *A. vera* gel materials, which was higher for the precipitated polysaccharides and some of the isolated polysaccharide fractions when compared to that of the *A. vera* gel material.

Chapter 4

4.2 Future recommendations

The following aspects are recommended for further investigation in future studies:

- Further studies should be done on the modulation effects of *A. vera* gel materials on the bioavailability of a series of drugs representative from the different classes of the Biopharmaceutical Classification System.
- The mechanism of action of *A. vera* gel materials on the transepithelial electrical resistance (TEER) of cell monolayers should be further investigated. This can be done by means of immunofluorescent staining of the tight junctions with antibodies raised against occludin, tricellulin and claudins and analysed by means of confocal laser scanning microscopy.
- The drug absorption enhancing capacity of the different aloe leaf materials should be determined by conducting *in vitro* transport studies using model compounds such as FITC-dextran molecules with a wide range of molecular weights (i.e. ranging from 3 kDa to 40 kDa).
- The reversibility of the membrane permeation enhancing effects of the aloe materials should be investigated on different models (e.g. cell cultures and excised tissues) by measuring transepithelial electrical resistance over an extended period of time after removal of the materials.
- The aloe materials should be included in solid oral dosage forms and investigated for macromolecular drug delivery across a suitable *in vitro* model. These studies should then be followed up by *in vivo* studies to determine if these dosage forms can deliver drugs at therapeutically acceptable levels.

4.3 References

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Appendix A

Chemical characterization of Aloe gel materials

A1 Quantitative ^{1}H-NMR

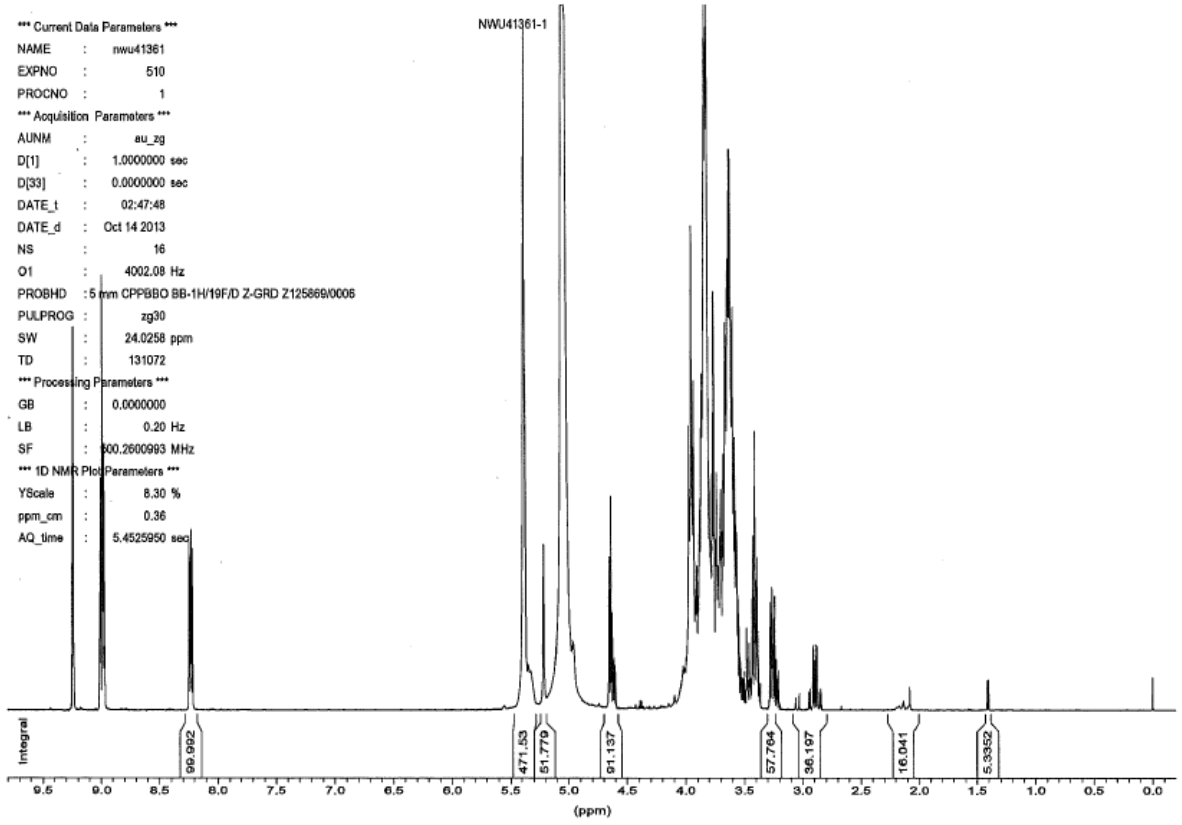

Figure A.1: 1H-NMR spectrum of *Aloe vera* gel (AVG)

Appendix A

Table A.1: Quantities (% w/w dry mass) of marker molecules in the *A. vera* gel material (AVG) as determined by quantitative ^{1}H-NMR spectrometry

Aloe vera gel (AVG)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	1.0	70.2	Fresh Aloe Vera
Glucose	19.3	1354.5	Fresh Aloe Vera
Malic acid	1.7	116.8	Fresh Aloe Vera
Lactic acid	0.1	9.3	Degradation (bacterial)
Citric acid	0.2	11.7	WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	63.4	4440.9	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Other**	Not detected		Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.
**Organic and inorganic compounds not to be quantified by NMR analysis.

Appendix A

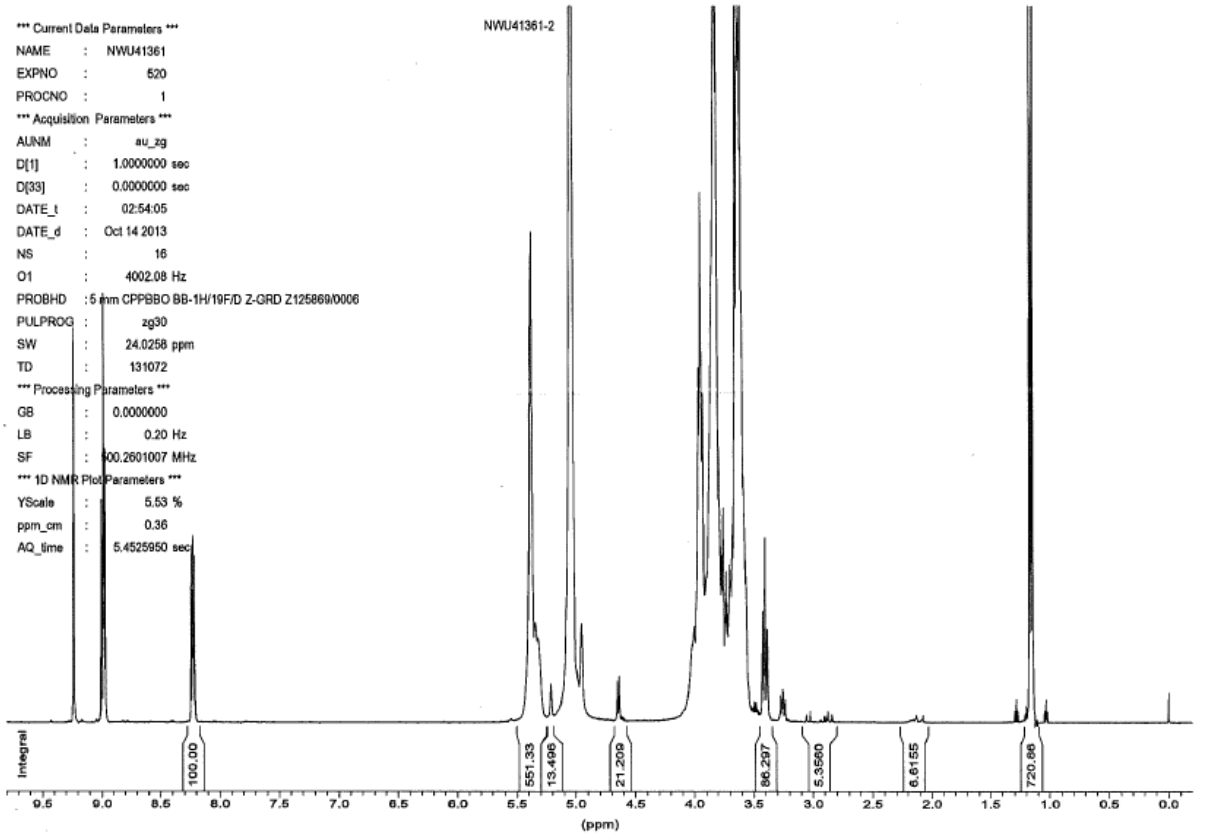

Figure A.2: 1H-NMR spectrum of crude precipitated polysaccharides (CPP)

Appendix A

Table A.2: Quantities (% w/w dry mass) of marker molecules in the crude precipitated polysaccharide material (CPP) as determined by quantitative ^{1}H-NMR spectrometry

Crude precipitated polysaccharides (CPP)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	0.4	27.9	Fresh Aloe Vera
Glucose	4.9	340	Fresh Aloe Vera
Malic acid	Detected		Fresh Aloe Vera
Lactic acid	Not detected		Degradation (bacterial)
Citric acid	Detected		WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	71.4	5000.3	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Ethanol	8.8	618.2	Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.

Appendix A

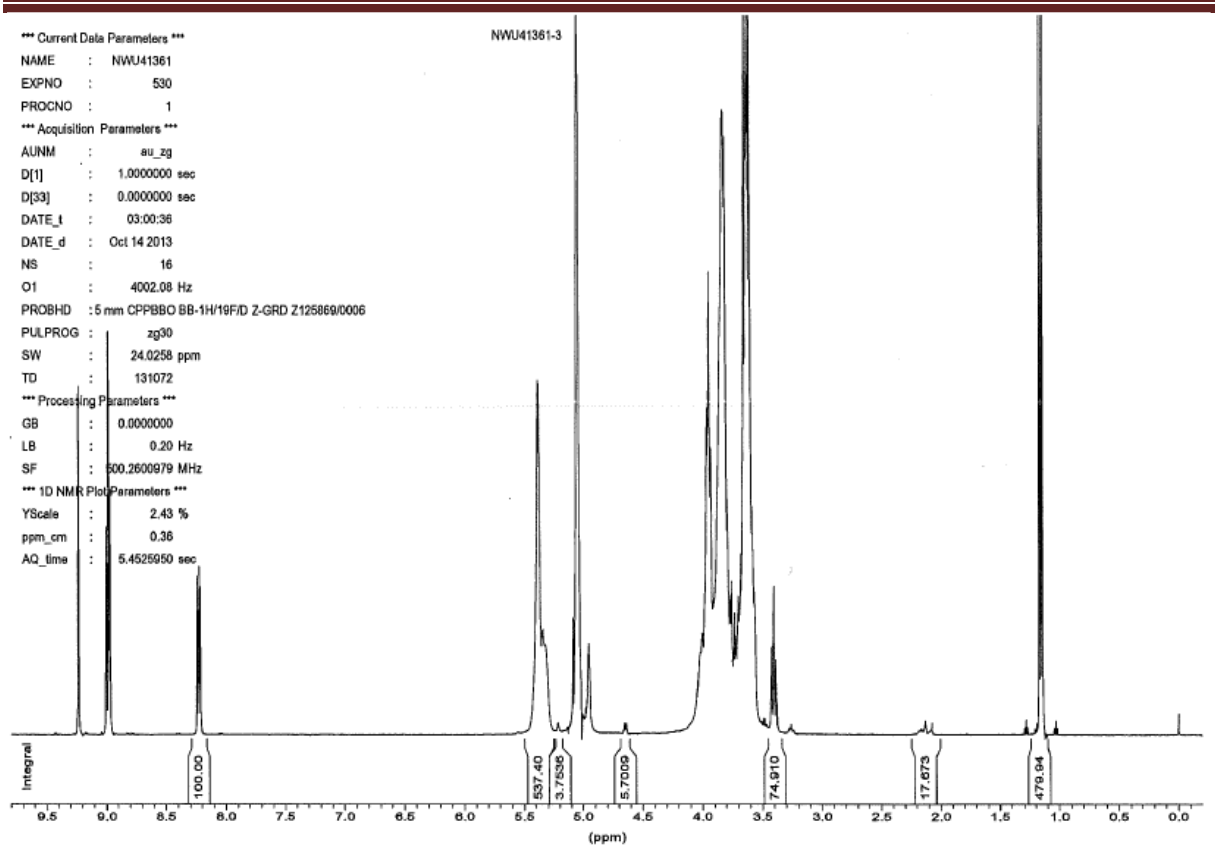

Figure A.3: 1H-NMR spectrum of polysaccharide molecular weight fraction 1 (MWF1)

Appendix A

Table A.3: Quantities (% w/w dry mass) of marker molecules in polysaccharide molecular weight fraction 1 (MWF1) as determined by quantitative ^{1}H-NMR spectrometry

Polysaccharide molecular weight fraction 1 (MWF1)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	1.1	77.2	Fresh Aloe Vera
Glucose	1.4	97.9	Fresh Aloe Vera
Malic acid	Not detected		Fresh Aloe Vera
Lactic acid	Not detected		Degradation (bacterial)
Citric acid	Not detected		WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	72.10	5046.85	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Ethanol	6.1	426.2	Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.

Appendix A

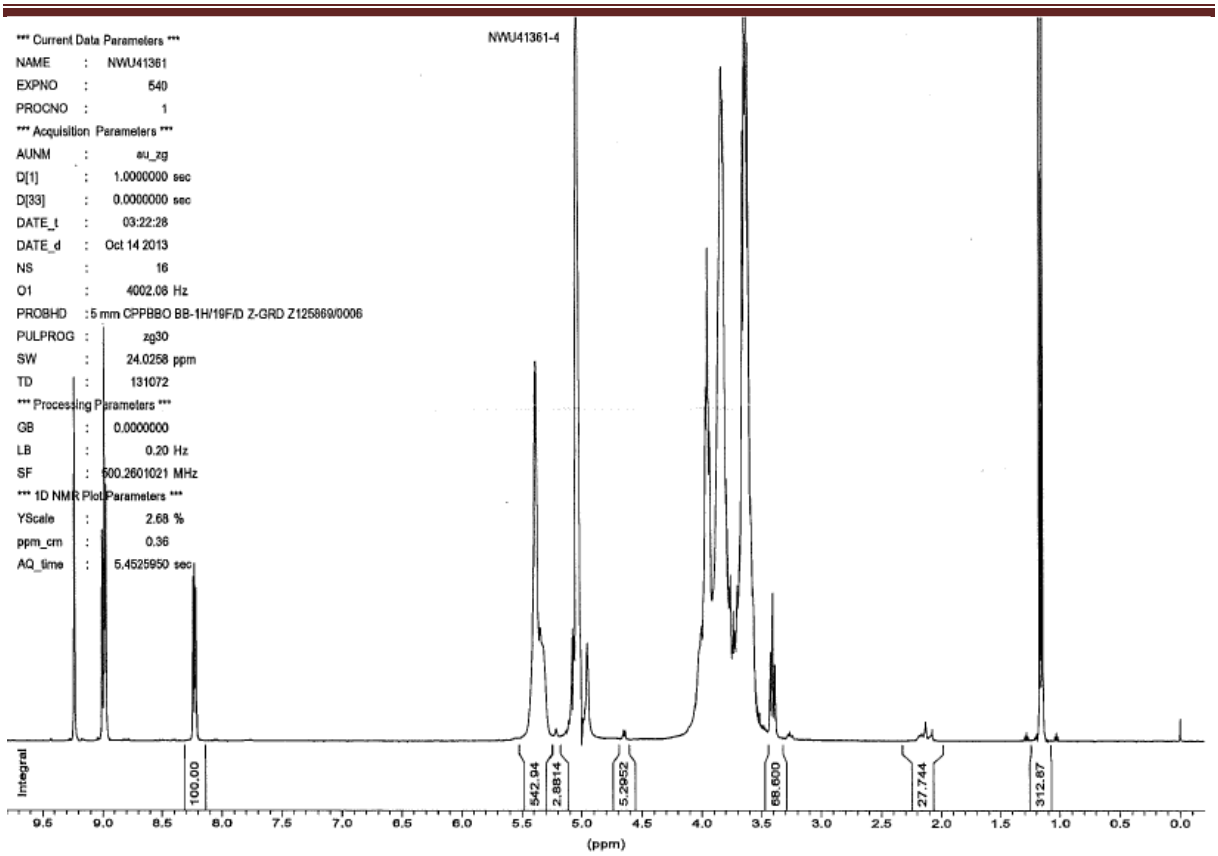

Figure A.4: 1H-NMR spectrum of polysaccharide molecular weight fraction 2 (MWF2)

Appendix A

Table A.4: Quantities (% w/w dry mass) of marker molecules in polysaccharide molecular weight fraction 2 (MWF2) as determined by quantitative ^{1}H-NMR spectrometry

Polysaccharide molecular weight fraction 2 (MWF2)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	1.7	121.4	Fresh Aloe Vera
Glucose	1.1	75.3	Fresh Aloe Vera
Malic acid	Not detected		Fresh Aloe Vera
Lactic acid	Not detected		Degradation (bacterial)
Citric acid	Not detected		WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	73	5110.5	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Ethanol	4.0	278.5	Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.

Appendix A

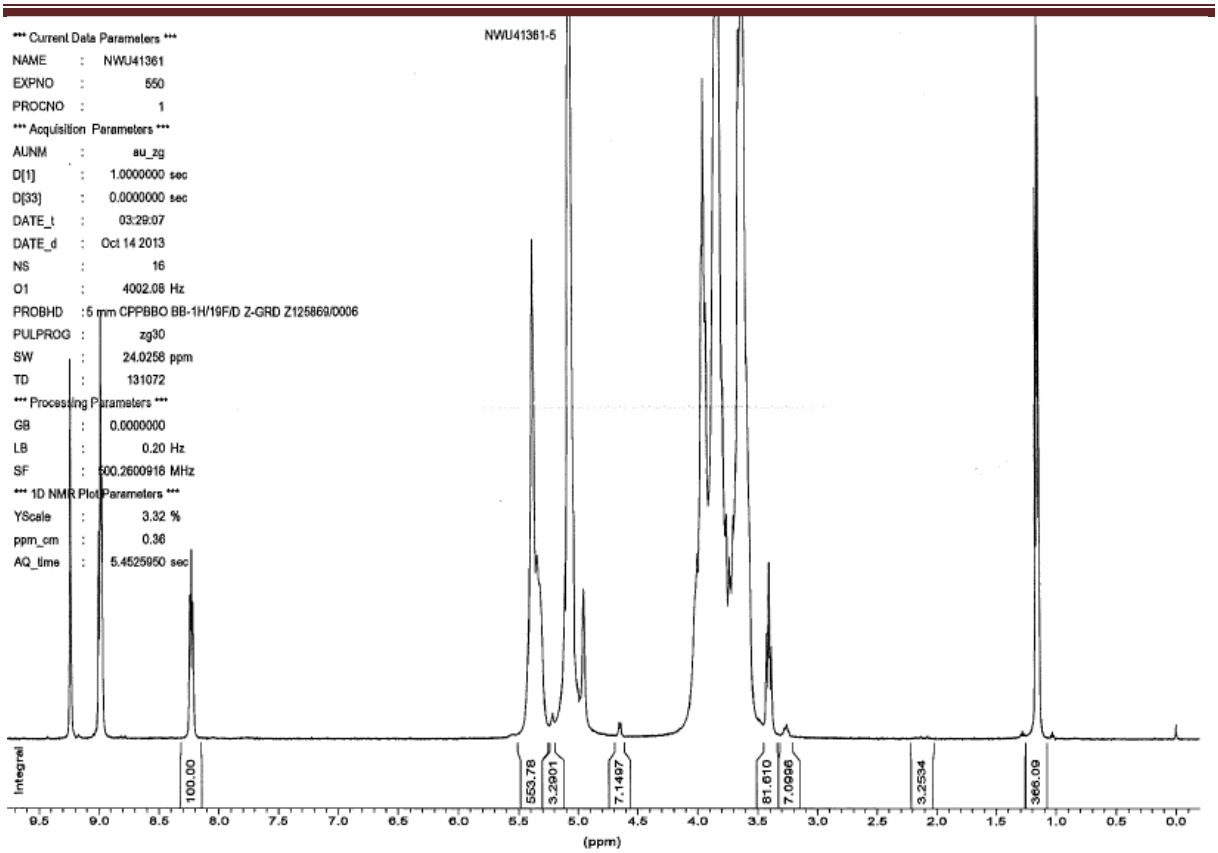

Figure A.5: 1H-NMR spectrum of polysaccharide molecular weight fraction 3 (MWF3)

Appendix A

Table A.5: Quantities (% w/w dry mass) of marker molecules in polysaccharide molecular weight fraction 3 (MWF3) as determined by quantitative ^{1}H-NMR spectrometry

Polysaccharide molecular weight fraction 3 (MWF3)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	Detected		Fresh Aloe Vera
Glucose	Detected		Fresh Aloe Vera
Malic acid	Not detected		Fresh Aloe Vera
Lactic acid	Not detected		Degradation (bacterial)
Citric acid	Not detected		WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	74.5	5212.4	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Ethanol	4.7	325.8	Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.

Appendix A

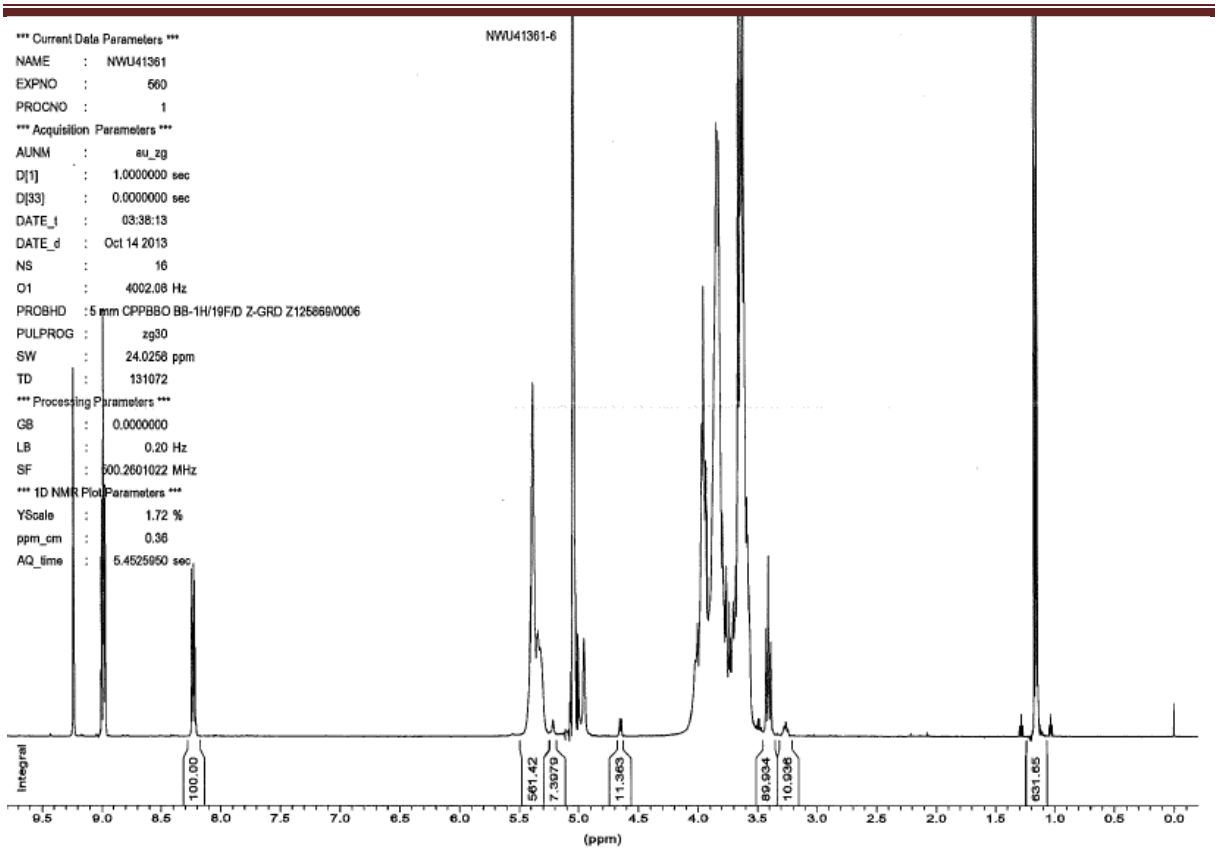

Figure A.6: 1H-NMR spectrum of polysaccharide molecular weight fraction (MWF4)

Appendix A

Table A.6: Quantities (% w/w dry mass) of marker molecules in polysaccharide molecular weight fraction 4 (MWF4) as determined by quantitative ^{1}H-NMR spectrometry

Polysaccharide molecular weight fraction 4 (MWF4)			
	Content [1%]*	**Content [mg/l]***	**Origin of component**
Aloverose	Not detected		Fresh Aloe Vera
Glucose	2.7		Fresh Aloe Vera
Malic acid	Not detected		Fresh Aloe Vera
Lactic acid	Not detected		Degradation (bacterial)
Citric acid	Not detected		WLM or added acidifier
WLM	Not detected		Whole leaf marker (WLM)
Maltodextrin	72.7	5091.7	Formulation aid for drying
Acetic acid	Not detected		Degradation (Hydrolysis)
Succinic acid	Not detected		Degradation (Enzymatic)
Fumaric acid	Not detected		Degradation (Enzymatic)
Sodium benzoate	Not detected		Added preservative
Potassium sorbate	Not detected		Added preservative
Ethanol	7.7	541.7	Unknown

*The content data [%] refer to dry matter. The content data [mg/l] refer to dry weight 0.7 [%] for powders.

Appendix B

In vitro studies: Data

Data obtained of the effect of the *A. vera* gel, precipitated polysaccharides and the different polysaccharide molecular weight fractions on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers and the metabolism of indinavir in LS180 cells, is specified in this chapter.

B1 Transepithelial electrical resistance (TEER)

Table B.1: Transepithelial electrical resistance of 0.1% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	860	720	710	720	140	140	130	130	140	120
SLS 2	2800	2670	2370	2280	180	190	170	180	180	180
SLS 3	2220	2080	2050	2150	160	150	140	130	130	120
Media 1	820	1300	1070	1420	860	810	520	500	390	360
Media 2	1350	1400	1770	1940	1030	1300	880	930	860	880
Media 3	3860	3680	3020	3530	2150	2170	1940	1840	2080	2210
AVG 1	4420	4190	4210	4040	3120	3010	2920	2840	2810	2820
AVG 2	3750	3570	3550	2380	2680	2650	2460	2480	2450	2480
AVG 3	3530	3760	3350	3650	2120	2420	1500	560	2520	2680
CPP 1	4300	4200	4170	3890	3150	2800	2880	2630	2710	2530
CPP 2	3520	3320	3240	3190	2400	2370	2300	2300	2330	2370
CPP 3	1220	1340	1640	1750	660	610	560	600	830	580
MWF 1.1	940	1630	1850	1870	850	760	810	750	1190	1230
MWF 1.2	3710	3700	3550	3120	1760	2400	2630	2560	2580	2330
MWF 1.3	3490	3180	3550	3510	1940	1920	2170	1710	2090	1940
MWF 2.1	3660	3350	2610	2710	1930	1740	1770	1910	1970	1770
MWF 2.2	4450	3830	3640	3610	2920	2580	2560	2580	2570	2580
MWF 2.3	4480	4010	4040	3850	2910	2790	2780	2790	2710	2770
MWF 3.1	1920	1790	1770	1690	1430	1400	1460	1120	1550	1560
MWF 3.2	4480	4010	3840	3690	2660	2950	2900	2820	2840	2910
MWF 3.3	3520	3360	2420	2680	1680	1760	1480	1640	1530	1680
MWF 4.1	3190	2740	2860	2660	2150	2040	2170	2210	2210	2190
MWF 4.2	4500	3940	3690	3650	2280	2830	2600	2650	2640	2610
MWF 4.3	3980	3490	3420	3390	2370	2380	2380	2340	2410	2410

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.2: Transepithelial electrical resistance of 0.5% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	860	720	710	720	140	140	130	130	140	120
SLS 2	2800	2670	2370	2280	180	190	170	180	180	180
SLS 3	2220	2080	2050	2150	160	150	140	130	130	120
Media 1	820	1300	1070	1420	860	810	520	500	390	360
Media 2	1350	1400	1770	1940	1030	1300	880	930	860	880
Media 3	3860	3680	3020	3530	2150	2170	1940	1840	2080	2210
AVG 1	2770	2450	2430	2460	2120	2030	1850	1680	1660	1720
AVG 2	2030	2070	1430	1860	1800	1200	1480	1330	1250	1370
AVG 3	4080	3830	3860	3910	2670	2270	2120	2160	1890	2170
CPP 1	3660	3740	3780	3810	2470	2320	2150	2130	2050	1880
CPP 2	2860	2630	720	650	580	560	640	560	670	700
CPP 3	3800	3500	3300	3210	2220	2200	2100	2120	2150	2150
MWF 1.1	4100	3940	3650	3730	2230	2410	2380	2220	1830	2050
MWF 1.2	5040	4770	4450	4400	3050	2940	2750	2630	2620	2590
MWF 1.3	3720	3960	3740	3640	2520	2660	2670	2550	2590	2600
MWF 2.1	3890	3640	3470	3450	2830	2670	2430	2320	2340	1930
MWF 2.2	2050	1880	2150	2280	1890	1590	1820	1760	1660	1770
MWF 2.3	4380	4130	3870	3770	1880	1800	1670	1460	1860	1910
MWF 3.1	4340	4630	4470	4470	3110	3040	2870	2620	2680	2480
MWF 3.2	3460	1810	2030	1960	1770	1700	1730	1880	1330	1560
MWF 3.3	4690	4410	4140	4150	3250	3020	2170	2700	2710	2640
MWF 4.1	4550	4390	4120	4150	2850	2860	2530	2520	2580	2550
MWF 4.2	2680	2260	2240	2760	1610	1010	790	790	710	720
MWF 4.3	660	670	680	650	610	590	590	560	580	590

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.3: Transepithelial electrical resistance of 1.0% w/w of *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	860	720	710	720	140	140	130	130	140	120
SLS 2	2800	2670	2370	2280	180	190	170	180	180	180
SLS 3	2220	2080	2050	2150	160	150	140	130	130	120
Media 1	820	1300	1070	1420	860	810	520	500	390	360
Media 2	1350	1400	1770	1940	1030	1300	880	930	860	880
Media 3	3860	3680	3020	3530	2150	2170	1940	1840	2080	2210
AVG 1	2420	1920	2210	2160	1830	1820	1690	1570	1280	1560
AVG 2	3750	3680	3730	3570	2520	2410	1930	2180	1940	1990
AVG 3	3800	3530	3550	3600	2670	2660	2550	2420	2370	2350
CPP 1	3830	3770	3820	3780	2970	2400	2260	2110	2180	2180
CPP 2	900	2220	2120	2300	1150	840	1400	930	1250	950
CPP 3	1240	2050	2200	2150	1170	1150	1100	1040	1390	1350
MWF 1.1	2700	2530	2540	2560	2040	1500	1570	1520	1300	1330
MWF 1.2	4340	3950	3780	3730	2620	2540	2350	2310	1970	1990
MWF 1.3	670	1020	1020	1020	590	580	670	560	660	570
MWF 2.1	910	890	890	950	830	860	730	740	760	770
MWF 2.2	620	830	740	650	600	600	760	560	660	730
MWF 2.3	2980	3210	2850	3140	2190	2060	1890	1950	2000	2150
MWF 3.1	3520	2730	2460	3080	2650	880	760	720	720	710
MWF 3.2	3520	3420	3600	3590	2120	1870	1810	1850	1830	1900
MWF 3.3	3890	3090	3130	3190	1460	1400	1590	1520	1270	1640
MWF 4.1	3690	3490	3420	3410	2400	2470	2310	2240	2030	2060
MWF 4.2	3910	3220	2900	3380	2500	2360	2330	2180	2320	2050
MWF 4.3	4250	3720	3660	3560	2480	2330	2020	1970	1930	1930

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.4: Calculated transepithelial electrical resistance measurements of 0.1% w/w of *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	283.8	237.6	234.3	237.6	46.2	46.2	42.9	42.9	46.2	39.6
SLS 2	924	881.1	782.1	752.4	59.4	62.7	56.1	59.4	59.4	59.4
SLS 3	732.6	686.4	676.5	709.5	52.8	49.5	46.2	42.9	42.9	39.6
Media 1	270.6	429	353.1	468.6	283.8	267.3	171.6	165	128.7	118.8
Media 2	445.5	462	584.1	640.2	339.9	429	290.4	306.9	283.8	290.4
Media 3	1273.8	1214.4	996.6	1164.9	709.5	716.1	640.2	607.2	686.4	729.3
AVG 1	1458.6	1382.7	1389.3	1333.2	1029.6	993.3	963.6	937.2	927.3	930.6
AVG 2	1237.5	1178.1	1171.5	785.4	884.4	874.5	811.8	818.4	808.5	818.4
AVG 3	1164.9	1240.8	1105.5	1204.5	699.6	798.6	495	184.8	831.6	884.4
CPP 1	1419	1386	1376.1	1283.7	1039.5	924	950.4	867.9	894.3	834.9
CPP 2	1161.6	1095.6	1069.2	1052.7	792	782.1	759	759	768.9	782.1
CPP 3	402.6	442.2	541.2	577.5	217.8	201.3	184.8	198	273.9	191.4
MWF 1.1	310.2	537.9	610.5	617.1	280.5	250.8	267.3	247.5	392.7	405.9
MWF 1.2	1224.3	1221	1171.5	1029.6	580.8	792	867.9	844.8	851.4	768.9
MWF 1.3	1151.7	1049.4	1171.5	1158.3	640.2	633.6	716.1	564.3	689.7	640.2
MWF 2.1	1207.8	1105.5	861.3	894.3	636.9	574.2	584.1	630.3	650.1	584.1
MWF 2.2	1468.5	1263.9	1201.2	1191.3	963.6	851.4	844.8	851.4	848.1	851.4
MWF 2.3	1478.4	1323.3	1333.2	1270.5	960.3	920.7	917.4	920.7	894.3	914.1
MWF 3.1	633.6	590.7	584.1	557.7	471.9	462	481.8	369.6	511.5	514.8
MWF 3.2	1478.4	1323.3	1267.2	1217.7	877.8	973.5	957	930.6	937.2	960.3
MWF 3.3	1161.6	1108.8	798.6	884.4	554.4	580.8	488.4	541.2	504.9	554.4
MWF 4.1	1052.7	904.2	943.8	877.8	709.5	673.2	716.1	729.3	729.3	722.7
MWF 4.2	1485	1300.2	1217.7	1204.5	752.4	933.9	858	874.5	871.2	861.3
MWF 4.3	1313.4	1151.7	1128.6	1118.7	782.1	785.4	785.4	772.2	795.3	795.3

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.5: Calculated transepithelial electrical resistance measurements of 0.5% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	283.8	237.6	234.3	237.6	46.2	46.2	42.9	42.9	46.2	39.6
SLS 2	924	881.1	782.1	752.4	59.4	62.7	56.1	59.4	59.4	59.4
SLS 3	732.6	686.4	676.5	709.5	52.8	49.5	46.2	42.9	42.9	39.6
Media 1	270.6	429	353.1	468.6	283.8	267.3	171.6	165	128.7	118.8
Media 2	445.5	462	584.1	640.2	339.9	429	290.4	306.9	283.8	290.4
Media 3	1273.8	1214.4	996.6	1164.9	709.5	716.1	640.2	607.2	686.4	729.3
AVG 1	914.1	808.5	801.9	811.8	699.6	669.9	610.5	554.4	547.8	567.6
AVG 2	669.9	683.1	471.9	613.8	594	396	488.4	438.9	412.5	452.1
AVG 3	1346.4	1263.9	1273.8	1290.3	881.1	749.1	699.6	712.8	623.7	716.1
CPP 1	1207.8	1234.2	1247.4	1257.3	815.1	765.6	709.5	702.9	676.5	620.4
CPP 2	943.8	867.9	237.6	214.5	191.4	184.8	211.2	184.8	221.1	231
CPP 3	1254	1155	1089	1059.3	732.6	726	693	699.6	709.5	709.5
MWF 1.1	1353	1300.2	1204.5	1230.9	735.9	795.3	785.4	732.6	603.9	676.5
MWF 1.2	1663.2	1574.1	1468.5	1452	1006.5	970.2	907.5	867.9	864.6	854.7
MWF 1.3	1227.6	1306.8	1234.2	1201.2	831.6	877.8	881.1	841.5	854.7	858
MWF 2.1	1283.7	1201.2	1145.1	1138.5	933.9	881.1	801.9	765.6	772.2	636.9
MWF 2.2	676.5	620.4	709.5	752.4	623.7	524.7	600.6	580.8	547.8	584.1
MWF 2.3	1445.4	1362.9	1277.1	1244.1	620.4	594	551.1	481.8	613.8	630.3
MWF 3.1	1432.2	1527.9	1475.1	1475.1	1026.3	1003.2	947.1	864.6	884.4	818.4
MWF 3.2	1141.8	597.3	669.9	646.8	584.1	561	570.9	620.4	438.9	514.8
MWF 3.3	1547.7	1455.3	1366.2	1369.5	1072.5	996.6	716.1	891	894.3	871.2
MWF 4.1	1501.5	1448.7	1359.6	1369.5	940.5	943.8	834.9	831.6	851.4	841.5
MWF 4.2	884.4	745.8	739.2	910.8	531.3	333.3	260.7	260.7	234.3	237.6
MWF 4.3	217.8	221.1	224.4	214.5	201.3	194.7	194.7	184.8	191.4	194.7

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.6: Calculated transepithelial electrical resistance measurements of 1.0% w/w of *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	283.8	237.6	234.3	237.6	46.2	46.2	42.9	42.9	46.2	39.6
SLS 2	924	881.1	782.1	752.4	59.4	62.7	56.1	59.4	59.4	59.4
SLS 3	732.6	686.4	676.5	709.5	52.8	49.5	46.2	42.9	42.9	39.6
Media 1	270.6	429	353.1	468.6	283.8	267.3	171.6	165	128.7	118.8
Media 2	445.5	462	584.1	640.2	339.9	429	290.4	306.9	283.8	290.4
Media 3	1273.8	1214.4	996.6	1164.9	709.5	716.1	640.2	607.2	686.4	729.3
AVG 1	798.6	633.6	729.3	712.8	603.9	600.6	557.7	518.1	422.4	514.8
AVG 2	1237.5	1214.4	1230.9	1178.1	831.6	795.3	636.9	719.4	640.2	656.7
AVG 3	1254	1164.9	1171.5	1188	881.1	877.8	841.5	798.6	782.1	775.5
CPP 1	1263.9	1244.1	1260.6	1247.4	980.1	792	745.8	696.3	719.4	719.4
CPP 2	297	732.6	699.6	759	379.5	277.2	462	306.9	412.5	313.5
CPP 3	409.2	676.5	726	709.5	386.1	379.5	363	343.2	458.7	445.5
MWF 1.1	891	834.9	838.2	844.8	673.2	495	518.1	501.6	429	438.9
MWF 1.2	1432.2	1303.5	1247.4	1230.9	864.6	838.2	775.5	762.3	650.1	656.7
MWF 1.3	221.1	336.6	336.6	336.6	194.7	191.4	221.1	184.8	217.8	188.1
MWF 2.1	300.3	293.7	293.7	313.5	273.9	283.8	240.9	244.2	250.8	254.1
MWF 2.2	204.6	273.9	244.2	214.5	198	198	250.8	184.8	217.8	240.9
MWF 2.3	983.4	1059.3	940.5	1036.2	722.7	679.8	623.7	643.5	660	709.5
MWF 3.1	1161.6	900.9	811.8	1016.4	874.5	290.4	250.8	237.6	237.6	234.3
MWF 3.2	1161.6	1128.6	1188	1184.7	699.6	617.1	597.3	610.5	603.9	627
MWF 3.3	1283.7	1019.7	1032.9	1052.7	481.8	462	524.7	501.6	419.1	541.2
MWF 4.1	1217.7	1151.7	1128.6	1125.3	792	815.1	762.3	739.2	669.9	679.8
MWF 4.2	1290.3	1062.6	957	1115.4	825	778.8	768.9	719.4	765.6	676.5
MWF 4.3	1402.5	1227.6	1207.8	1174.8	818.4	768.9	666.6	650.1	636.9	636.9

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.7: Normalized percentages of transepithelial electrical resistance measurements of 0.1% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	119.4444	100	98.61111	100	19.44444	19.44444	18.05556	18.05556	19.44444	16.66667
SLS 2	122.807	117.1053	103.9474	100	7.894737	8.333333	7.45614	7.894737	7.894737	7.894737
SLS 3	103.2558	96.74419	95.34884	100	7.44186	6.976744	6.511628	6.046512	6.046512	5.581395
Media 1	97.222	97.222	97.0833	100	78.4722	77.6388	71.3194	72.5694	69.0972	68.75
Media 2	90.6976	94.5736	96.124	100	77.5193	70.155	61.6279	67.6744	61.4728	64.7286
Media 3	91.6535	95.2755	96.8503	100	88.1889	79.2913	68.3464	74.0157	68.6614	71.1811
AVG 1	109.4059	103.7129	104.2079	100	77.22772	74.50495	72.27723	70.29703	69.55446	69.80198
AVG 2	157.563	150	149.1597	100	112.605	111.3445	103.3613	104.2017	102.9412	104.2017
AVG 3	96.71233	103.0137	91.78082	100	58.08219	66.30137	41.09589	15.34247	69.0411	73.42466
CPP 1	110.5398	107.9692	107.1979	100	80.97686	71.97943	74.03599	67.60925	69.66581	65.03856
CPP 2	110.3448	104.0752	101.5674	100	75.23511	74.29467	72.10031	72.10031	73.04075	74.29467
CPP 3	69.71429	76.57143	93.71429	100	37.71429	34.85714	32	34.28571	47.42857	33.14286
MWF 1.1	50.26738	87.16578	98.93048	100	45.45455	40.64171	43.31551	40.10695	63.63636	65.7754
MWF 1.2	118.9103	118.5897	113.7821	100	56.41026	76.92308	84.29487	82.05128	82.69231	74.67949
MWF 1.3	99.4302	90.59829	101.1396	100	55.27066	54.70085	61.82336	48.71795	59.54416	55.27066
MWF 2.1	135.0554	123.6162	96.30996	100	71.21771	64.20664	65.31365	70.4797	72.69373	65.31365
MWF 2.2	123.2687	106.0942	100.831	100	80.88643	71.46814	70.91413	71.46814	71.19114	71.46814
MWF 2.3	116.3636	104.1558	104.9351	100	75.58442	72.46753	72.20779	72.46753	70.38961	71.94805
MWF 3.1	113.6095	105.9172	104.7337	100	84.61538	82.84024	86.39053	66.27219	91.71598	92.30769
MWF 3.2	121.4092	108.6721	104.065	100	72.08672	79.9458	78.59079	76.42276	76.96477	78.86179
MWF 3.3	131.3433	125.3731	90.29851	100	62.68657	65.67164	55.22388	61.19403	57.08955	62.68657
MWF 4.1	119.9248	103.0075	107.5188	100	80.82707	76.69173	81.57895	83.08271	83.08271	82.33083
MWF 4.2	123.2877	107.9452	101.0959	100	62.46575	77.53425	71.23288	72.60274	72.32877	71.50685
MWF 4.3	117.4041	102.9499	100.885	100	69.9115	70.20649	70.20649	69.02655	71.09145	71.09145

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.8: Normalized percentages of transepithelial electrical resistance measurements of 0.5% w/w of *Aloe vera* material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	119.4444	100	98.61111	100	19.44444	19.44444	18.05556	18.05556	19.44444	16.66667
SLS 2	122.807	117.1053	103.9474	100	7.894737	8.333333	7.45614	7.894737	7.894737	7.894737
SLS 3	103.2558	96.74419	95.34884	100	7.44186	6.976744	6.511628	6.046512	6.046512	5.581395
Media 1	97.222	97.222	97.0833	100	78.4722	77.6388	71.3194	72.5694	69.0972	68.75
Media 2	90.6976	94.5736	96.124	100	77.5193	70.155	61.6279	67.6744	61.4728	64.7286
Media 3	91.6535	95.2755	96.8503	100	88.1889	79.2913	68.3464	74.0157	68.6614	71.1811
AVG 1	112.6016	99.5935	98.78049	100	86.17886	82.52033	75.20325	68.29268	67.47967	69.9187
AVG 2	109.1398	111.2903	76.88172	100	96.77419	64.51613	79.56989	71.50538	67.2043	73.65591
AVG 3	104.3478	97.95396	98.72123	100	68.28645	58.05627	54.21995	55.24297	48.3376	55.49872
CPP 1	96.06299	98.16273	99.2126	100	64.8294	60.89239	56.43045	55.90551	53.80577	49.34383
CPP 2	440	404.6154	110.7692	100	89.23077	86.15385	98.46154	86.15385	103.0769	107.6923
CPP 3	118.3801	109.0343	102.8037	100	69.15888	68.53583	65.42056	66.04361	66.97819	66.97819
MWF 1.1	109.9196	105.63	97.85523	100	59.78552	64.61126	63.80697	59.51743	49.06166	54.95979
MWF 1.2	114.5455	108.4091	101.1364	100	69.31818	66.81818	62.5	59.77273	59.54545	58.86364
MWF 1.3	102.1978	108.7912	102.7473	100	69.23077	73.07692	73.35165	70.05495	71.15385	71.42857
MWF 2.1	112.7536	105.5072	100.5797	100	82.02899	77.3913	70.43478	67.24638	67.82609	55.94203
MWF 2.2	89.91228	82.45614	94.29825	100	82.89474	69.73684	79.82456	77.19298	72.80702	77.63158
MWF 2.3	116.1804	109.5491	102.6525	100	49.86737	47.74536	44.29708	38.72679	49.33687	50.66313
MWF 3.1	97.09172	103.5794	100	100	69.57494	68.00895	64.20582	58.61298	59.95526	55.48098
MWF 3.2	176.5306	92.34694	103.5714	100	90.30612	86.73469	88.26531	95.91837	67.85714	79.59184
MWF 3.3	113.012	106.2651	99.75904	100	78.31325	72.77108	52.28916	65.06024	65.3012	63.61446
MWF 4.1	109.6386	105.7831	99.27711	100	68.6747	68.91566	60.96386	60.72289	62.16867	61.44578
MWF 4.2	97.10145	81.88406	81.15942	100	58.33333	36.5942	28.62319	28.62319	25.72464	26.08696
MWF 4.3	101.5385	103.0769	104.6154	100	93.84615	90.76923	90.76923	86.15385	89.23077	90.76923

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

Table B.9: Normalized percentages of transepithelial electrical resistance measurements of 1.0% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS 1	119.4444	100	98.61111	100	19.44444	19.44444	18.05556	18.05556	19.44444	16.66667
SLS 2	122.807	117.1053	103.9474	100	7.894737	8.333333	7.45614	7.894737	7.894737	7.894737
SLS 3	103.2558	96.74419	95.34884	100	7.44186	6.976744	6.511628	6.046512	6.046512	5.581395
Media 1	97.222	97.222	97.0833	100	78.4722	77.6388	71.3194	72.5694	69.0972	68.75
Media 2	90.6976	94.5736	96.124	100	77.5193	70.155	61.6279	67.6744	61.4728	64.7286
Media 3	91.6535	95.2755	96.8503	100	88.1889	79.2913	68.3464	74.0157	68.6614	71.1811
AVG 1	112.037	88.88889	102.3148	100	84.72222	84.25926	78.24074	72.68519	59.25926	72.22222
AVG 2	105.042	103.0812	104.4818	100	70.58824	67.507	54.06162	61.06443	54.34174	55.7423
AVG 3	105.5556	98.05556	98.61111	100	74.16667	73.88889	70.83333	67.22222	65.83333	65.27778
CPP 1	101.3228	99.73545	101.0582	100	78.57143	63.49206	59.78836	55.82011	57.67196	57.67196
CPP 2	39.13043	96.52174	92.17391	100	50	36.52174	60.86957	40.43478	54.34783	41.30435
CPP 3	57.67442	95.34884	102.3256	100	54.4186	53.48837	51.16279	48.37209	64.65116	62.7907
MWF 1.1	105.4688	98.82813	99.21875	100	79.6875	58.59375	61.32813	59.375	50.78125	51.95313
MWF 1.2	116.3539	105.8981	101.3405	100	70.24129	68.09651	63.00268	61.93029	52.81501	53.35121
MWF 1.3	65.68627	100	100	100	57.84314	56.86275	65.68627	54.90196	64.70588	55.88235
MWF 2.1	95.78947	93.68421	93.68421	100	87.36842	90.52632	76.84211	77.89474	80	81.05263
MWF 2.2	95.38462	127.6923	113.8462	100	92.30769	92.30769	116.9231	86.15385	101.5385	112.3077
MWF 2.3	94.90446	102.2293	90.76433	100	69.74522	65.6051	60.19108	62.10191	63.69427	68.47134
MWF 3.1	114.2857	88.63636	79.87013	100	86.03896	28.57143	24.67532	23.37662	23.37662	23.05195
MWF 3.2	98.05014	95.26462	100.2786	100	59.05292	52.08914	50.41783	51.53203	50.97493	52.92479
MWF 3.3	121.9436	96.8652	98.11912	100	45.76803	43.88715	49.84326	47.6489	39.81191	51.41066
MWF 4.1	108.2111	102.346	100.2933	100	70.38123	72.43402	67.74194	65.68915	59.53079	60.41056
MWF 4.2	115.6805	95.26627	85.79882	100	73.9645	69.82249	68.93491	64.49704	68.63905	60.65089
MWF 4.3	119.382	104.4944	102.809	100	69.66292	65.44944	56.74157	55.33708	54.21348	54.21348

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

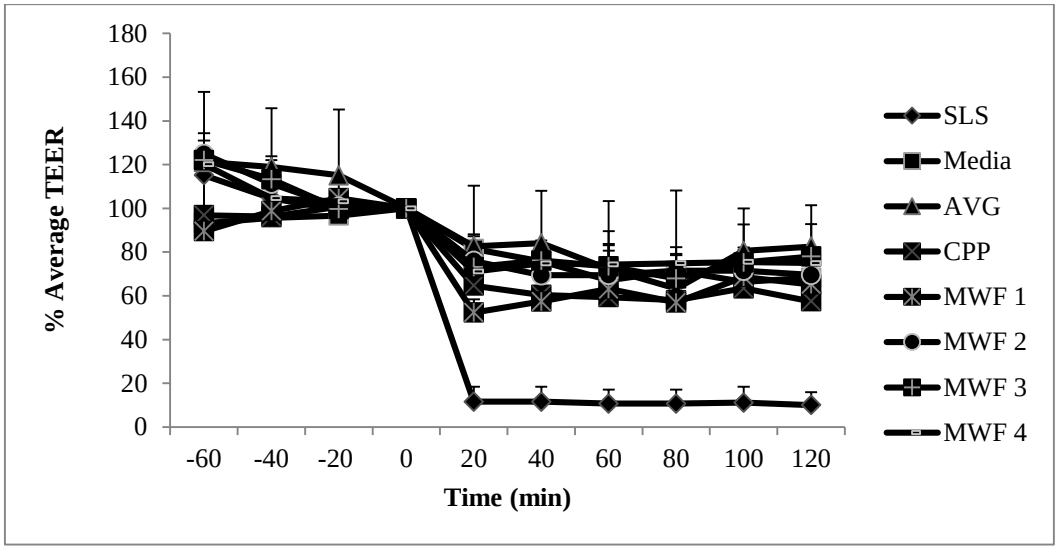

Figure B.1: Percentage transepithelial electrical resistance of 0.1% w/w *Aloe vera* weight fractions. SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions

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Table B.10: Average percentages of transepithelial electrical resistance measurements of 0.1% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	115.1691	104.6165	99.30244	100	11.59368	11.58484	10.67444	10.6656	11.12856	10.0476
Media	93.19103	95.69037	96.68587	100	81.39347	75.69503	67.0979	71.41983	66.41047	68.2199
AVG	121.2271	118.9089	115.0495	100	82.63832	84.05029	72.24482	63.28039	80.51224	82.47611
CPP	96.86632	96.20527	100.8265	100	64.64209	60.37708	59.37877	57.99843	63.37838	57.49203
MWF 1	89.53595	98.7846	104.6174	100	52.37849	57.42188	63.14458	56.95873	68.62428	65.24185
MWF 2	124.8959	111.2888	100.692	100	75.89618	69.38077	69.47852	71.47179	71.42482	69.57662
MWF 3	122.1207	113.3208	99.69909	100	73.12956	76.15256	73.40173	67.96299	75.25677	77.95202
MWF 4	120.2055	104.6342	103.1665	100	71.06811	74.81082	74.33944	74.904	75.50097	74.97637

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Table B.11: Standard deviation of average transepithelial electrical resistance measurements of 0.1% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	6.875039	3.713024	7.816708	0	15.34976	15.78177	10.69483	10.08903	14.73544	5.266842
Media	5.209951	5.873943	13.95235	0	13.66296	10.13605	12.11771	8.559429	7.580996	10.31689
AVG	32.10151	26.992799	30.18671	0	27.66119	23.99077	31.13274	44.84323	19.42572	18.90189
CPP	23.51455	17.11451	6.77229	0	23.49621	22.13121	23.73045	20.65822	13.91563	21.5889
MWF 1	35.3749	17.23741	8.013331	0	6.02332	18.2931	20.52161	22.15321	12.35389	9.71541
MWF 2	9.451501	10.71981	4.314231	0	4.841891	4.508705	3.664424	0.993919	1.169699	3.699624
MWF 3	8.888288	10.52813	8.148008	0	11.00154	9.191391	16.21835	7.753883	17.37628	14.8315
MWF 4	2.951799	2.867567	3.770634	0	9.235138	4.009659	6.290568	7.305186	6.595055	6.372529

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

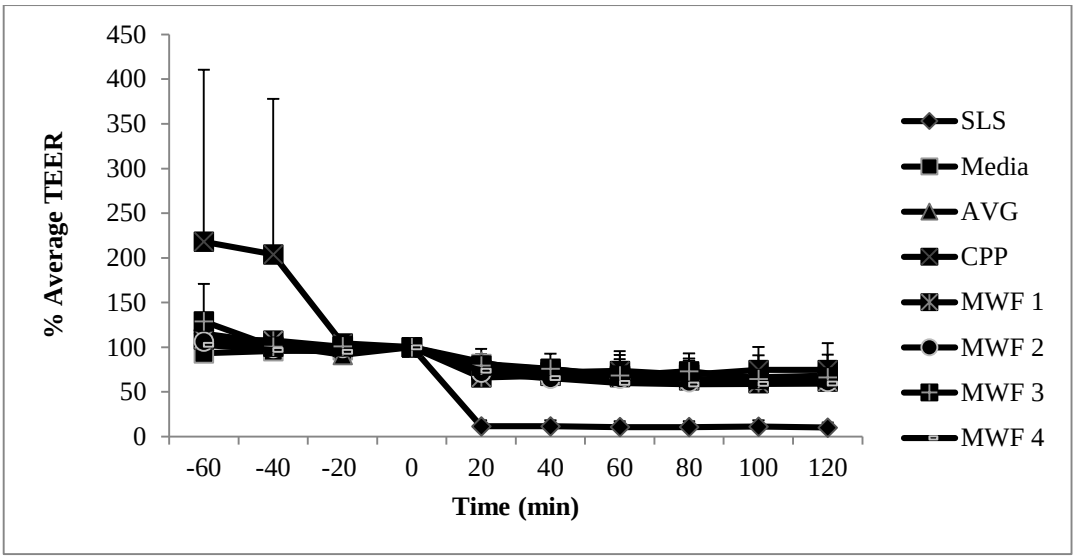

Figure B.2: Percentage transepithelial electrical resistance of 0.5% w/w *Aloe vera* weight fractions. SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions

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Table B.12: Average percentages of transepithelial electrical resistance measurements of 0.5% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	115.1691	104.6165	99.30244	100	11.59368	11.58484	10.67444	10.6656	11.12856	10.0476
Media	93.19103	95.69037	96.68587	100	81.39347	75.69503	67.0979	71.41983	66.41047	68.2199
AVG	108.6964	102.9459	91.46115	100	83.7465	68.36424	69.66436	65.01368	61.00719	66.35778
CPP	218.1477	203.9375	104.2619	100	74.40635	71.86069	73.43752	69.36766	74.6203	74.67144
MWF 1	108.8876	107.6101	100.5796	100	66.11149	68.16879	66.55287	63.11503	59.92032	61.75066
MWF 2	106.2821	99.17082	99.17683	100	71.59703	64.95783	64.85214	61.05538	63.32332	61.41225
MWF 3	128.8781	100.7305	101.1102	100	79.39811	75.83824	68.25343	73.19719	64.3712	66.22909
MWF 4	102.7595	96.9147	95.0173	100	73.61806	65.42637	60.11876	58.49998	59.04136	59.43399

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Table B.13: Standard deviation of average transepithelial electrical resistance measurements of 0.5% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's).

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	34.22706	1.770149	5.201578	0	16.01764	11.55498	5.741164	9.511368	7.202473	10.74331
Media	25.48302	6.108146	4.088696	0	14.82563	17.13928	7.339209	11.78225	13.62795	15.31513
AVG	4.144724	7.272806	12.62619	0	14.39879	12.67788	13.55228	8.612805	10.97305	9.588067
CPP	192.4535	173.8772	5.914687	0	13.01955	12.95478	22.13271	15.39569	25.50907	29.92534
MWF 1	6.238176	1.725412	2.493081	0	5.478624	4.391465	5.924066	6.011496	11.05086	8.605604
MWF 2	14.27983	14.61573	4.350234	0	18.82341	15.38992	18.40991	19.96643	12.36601	14.29219
MWF 3	42.02896	7.383488	2.134928	0	10.40808	9.732368	18.32643	19.93942	4.032199	12.26624
MWF 4	6.357116	13.08706	12.29451	0	18.26521	27.25555	31.08164	28.82968	31.86836	32.38803

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Appendix B

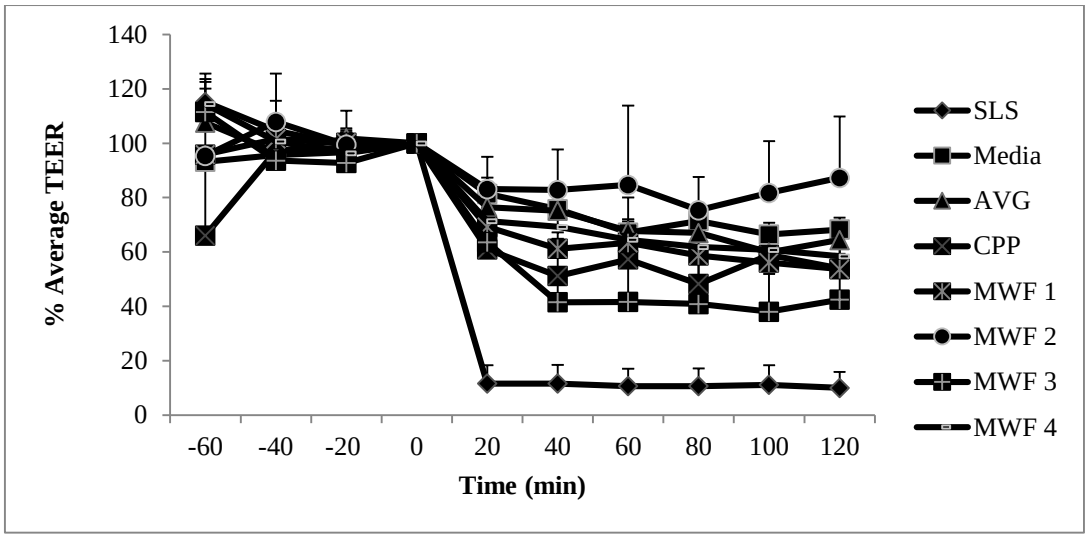

Figure B.3: Percentage transepithelial electrical resistance of 1.0% w/w *Aloe vera* weight fractions. SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions

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Table B.14: Average percentages of transepithelial electrical resistance measurements of 1.0% w/w *Aloe vera* gel material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	115.1691	104.6165	99.30244	100	11.59368	11.58484	10.67444	10.6656	11.12856	10.0476
Media	93.19103	95.69037	96.68587	100	81.39347	75.69503	67.0979	71.41983	66.41047	68.2199
AVG	107.5449	96.67523	101.8026	100	76.49237	75.21838	67.7119	66.99061	59.81144	64.4141
CPP	66.04253	97.20201	98.51923	100	60.99668	51.16739	57.27357	48.20899	58.89032	53.92233
MWF 1	95.8363	101.5754	100.1864	100	69.25731	61.18434	63.33903	58.73575	56.10072	53.72889
MWF 2	95.35952	107.8686	99.43157	100	83.14045	82.81303	84.65209	75.3835	81.74424	87.27722
MWF 3	111.4265	93.58873	92.75593	100	63.61997	41.5159	41.64547	40.85252	38.05449	42.46247
MWF 4	114.4245	100.7022	96.30035	100	71.33622	69.23531	64.47281	61.84109	60.79444	58.42498

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions.

Table B.15: Standard deviation of average transepithelial electrical resistance measurements of 1.0% w/w *Aloe vera* material (AVG), crude precipitated polysaccharides (CPP) and polysaccharide molecular weight fractions (MWF's)

	Time									
Sample	**-60 min**	**-40 min**	**-20 min**	**0 min**	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**	**120 min**
SLS	10.45329	10.93743	4.340753	0	6.802731	6.84033	6.409654	6.466263	7.260811	5.847814
Media	3.523486	1.372076	0.500343	0	5.904273	4.868436	4.964913	3.323274	4.281693	3.258749
AVG	3.898795	7.196154	2.968673	0	7.348407	8.454892	12.3881	5.813841	5.765664	8.273841
CPP	31.92945	2.27105	5.531624	0	15.37969	13.63414	5.319632	7.693958	5.25861	11.22322
MWF 1	26.67189	3.789152	1.073079	0	10.95537	6.048367	2.198457	3.557506	7.521351	1.991656
MWF 2	0.443041	17.6915	12.56848	0	11.86057	14.92911	29.16122	12.22103	18.9823	22.57135
MWF 3	12.20063	4.362902	11.21155	0	20.52025	11.93682	14.69939	15.2586	13.88283	16.82704
MWF 4	5.690356	4.828673	9.181173	0	2.304319	3.529117	6.721962	5.664089	7.295333	3.649239

SLS = Sodium Lauryl Sulphate; Media = DMEM; AVG = *Aloe vera* gel; CPP = Crude precipitated polysaccharide; MWF 1-4 = four different molecular weight fractions

B2 Metabolism study

Appendix B

Table B.16: Indinavir and M6 concentration ratios for *A. vera g*el (AVG), crude precipitated polysaccharide (CPP), various *A. vera* weight fractions (MWF 1-4), indinavir (negative control) and indinavir together with ketoconazole (positive control), at concentrations of 0.1% w/v

Sample	Ind P.Area	M6 P.Area	M6/Indinavir	Average M6/Ind
AVG1	5711000	6806	0.00119	0.00128
AVG2	4011000	5456	0.00136	
AVG3	5300000	6770	0.00128	
CPP1	3803000	5548	0.00146	0.00102
CPP2	2145000	1749	0.00082	
CPP3	3550000	2761	0.00078	
MWF1.1	3093000	15510	0.00501	0.00391
MWF1.2	2205000	10800	0.00490	
MWF1.3	1269000	2322	0.00183	
MWF2.1	3778000	4676	0.00124	0.00570
MWF2.2	3759000	13510	0.00359	
MWF2.3	2464000	30260	0.01228	
MWF3.1	1651000	7642	0.00463	0.00332
MWF3.2	1206000	4541	0.00377	
MWF3.3	511100	793.8	0.00155	
MWF4.1	622000	1598	0.00257	0.00187
MWF4.2	315600	282.2	0.00089	
MWF4.3	208000	446.5	0.00215	
Indinavir	636900	741	0.00116	0.00199
Indinavir	879100	1056	0.00120	
Indinavir	401400	561.2	0.00140	
Indinavir	475200	662	0.00139	
Indinavir	314300	1330	0.00423	
Indinavir	605900	1529	0.00252	
Ind + Ket	262500	5706	0.02174	0.00742
Ind + Ket	238800	1102	0.00461	
Ind + Ket	180200	923.4	0.00512	
Ind + Ket	68330	307.7	0.00450	
Ind + Ket	117400	310.5	0.00264	
Ind + Ket	100800	592.2	0.00588	

Appendix B

Table B.17: Indinavir and M6 concentration ratios for *A. vera g*el (AVG), crude precipitated polysaccharide (CPP), various *A. vera* weight fractions (MWF 1-4), indinavir (negative control) and indinavir together with ketoconazole (positive control), at concentrations of 0.5% w/v

Sample Name	Indinavir P.Area	M6 P.Area	M6/Indinavir	Average M6/ Ind
AVG1	6191000	8954	0.00145	0.00153
AVG2	4638000	7853	0.00169	
AVG3	3329000	4830	0.00145	
CPP1	3015000	41890	0.01389	0.00859
CPP2	3267000	31190	0.00955	
CPP3	1925000	4500	0.00234	
MWF1.1	3412000	2450	0.00072	0.00080
MWF1.2	4026000	2979	0.00074	
MWF1.3	2058000	1916	0.00093	
MWF2.1	2247000	2857	0.00127	0.00153
MWF2.2	2551000	2845	0.00112	
MWF2.3	2534000	5611	0.00221	
MWF3.1	2142000	3718	0.00174	0.00181
MWF3.2	2142000	3502	0.00163	
MWF3.3	1566000	3246	0.00207	
MWF4.1	642300	11220	0.01747	0.01798
MWF4.2	8847	281	0.03176	
MWF4.3	164400	774.1	0.00471	
Indinavir	636900	741	0.00116	0.00199
Indinavir	879100	1056	0.00120	
Indinavir	401400	561.2	0.00140	
Indinavir	475200	662	0.00139	
Indinavir	314300	1330	0.00423	
Indinavir	605900	1529	0.00252	
Ind + Ket	262500	5706	0.02174	0.00742
Ind + Ket	238800	1102	0.00461	
Ind + Ket	180200	923.4	0.00512	
Ind + Ket	68330	307.7	0.00450	
Ind + Ket	117400	310.5	0.00264	
Ind + Ket	100800	592.2	0.00588	

Appendix B

Table B.18: Indinavir and M6 concentration ratios for *A. vera g*el (AVG), crude precipitated polysaccharide (CPP), various *A. vera* weight fractions (MWF 1-4), indinavir (negative control) and indinavir together with ketoconazole (positive control), at concentrations of 1.0% w/v

Sample Name	Indinavir P.Area	M6 P.Area	M6/Indinavir	Average M6/ Ind
AVG1	2697000	4011	0.00149	0.00248
AVG2	3398000	11630	0.00342	
AVG3	1867000	4702	0.00252	
CPP1	12260000	19720	0.00161	0.00330
CPP2	7673000	26970	0.00351	
CPP3	7428000	35450	0.00477	
MWF1.1	7529000	8575	0.00114	0.00230
MWF1.2	6722000	32480	0.00483	
MWF1.3	2745000	2535	0.00092	
MWF2.1	3661000	4690	0.00128	0.00165
MWF2.2	2349000	3225	0.00137	
MWF2.3	1177000	2707	0.00230	
MWF3.1	1496000	8687	0.00581	0.00605
MWF3.2	3151000	30170	0.00957	
MWF3.3	2787000	7677	0.00275	
MWF4.1	164800	278.6	0.00169	0.00149
MWF4.2	194200	322.7	0.00166	
MWF4.3	283500	320.5	0.00113	
Indinavir	636900	741	0.00116	0.00199
Indinavir	879100	1056	0.00120	
Indinavir	401400	561.2	0.00140	
Indinavir	475200	662	0.00139	
Indinavir	314300	1330	0.00423	
Indinavir	605900	1529	0.00252	
Ind + Ket	262500	5706	0.02174	0.00742
Ind + Ket	238800	1102	0.00461	
Ind + Ket	180200	923.4	0.00512	
Ind + Ket	68330	307.7	0.00450	
Ind + Ket	117400	310.5	0.00264	
Ind + Ket	100800	592.2	0.00588	

Appendix C

In vivo study: Data

The area under the curve (AUC) and the maximum plasma concentration (C_{max}) values were determined as the bioavailability parameters from the *in vivo* study in Sprague-Dawley rats. The data obtained from this study is related in this appendix.

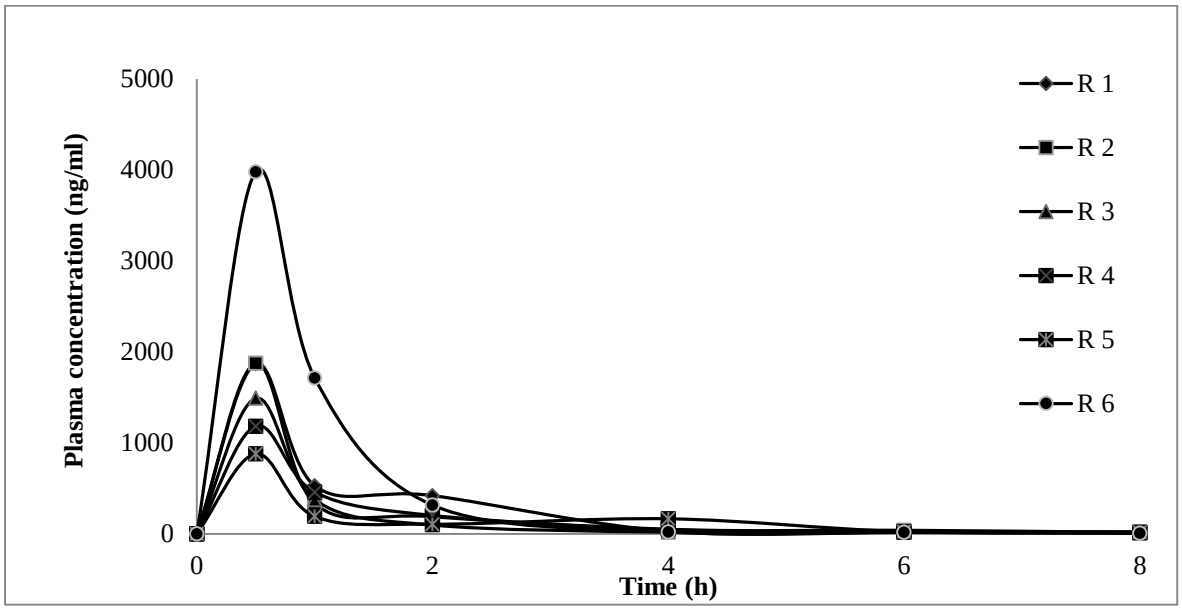

Figure C.1: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) administered with *Aloe vera* gel (AVG) (5%w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

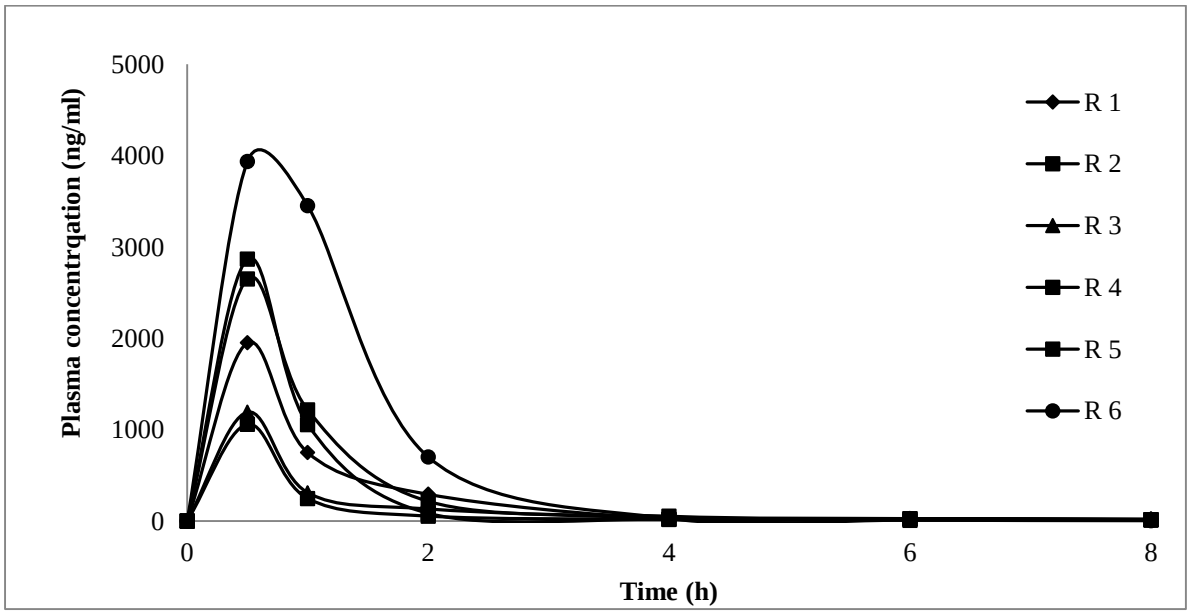

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Figure C.2: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) administered with crude precipitated polysaccharides (CPP) (5%w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

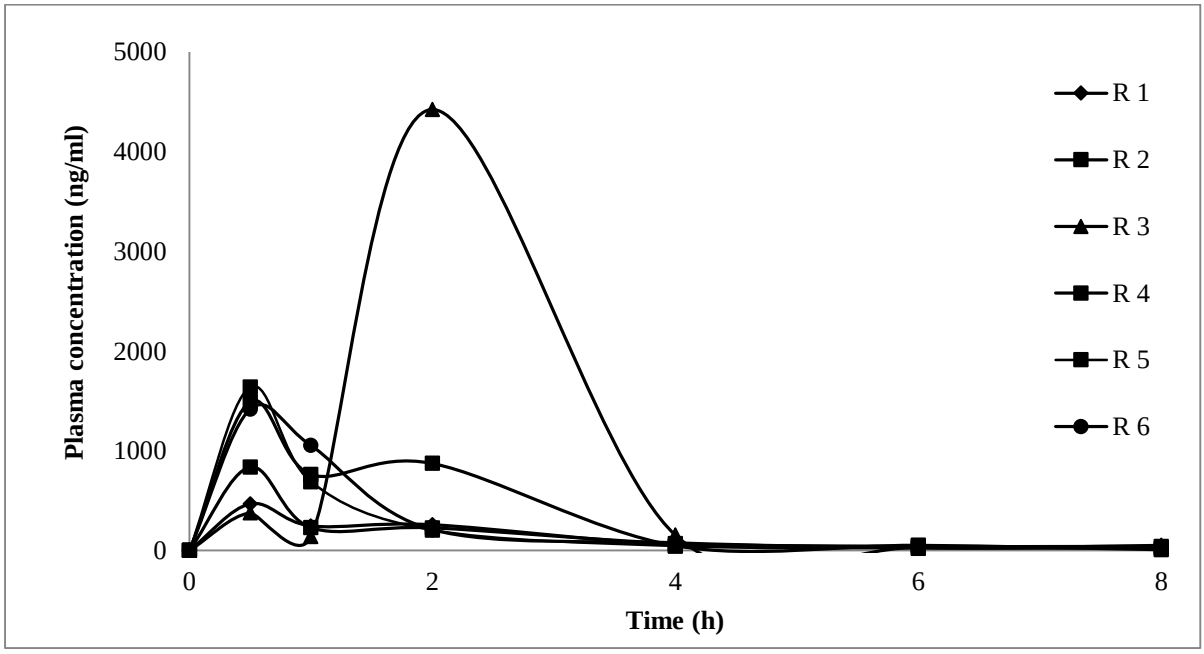

Figure C.3: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) administered with polysaccharide molecular weight fraction 1 (MWF1) (5%w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

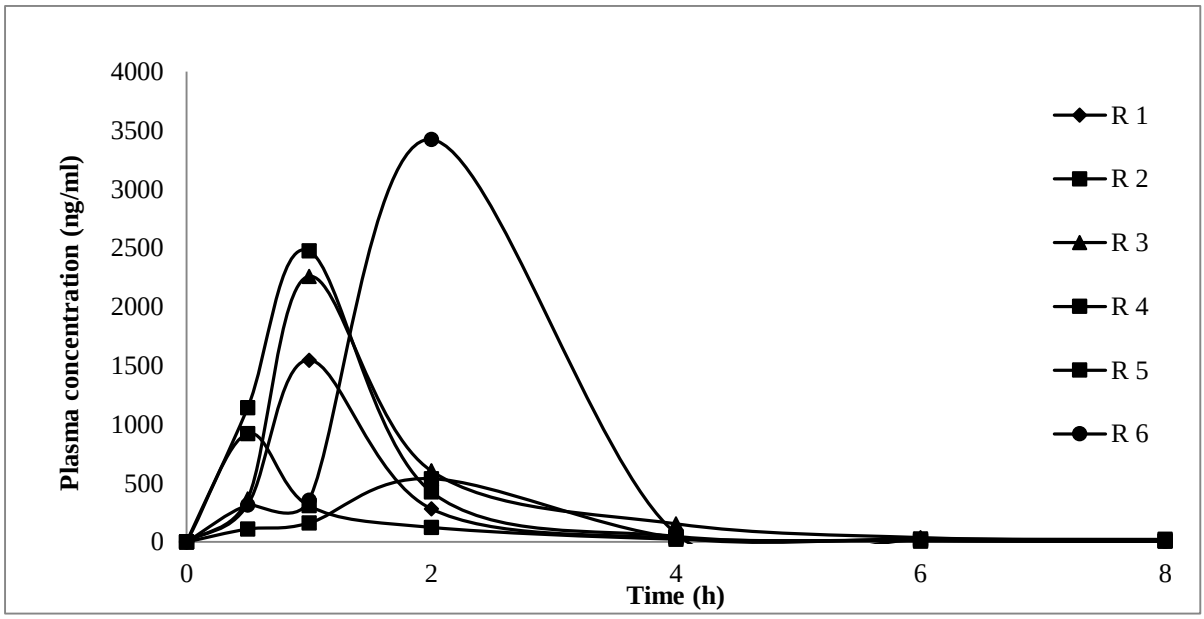

Figure C.4: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) administered with polysaccharide molecular weight fraction 2 (MWF2) (5%w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

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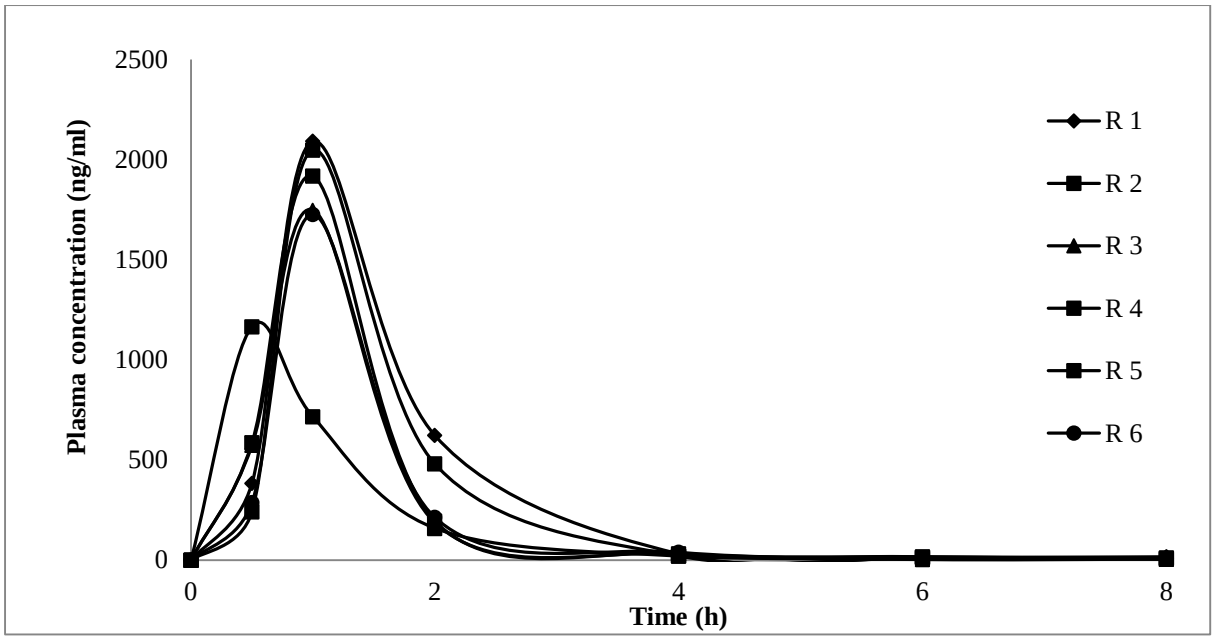

Figure C.5: Plasma concentration (ng/ml) time curve of indinavir (40 mg/kg) administered with polysaccharide molecular weight fraction 3 (MWF3) (5% w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

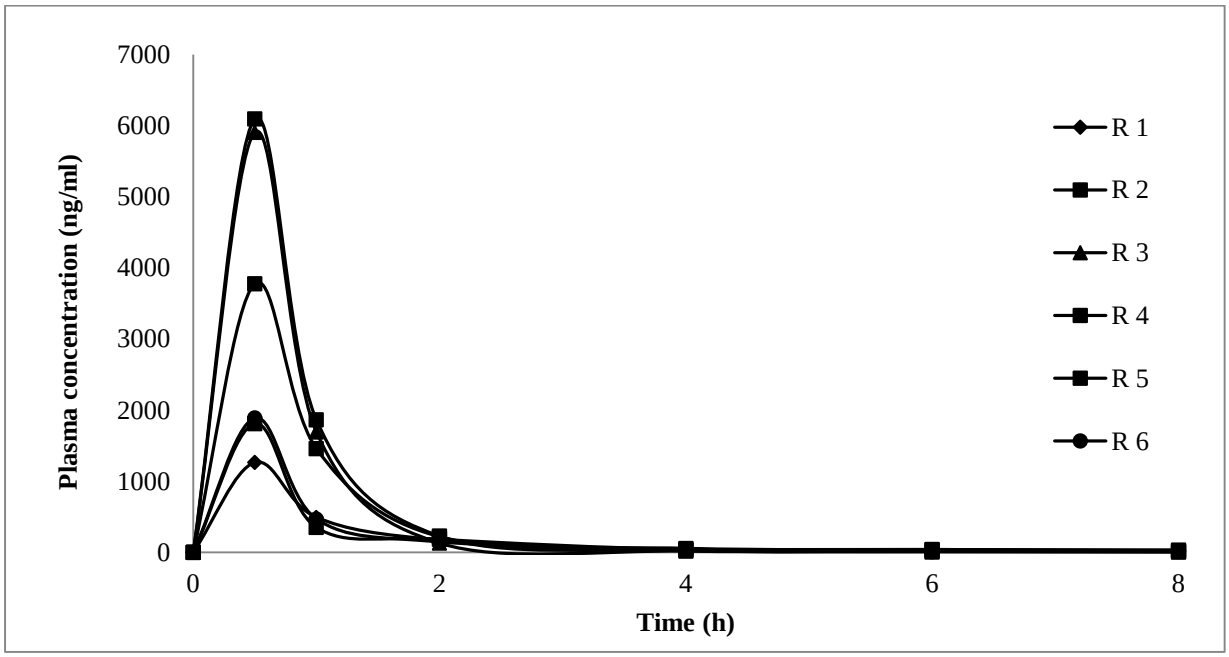

Figure C.6: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) administered with polysaccharide molecular weight fraction 4 (MWF4) (5%w/v) in Sprague-Dawley rats. R1 – R6 = number of repeats

Appendix C

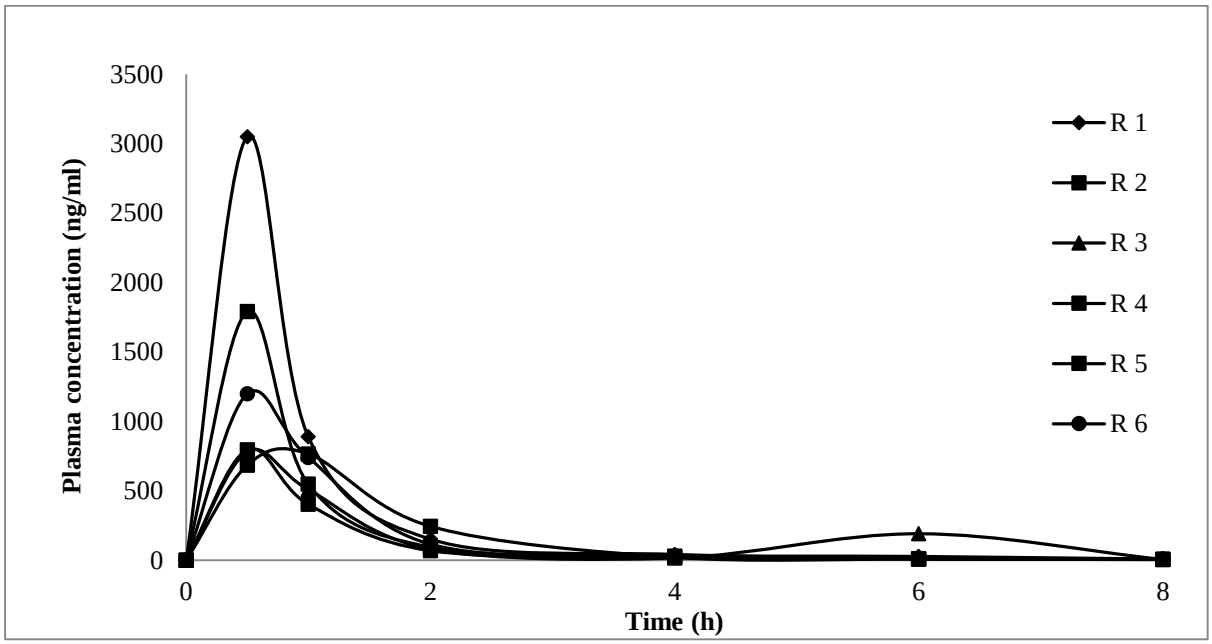

Figure C.7: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) alone administered to Sprague-Dawley rats. R1 – R6 = number of repeats

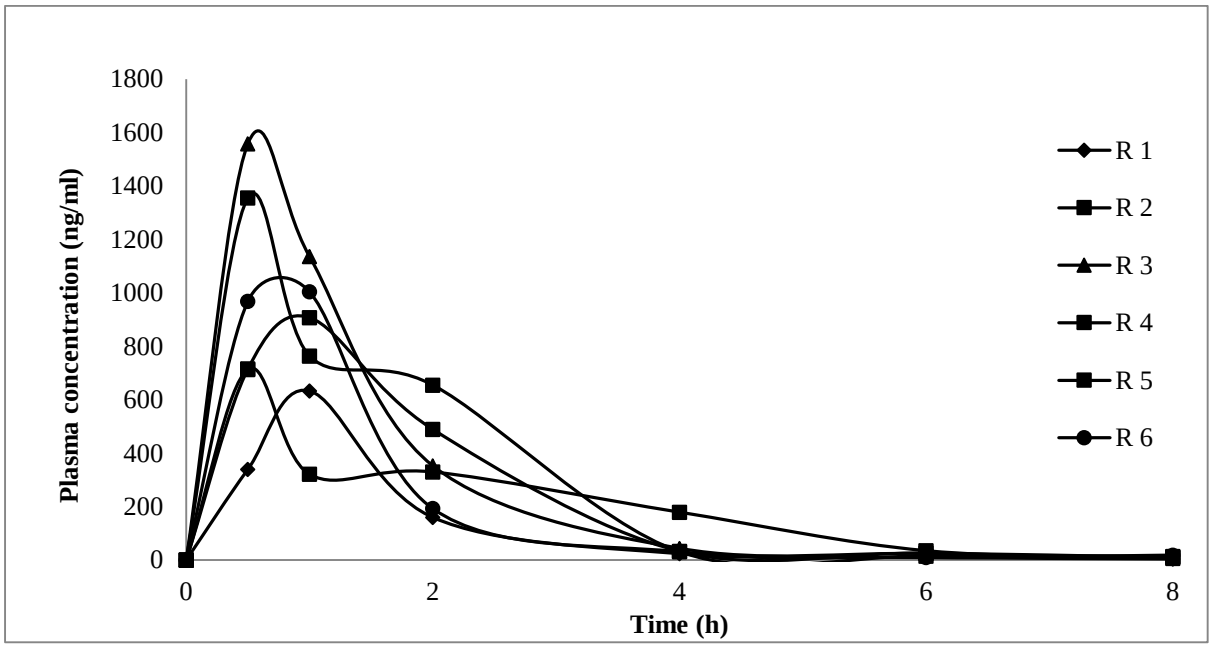

Figure C.8: Plasma concentration (ng/ml) time curve of indinavir (40mg/kg) and verapamil (9mg/kg) administered to Sprague-Dawley rats as the positive control group. R1 – R6 = number of repeats

Appendix C

Table C.1: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats with the *Aloe vera* gel (AVG) (5% w/v) material. R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	1868	1876	1490	1182	878.7	3980	1879	1100	449
1	529.2	336.0	377.3	458.7	192.1	1714	601.2	557	227
2	420.2	189.2	97.47	202.8	106.9	315.8	222.1	125	51.1
4	27.76	47.48	19.55	51.14	166.2	21.24	55.56	56	22.8
6	15.43	40.14	25.27	19.11	25.07	16.43	23.58	9	3.73
8	21.42	16.48	20.51	9.059	16.51	5.781	14.96	6	2.56

Table C.2: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats with the crude precipitated polysaccharides (CPP) (5% w/v) material. R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	1952	2650	1193	1061	2868	3936	2277	1095	447
1	751.3	1216	311.3	247.6	1058	3453	1173	1182	483
2	295.5	215.9	132.2	54.20	85.73	702.8	247.7	240	97.9
4	26.24	52.66	32.80	18.24	20.69	28.05	29.78	12	5.05
6	29.88	26.62	22.65	9.056	27.55	9.978	20.96	9	3.74
8	24.49	14.08	12.03	15.51	16.54	4.061	14.45	7	2.71

Table C.3: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats with the polysaccharide molecular weight fraction 1 (MWF1) (5% w/v). R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	467.1	835.1	376.3	1494	1639	1418	1038	551	225
1	246.6	229.0	139.9	763.2	689.9	1055	520.6	368	150
2	258.1	226.3	4422	873.9	204.2	208.4	1032	1681	686
4	57.69	70.12	155.6	47.81	44.22	76.56	75.33	41	16.8
6	41.24	40.16	28.40	52.10	22.15	29.22	35.55	11	4.46

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8	32.06	40.70	51.64	7.997	21.51	29.13	30.51	15	6.17

Table C.4: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats, with the polysaccharide molecular weight fraction 2 (MWF2) (5% w/v). R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	316.3	920.9	366.1	109.4	1141	312.7	527.7	406	166
1	1545	306.9	2257	159.8	2474	352.8	1183	1045	426
2	281.6	123.1	604.1	536.6	425.6	3421	898.7	1248	509
4	30.95	23.03	153.6	37.42	46.79	72.59	60.73	49	19.8
6	17.49	6.907	36.58	24.92	13.88	15.96	19.29	10	4.19
8	15.84	4.395	10.37	21.87	8.290	5.571	11.06	7	2.72

Table C.5: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats, with the polysaccharide molecular weight fraction 3 (MWF3) (5% w/v). R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	383.6	1165	572.7	585.9	241.3	287.0	539.3	338	138
1	2091	715.2	1747	1917	2047	1725	1707	508	208
2	622.7	158.5	187.0	194.8	480.1	213.3	309.4	194	79.0
4	30.92	19.6	29.26	32.27	20.13	39.08	28.54	8	3.07
6	12.02	15.4	3.266	16.21	9.446	8.789	10.86	5	1.95
8	16.53	11.5	4.613	3.700	9.870	9.703	9.311	5	1.92

Table C.6: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) administered to rats with the polysaccharide molecular weight fraction 4 (MWF4) (5% w/v). R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	1264	6094	5906	1808	3779	1889	3457	2147	876
1	493.5	1862	1692	350.3	1458	470.8	1054	689	281
2	174.8	228.3	130.1	184.0	219.0	147.4	180.6	39	15.8
4	33.92	28.27	17.16	39.37	56.02	29.64	34.06	13	5.32
6	17.24	7.436	18.02	40.34	38.17	11.86	22.18	14	5.63

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8	11.87	4.098	8.755	20.84	33.44	23.19	17.03	11	4.41

Table C.7: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) alone administered to rats. R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	3050	1791	778.9	683.7	794.5	1198	1383	914	373
1	889.4	548.7	509.0	766.1	402.7	740.9	642.8	184	75.3
2	151.9	94.64	81.12	244.8	68.67	115.7	126.1	65	26.6
4	41.89	30.50	16.28	21.47	22.13	19.73	25.33	9	3.83
6	27.31	11.70	190.8	9.479	9.543	11.34	43.36	73	29.6
8	8.285	6.025	5.839	6.820	4.704	9.761	6.906	2	0.749

Table C.8: Maximum plasma concentration (C_{max}) values for indinavir (40 mg/kg) and verapamil (9 mg/kg) administered to rats. R1- R6 = number of repeats; STDEV = standard deviation; SEM = standard error of mean

	Concentration (ng/ml)								
Time (h)	**R 1**	**R 2**	**R 3**	**R 4**	**R 5**	**R 6**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0	0
0.5	339.0	1355	1558	713.0	714.6	968.2	941.3	451	184
1	632.4	763.7	1136	320.6	907.4	1004	794.0	292	119
2	159.3	654.6	352.2	329.5	488.9	192.3	362.8	186	75.9
4	23.65	30.61	43.45	179.0	32.59	31.07	56.73	60	24.6
6	9.382	14.70	23.72	34.94	25.81	9.069	19.60	10	4.20
8	3.850	13.21	6.070	10.91	9.208	18.39	10.27	5	2.12

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Table C.9: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats with the *Aloe vera* gel (AVG) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.5	1.6	3.1	1.6	1.8	1.3	1.8
C_{max} (µg/L)	1868	1876	1490	1182	878.7	3980	1879
T_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	N/A
$AUC_{0-\infty}$ (min. µmol/L)	206.7	166.4	134.6	137.8	116.0	375.5	190.0

Table C.10: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats with the crude precipitated polysaccharides (CPP) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.9	1.6	1.8	1.8	1.4	1.5	1.7
C_{max} (µg/L)	1952	2650	1193	1061	2868	3936	2277
T_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	N/A
$AUC_{0-\infty}$ (min. µmol/L)	213.7	270.3	115.6	88.75	244.7	557.3	248.0

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Table C.11: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats with the polysaccharide molecular weight fraction 1 (MWF1) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	2.1	2.4	2.5	1.5	1.9	2.0	2.1
C_{max} (µg/L)	467.1	835.1	4422	1494	1639	1418	1713
T_{max} (h)	0.5	0.5	2.0	0.5	0.5	0.5	N/A
$AUC_{0-\infty}$ (min. µmol/L)	111	130	736	279	181	209	274

Table C.12: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats, with the polysaccharide molecular weight fraction 2 (MWF2) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.5	1.7	1.0	1.4	1.6	1.1	1.4
C_{max} (µg/L)	1545	920.9	2257	536.6	2474	3421	1859
T_{max} (h)	1.0	0.5	1.0	2.0	1.0	2.0	N/A
$AUC_{0-\infty}$ (min. µmol/L)	184	93	312	114	314	561	263

Appendix C

Table C.13: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats with the polysaccharide molecular weight fraction 3 (MWF3) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.2	1.7	1.0	1.1	1.1	1.3	1.2
C_{max} (µg/L)	2091	1165	1747	1917	2047	1725	1782
T_{max} (h)	1.0	0.5	1.0	1.0	1.0	1.0	N/A
$AUC_{0-\infty}$ (min. µmol/L)	276	143	191	208	241	184	207

Table C.14: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats with the polysaccharide molecular weight fraction 4 (MWF4) (5% w/v) material. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.6	1.4	1.7	2.1	2.3	2.1	1.9
C_{max} (µg/L)	1264	6094	5906	1808	3779	1889	3457
T_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	N/A
$AUC_{0-\infty}$ (min. µmol/L)	137	476	442	165	356	166	290

Appendix C

Table C.15: Area under the curve (AUC) values for indinavir (40 mg/kg) alone administered to rats. R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.5	1.5	1.4	1.2	1.6	1.7	1.5
C_{max} (µg/L)	3050	1791	778.9	766.1	794.5	1198	1396
T_{max} (h)	0.5	0.5	0.5	1.0	0.5	0.5	N/A
$AUC_{0-\infty}$ (min. µmol/L)	252.7	151.8	129.5	133.3	86.11	139.3	148.8

Table C.16: Area under the curve (AUC) values for indinavir (40 mg/kg) administered to rats, with verapamil (9 mg/kg). R1- R6 = number of repeats; $t_{1/2}$ = half-life of indinavir; C_{max} = maximum concentration; T_{max} = time at which C_{max} was observed

Parameters	R 1	R 2	R 3	R 4	R 5	R 6	Mean
Nominal Dose (mg/kg)	40	40	40	40	40	40	40
Apparent $t_{1/2}$ (h)	1.1	1.1	1.1	1.2	1.1	1.2	1.1
C_{max} (µg/L)	632.4	1355	1558	713	907.4	1004	1028
T_{max} (h)	1.0	0.5	0.5	0.5	1.0	1.0	N/A
$AUC_{0-\infty}$ (min. µmol/L)	93.75	230.4	225.7	151.4	186.9	161.8	175.0

Appendix D

Protein and Peptide Letters: Guide for authors

D1. ONLINE MANUSCRIPT SUBMISSION

An online submission and tracking service *via* Internet facilitates a speedy and cost-effective submission of manuscripts. The full manuscript has to be submitted online *via* Bentham's Content Management System (CMS) at bsp-cms.eurekaselect.com / **View Submission Instructions**

Manuscripts must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The principal/corresponding author will be required to submit a Copyright Letter along with the manuscript, on behalf of all the co-authors (if any). The author(s) will confirm that the manuscript (or any part of it) has not been published previously or is not under consideration for publication elsewhere. Furthermore, any illustration, structure or table that has been published elsewhere must be reported, and copyright permission for reproduction must be obtained.

For all online submissions, please provide soft copies of all the materials (main text in MS Word or Tex/LaTeX), figures/illustrations in TIFF, PDF or JPEG, and chemical structures drawn in ChemDraw (CDX)/ISISDraw (TGF) as separate files, while a PDF version of the entire manuscript must also be included, embedded with all the figures/illustrations/ tables/ chemical structures etc. It is advisable that the document files related to a manuscript submission should always have the name of the corresponding author as part of the file name, *i.e.*, Cilli MS text.doc ,Cilli MS Figure 1, *etc.*

It is imperative that before submission, authors should carefully proofread the files for special characters, mathematical symbols, Greek letters,equations, tables, references and images, to ensure that they appear in proper format.

References, figures, tables, chemical structures etc. should be referred to in the text at the appropriate place where they have been first discussed. Figure legends/captions should also be provided.

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A successful electronic submission of a manuscript will be followed by a system-generated acknowledgement to the principal/corresponding author. Any queries therein should be addressed to manuscript@benthamscience.org

D1.1 Editorial Policies

The editorial policies of *Bentham Science* Publishers on publication ethics, peer-review, plagiarism, copyrights/ licenses,errata/corrections and article retraction/ withdrawal can be viewed at Editorial Policy

D2 MANUSCRIPTS PUBLISHED

The Journal publishes peer-reviewed mini-reviews, letters and research papers written in English. Single topic/thematic issues may also be considered for publication.

D2.1 Single Topic Issues

These special issues are peer-reviewed and may contain invited or uninvited review/mini-review articles. A Single Topic Issue Editor will offer a short perspective and co-ordinate the solicitation of manuscripts (at least 10) for full-length thematic issues from leading scientists. Authors interested in editing a single topic issue in an emerging topic of outstanding developments in all important aspects of protein and peptide research, including structural studies, advances in recombinant expression, function, synthesis, enzymology, immunology, molecular modeling, drug design medicinal chemistry or rational drug design may submit their proposal to the Editor-in-Chief at ppl@benthamscience.org for consideration.

D2.2 Conference Proceedings

For proposals to publish conference proceedings in this journal, please contact us at proceedings@benthamscience.org for consideration.

D3 MANUSCRIPT LENGTH

D3.1 Mini-Reviews/ Research Articles/ Letters

Appendix D

All manuscript types should be between 3000 and 8000 words excluding figures, structures,photographs, schemes and tables.

There is no restriction on the number of figures, tables or additional files e.g. video clips, animation and datasets, that can be included with each article online. Authors should include all relevant supporting data with each article (Refer to Supplementary Material section).

D4 MANUSCRIPT PREPARATION

The manuscript should be written in English in a clear, direct and active style. All pages must be numbered sequentially, facilitating in the reviewing and editing of the manuscript.

D5 MICROSOFT WORD TEMPLATE

It is advisable that authors prepare their manuscript using the template available on the Web, which will assist in preparation of the manuscript according to Journal's Format. Download the Template.

Our contracted service provider Eureka Science can, if needed, provide professional assistance to authors for the improvement of English language and figures in manuscripts.

D6 MANUSCRIPT SECTIONS FOR PAPERS

Manuscripts may be divided into the following sections:

- Copyright Letter
- Title
- Title Page
- Structured Abstract
- Graphical Abstract
- Keywords
- Text Organization
- Conclusion
- List of Abbreviations (if any)
- Conflict of Interest

Appendix D

- Acknowledgements
- References
- Appendices
- Figures/Illustrations (if any)
- Chemical Structures (if any)
- Tables (if any)
- Supportive/Supplementary Material (if any)

D6.1 Copyright Letter

It is mandatory that a signed copyright letter should also be submitted along with the manuscript by the author to whom correspondence is to be addressed, delineating the scope of the submitted article declaring the potential competing interests, acknowledging contributions from authors and funding agencies, and certifying that the paper is prepared according to the ***'Instructions for Authors'***. All inconsistencies in the text and in the reference section and any typographical errors must be carefully checked and corrected before the submission of the manuscript. The article should not contain any such material or information that may be unlawful, defamatory, fabricated, plagiarized, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the copyright agreement. The authors acknowledge that the publishers have the legal right to take appropriate action against the authors for any such violation of the terms and conditions as laid down in the copyright agreement. Download the Copyright letter

D6.2 Title

The title of the article should be precise and brief and must not be more than 120 characters. Authors should avoid the use of non-standard abbreviations. The title must be written in title case except for articles, conjunctions and prepositions.

Authors should also provide a short 'running title'. Title, running title, byline, correspondent, footnote and key words should be written as presented in original manuscripts.

D6.3 Title Page

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Title page should include paper title, author(s) full name and affiliation, corresponding author(s) names complete affiliation/address, along with phone, fax and email.

D6.4 Structured Abstract

The abstract of an article should be its clear, concise and accurate summary, having no more than 250 words, and including the explicit sub-headings (as in-line or run-in headings in bold). Use of abbreviations should be avoided and the references should not be cited in the abstract. Ideally, each abstract should include the following sub-headings, but these may vary according to requirements of the article.

- Background
- Objective
- Method
- Results
- Conclusion

D6.5 Graphical Abstract

A graphic must be included with each manuscript for use in the Table of Contents (TOC). This must be submitted separately as an electronic file (preferred file types are EPS, PDF, TIFF, Microsoft Word, PowerPoint and CDX *etc*). A graphical abstract, not exceeding 30 words along with the illustration, helps to summarize the contents of the manuscript in a concise pictorial form. It is meant as an aid for the rapid viewing of the journals' contents and to help capture the readers' attention. The graphical abstract may feature a key structure, reaction, equation, *etc*. that the manuscript elucidates upon. It will be listed along with the manuscript title, authors' names and affiliations in the contents page, typeset within an area of 5 cm by 17 cm, but it will not appear in the article PDF file or in print.

Graphical Abstracts should be submitted as a separate file (must clearly mention graphical abstract within the file) online via Bentham's Content Management System by selecting the option “supplementary material”.

D6.6 Keywords

6 to 8 keywords must be provided.

D6.7 Text Organization

The main text should begin on a separate page and should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the Acknowledgements and Reference sections. For Letters/Research Articles, the manuscript should begin with the title page and abstract followed by the main text, which must be structured into separate sections as ***Introduction, Materials and Methods, Results, Discussion, Conclusion, List of abbreviations (if any), Conflict of Interest, Acknowledgements and References***. The Review Article should mention any previous important recent and old reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review. The authors are advised to present and discuss their observations in brief. Crystallization reports are no longer routinely published by the journal. Only reports on the first crystallization of proteins of substantial interest or reports on new crystallization techniques of wide application will be considered. Crystallization reports should include data describing the source of the protein, composition, pH and concentrations of precipitants and buffers etc., the method employed, crystal size and morphology, and time of growth etc. Unit cell dimensions, space-group assignments, resolution limit, specific volume and asymmetric unit contents must also be provided. The manuscript style must be uniform throughout the text and 10 pt Times New Roman fonts should be used. The full term for an abbreviation should precede its first appearance in the text unless it is a standard unit of measurement. The reference numbers should be given in square brackets in the text. Italics should be used for Binomial names of organisms (Genus and Species), for emphasis and for unfamiliar words or phrases. Non-assimilated words from Latin or other languages should also be italicized e.g. *per se, et al. etc*.

D6.8 Standard Protocol on Approvals, Registrations, Patient Consents & Animal Protection

Appendix D

All clinical investigations must be conducted according to the Declaration of Helsinki principles. For all manuscripts reporting data from studies involving human participants, formal review and approval by an appropriate institutional review board or ethics committee is required. For research involving animals, the authors should indicate whether the procedures followed were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdf; published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

A specific declaration of such approval must be made in the copyright letter and in a stand-alone paragraph at the end of the Methods section especially in the case of human studies where inclusion of a statement regarding obtaining the written informed consent from each subject or subject's guardian is a must. The original should be retained by the guarantor or corresponding author. Editors may request to provide the original forms by fax or email.

D6.9 Authentication of Cell Lines

The NIH acknowledges the misidentification and/or cross-contamination of cell cultures e.g. HeLa cells being used in a research study as a serious problem. In order to ensure the validation of the work and proper utilization of resources, it is a prerequisite that correct reagents be used in studies dealing with established human (tumor) cell lines that have been cultured for more than 4 years up to the date of submission of the manuscript. Cell lines such as short-term cultures of human tumors, murine cell lines (as a catalog of DNA profiles is not yet available) and tumor cell lines established in the course of the study that is being submitted, are presently exempt from this rule. To minimize the risk of working with misidentified and/or contaminated cell lines, tests such as isoenzyme analysis, karyotyping/cytogenetic analysis and, more recently, molecular techniques of DNA profiling may be carried out to authenticate cell cultures. These tests may help confirm or establish the identity profile for a cell line. *Bentham Science* recommends that all cell lines be authenticated prior to submitting a paper for review. Authors are therefore required to provide authentication of the origin and identity of the cells by performing cell profiling either in their own laboratory or by outsourcing an approved laboratory or cell bank. Authentication is required when a new line is established or acquired, before freezing a cell line, if the

performance of the line is not consistent or results are unexpected, if using more than one cell line, and before publication of the study.

The cell lines profile should be cross-checked with the profile of the donor tissue of other continuous cell lines such as provided by the authentic data bank such as www.dsmz.de/fp/cgi-bin/str.html, ATCC® etc.

D6.10 Greek Symbols and Special Characters

Greek symbols and special characters often undergo formatting changes and get corrupted or lost during preparation of manuscript for publication. To ensure that all special characters used are embedded in the text, these special characters should be inserted as a symbol but should not be a result of any format styling (*Symbol* font face) otherwise they will be lost during conversion to PDF/XML.

Authors are encouraged to consult reporting guidelines. These guidelines provide a set of recommendations comprising a list of items relevant to their specific research design. Chemical equations, chemical names, mathematical usage, unit of measurements, chemical and physical quantity and units must conform to SI and Chemical Abstracts or IUPAC.

All kinds of measurements should be reported only in International System of Units (SI).

D6.11 Conclusion

A small paragraph summarizing the contents of the article, presenting the final outcome of the research or proposing further study on the subject, may be given at the end of the article under the Conclusion section.

D6.12 List of Abbreviations

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

D6.13 Conflict of Interest

Appendix D

Financial contributions and any potential conflict of interest must be clearly acknowledged under the heading 'Conflict of Interest'. Authors must list the source(s) of funding for the study. This should be done for each author.

D6.14 Acknowledgements

All individuals listed as authors must have contributed substantially to the design, performance, analysis, or reporting of the work and are required to indicate their specific contribution. Anyone (individual/company/institution) who has substantially contributed to the study for important intellectual content, or who was involved in the articles drafting the manuscript or revising must also be acknowledged. Guest or honorary authorship based solely on position (e.g. research supervisor, departmental head) is discouraged.

The specific requirements for authorship have been defined by the International Committee of Medical Journal Editors (ICMJE; www.icmje.org). Examples of authors' contributions are: 'designed research/study', 'performed research/study', 'contributed important reagents', 'collected data', 'analyzed data', 'wrote paper' etc. This information must be included in the submitted manuscript as a separate paragraph under the heading Acknowledgements. The corresponding author is responsible for obtaining permission from all co-authors for the submission of any version of the manuscript and for any changes in the authorship.

D6.15 References

References must be listed in the ACS Style only. All references should be numbered sequentially [in square brackets] in the text and listed in the same numerical order in the reference section. The reference numbers must be finalized and the bibliography must be fully formatted before submission.

See below few examples of references listed in the ACS Style:

D6.15.1 Journal Reference

- [1] Zheng, X. Q.; Li, C.; Wang, J. An information-theoretic approach to the prediction of protein structural class. *J. Comput. Chem.*, **2010**, *31*, 1201-1206.

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- [2] Mohabatkar, H.; Mohammad Beigi, M.; Esmaeili, A. Prediction of GABA(A) receptor proteins using the concept of Chou's pseudo-amino acid composition and support vector machine. *J. Theor. Biol.*, **2011**, *281*, 18-23.

D6.15.2 Book Reference

- [3] Crabtree, R.H. The Organometallic Chemistry of the Transition Metals, 3rd ed.; Wiley and Sons: New York, **2001**.

D6.15.3 Book Chapter Reference

- [4] Wheeler, D.M.S.; Wheeler, M.M. In: *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier Science B. V: Amsterdam, **1994**; Vol. *14*, pp. 3-46.

D6.15.4 Conference Proceedings

- [5] Jakeman, D.L.; Withers, S.G.E. In: *Carbohydrate Bioengineering: Interdisciplinary Approaches*, Proceedings of the 4th Carbohydrate Bioengineering Meeting, Stockholm, Sweden, June 10-13, 2001; Teeri, T.T.; Svensson, B.; Gilbert, H.J.; Feizi, T., Eds.; Royal Society of Chemistry: Cambridge, UK, **2002**; pp. 3-8.

D6.15.5 URL (WebPage)

- [6] National Library of Medicine. Specialized Information Services: Toxicology and Environmental Health. sis.nlm.nih.gov/Tox/ToxMain.html (Accessed May 23, **2004**).

D6.15.6 Patent

- [7] Hoch, J.A.; Huang, S. Screening methods for the identification of novel antibiotics. U.S. Patent 6,043,045, March 28, 2000.

D6.15.7 Thesis

- [8] Mackel, H. *Capturing the Spectra of Silicon Solar Cells*. PhD Thesis, The Australian National University: Canberra, December **2004**.

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D6.15.8 E-citations

- [9] Citations for articles/material published exclusively online or in open access (free-to-view), must contain the accurate Web addresses (URLs) at the end of the reference(s), except those posted on an author's Web site (unless editorially essential), e.g. 'Reference: Available from: URL'.

Some important points to remember:

- All references must be complete and accurate.
- All authors must be cited and there should be no use of the phrase *et al*.
- Date of access should be provided for online citations.
- Journal names should be abbreviated according to the Index Medicus/MEDLINE.
- Punctuation should be properly applied as mentioned in the examples given above.
- Superscript in the in-text citations and reference section should be avoided.
- Abstracts, unpublished data and personal communications (which can only be included if prior permission has been obtained) should not be given in the references section. The details may however appear in the footnotes.
- The authors are encouraged to use a recent version of EndNote (version 5 and above) or Reference Manager (version 10) when formatting their reference list, as this allows references to be automatically extracted.

D6.16 Appendices

In case there is a need to present lengthy, but essential methodological details, appendices must be used, which can be a part of the article. An appendix must not exceed three pages (Times New Roman, 12 point fonts, 900 max. words per page). The information should be provided in a condensed form, ruling out the need of full sentences. A single appendix should be titled APPENDIX, while more than one can be titled APPENDIX A, APPENDIX B, and so on.

D6.17 Figures/Illustrations

Appendix D

All authors must strictly follow the guidelines below for preparing illustrations for publication in ***Protein & Peptide Letters***. If the figures are found to be sub-standard, then the manuscripts will be rejected and the authors offered the option of figure improvement professionally by **Eureka Science**. The costs for such improvement will be charged to the authors.

Illustrations should be provided as separate files, embedded in the text file, and must be numbered consecutively in the order of their appearance. Each figure should include only a single illustration which should be cropped to minimize the amount of space occupied by the illustration.

If a figure is in separate parts, all parts of the figure must be provided in a single composite illustration file.

Photographs should be provided with a scale bar if appropriate, as well as high-resolution component files.

D6.18 Scaling/Resolution

Line Art image type is normally an image based on lines and text. It does not contain tonal or shaded areas. The preferred file format should be TIFF or EPS, with the color mode being Monochrome 1-bit or RGB, in a resolution of 900-1200 dpi.

Halftone image type is a continuous tone photograph containing no text. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 300 dpi.

Combination image type is an image containing halftone , text or line art elements. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 500-900 dpi.

D6.19 Formats

Illustrations may be submitted in the following file formats:

- **Illustrator**
- **EPS** (preferred format for diagrams)
- **PDF** (also especially suitable for diagrams)
- **PNG** (preferred format for photos or images)
- **Microsoft Word** (version 5 and above; figures must be a single page)
- **PowerPoint** (figures must be a single page)
- **TIFF**
- **JPEG** (conversion should be done using the original file)
- **BMP**
- **CDX** (ChemDraw)
- **TGF** (ISISDraw)

Bentham Science does not process figures submitted in GIF format.

For TIFF or EPS figures with considerably large file size restricting the file size in online submissions is advisable. Authors may therefore convert to JPEG format before submission as this results in significantly reduced file size and upload time, while retaining acceptable quality. JPEG is a 'lossy' format. However, in order to maintain acceptable image quality, it is recommended that JPEG files are saved at High or Maximum quality.

Zipit or Stuffit tools should not be used to compress files prior to submission as the resulting compression through these tools is always negligible.

Please refrain from supplying:

1. Graphics embedded in word processor (spreadsheet, presentation) document.
2. Optimized files optimized for screen use (like GIF, BMP, PICT, WPG) because of the low resolution.
3. Files with too low a resolution.
4. Graphics that are disproportionately large for the content.

D6.20 Image Conversion Tools

Appendix D

There are many software packages, many of them freeware or shareware, capable of converting to and from different graphics formats, including PNG.

General tools for image conversion include Graphic Converter on the Macintosh, Paint Shop Pro, for Windows, and ImageMagick, available on Macintosh, Windows and UNIX platforms.

Bitmap images (e.g. screenshots) should not be converted to EPS as they result in a much larger file size than the equivalent JPEG, TIFF, PNG or BMP, and poor quality. EPS should only be used for images produced by vector-drawing applications such as Adobe Illustrator or CorelDraw. Most vector-drawing applications can be saved in, or exported as, EPS format. If the images were originally prepared in an Office application, such as Word or PowerPoint, original Office files should be directly uploaded to the site, instead of being converted to JPEG or another format of low quality.

D6.21 Color Figures/Illustrations

- **Color figures publication in the journal**: The cost for each individual page of color figures is **US$ 995**.
- Color figures should be supplied in CMYK and not RGB colors.

D6.22 Chemical Structures

Chemical structures must be prepared in ChemDraw (CDX) file and provided as separate file.

D6.23 Structure Drawing Preferences:

[As according to the ACS style sheet]

<u>Drawing Settings:</u>

Chain angle	120°
Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)

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Line width	0.6 pt (0.021cm, 0.0084in)
Margin width	1.6 pt (0.096cm)
Hash spacing	2.5 pt (0.088cm, 0.0347in)
Text settings:	
Font	Times New Roman
Size	8 pt
Under the Preference Choose:	
Units	points
Tolerances	3 pixels
Under Page Setup Use:	
Paper	US letter
Scale	100%

D6.24 Tables

- Data Tables should be submitted in Microsoft Word table format.
- Each table should include a title/caption being explanatory in itself with respect to the details discussed in the table. Detailed legends may then follow.
- Table number in bold font *i.e*. Table **1**, should follow a title. The title should be in small case with the first letter in caps. A full stop should be placed at the end of the title.
- Tables should be embedded in the text exactly according to their appropriate placement in the submitted manuscript.
- Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell are displayed as black lines.
- Tables should be numbered in Arabic numerals sequentially in order of their citation in the body of the text.
- If a reference is cited in both the table and text, please insert a lettered footnote in the table to refer to the numbered reference in the text.
- Tabular data provided as additional files can be submitted as an Excel spreadsheet.

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D6.25 Supportive/Supplementary Material

We do encourage to append supportive material, for example a PowerPoint file containing a talk about the study, a PowerPoint file containing additional screenshots, a Word, RTF, or PDF document showing the original instrument(s) used, a video, or the original data (SAS/SPSS files, Excel files, Access Db files etc.) provided it is inevitable or endorsed by the journal's Editor.

Supportive/Supplementary material intended for publication must be numbered and referred to in the manuscript but should not be a part of the submitted paper. In-text citations as well as a section with the heading "Supportive/Supplementary Material" before the "References" section should be provided. Here, list all Supportive/Supplementary Material and include a brief caption line for each file describing its contents.

Any additional files will be linked to the final published article in the form supplied by the author, but will not be displayed within the paper. They will be made available in exactly the same form as originally provided only on our Web site. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet). Supportive/ Supplementary material must be provided in a single zipped file not larger than 4 MB.

Authors must clearly indicate if these files are not for publication but meant for the reviewers'/editors' perusal only.

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D8 AUTHORS AND INSTITUTIONAL AFFILIATIONS

The author will be required to provide their full names, the institutional affiliations and the location, with an asterisk in front of the name of the principal/corresponding author. The corresponding author(s) should be designated and their complete address, business telephone and fax numbers and e-mail address must be stated to receive correspondence and galley proofs.

D9 PAGE CHARGES

No page charges will be levied to authors for the publication of their article.

D10 LANGUAGE AND EDITING

Manuscripts submitted containing language inconsistencies will not be published. Authors must seek professional assistance for correction of grammatical, scientific and typographical errors. Professional team available at Eureka Science may assist you in the English language editing of your article. Please contactEureka Science for a language editing quote at e-mail: info@eureka-science.com stating the total number of words of the article to be edited.

D11 언어 및 편집

영문 오타가 많은 원고는 출판되지 않을 것입니다. 영문 오타를 없애겠다는 조건으로 받은 원고는 영어 편집 전문회사인 유럽 공동 기술개발 기구로부터 가격 견적서가 보내 질 것입니다. 영어 작문에 어려움이 있는 비영어권 국가의 저자들은 원고를

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학술지에 제출하기 전에 영어 편집회사와 접촉할 것을 권합니다. 영어 편집 견적서를 받기 위해서 교정될 원고의 단어수를 적은 메일을 유럽 공동 기술개발 기구 메일인 info@eureka-science.com 로 보내시기 바랍니다.

D12 语言和编辑

含有很多英文印刷错误的提交稿将不予发表。接受发表的稿件其英文写作应是正确的；专业的语言编辑公司（尤里卡科学，可对稿件的英文润色提供报价。建议非英语国家、且英文写作欠佳的作者在投稿前先与语言编辑公司联系。请与尤里卡科学联系 info@eureka-science.com.

D13 EDITION ET LANGUE

Les manuscrits soumis avec plusieurs erreurs typographiques en Anglais ne seront pas publiés en l'état. Les manuscrits sont acceptés pour publication à la condition que l'anglais utilisé soit corrigé après la soumission et seront envoyés pour examen à Eureka Science, une société d'édition de langue professionnelle. Les auteurs en provenance de pays où la langue est différente de l'anglais et qui ont de médiocres compétences en anglais écrit, sont priés de contacter la société d'édition de langue avant de soumettre leur manuscrit à la revue. Merci de contacter Eureka Science à info@eureka-science.com pour un devis en indiquant le nombre total de mot de l'article à éditer.

D14 PROOF CORRECTIONS

Authors will receive page proofs of their accepted paper before publications. To avoid delays in publication, proofs should be checked immediately for typographical errors and returned within **48 hours**. Major changes are not acceptable at the proof stage. If unable to send corrections within **48 hours** due to some reason, the author(s) must at least send an acknowledgement on receiving the galley proofs or the article will be published exactly as received and the publishers will not be responsible for any error occurring in the published manuscript in this regard.

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The corresponding author will be solely responsible for ensuring that the revised version of the manuscript incorporating all the submitted corrections receives the approval of all the co-authors of the manuscript.

D15 REPRINTS

Printed reprints and e-prints may be ordered from the Publisher prior to publication of the article. First named authors may also order a personal print and online subscription of the journal at 50% off the normal subscription rate by contacting the subscription department at e-mail: subscriptions@benthamscience.org.

D16 OPEN ACCESS PLUS

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D17 FEATURED ARTICLE

Authors may opt to publicize their article(s) published with *Bentham Science* by highlighting their title(s) both at the journal's Homepage and the issue Contents page at a cost of **US$ 300**.

D18 REVIEWING AND PROMPTNESS OF PUBLICATION

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All manuscripts submitted for publication will be immediately subjected to peer-reviewing, usually in consultation with the members of the Editorial Advisory Board and a number of external referees. Authors may, however, provide in their Copyright Letter the contact details (including e-mail addresses) of four potential peer reviewers for their paper. Any peer reviewers suggested should not have recently published with any of the authors of the submitted manuscript and should not be members of the same research institution.

All peer-reviewing will be conducted *via* the Internet to facilitate rapid reviewing of the submitted manuscripts. Every possible effort will be made to assess the manuscripts quickly with the decision being conveyed to the authors in due course.

D19 COPYRIGHT

Authors who publish in *Bentham Science* print and online journals will transfer copyright to their work to *Bentham Science* Publishers. Submission of a manuscript to the respective journals implies that all authors have read and agreed to the content of the Copyright Letter or the Terms and Conditions. It is a condition of publication that manuscripts submitted to this journal have not been published and will not be simultaneously submitted or published elsewhere. Plagiarism is strictly forbidden, and by submitting the article for publication the authors agree that the publishers have the legal right to take appropriate action against the authors, if plagiarism or fabricated information is discovered. By submitting a manuscript the authors agree that the copyright of their article is transferred to the publishers if and when the article is accepted for publication. Once submitted to the journal, the author will not withdraw their manuscript at any stage prior to publication.

D20 SELF-ARCHIVING

By signing the Copyright Letter the authors retain the rights of self-archiving. Following are the important features of self-archiving policy of Bentham Science journals:

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2. Authors may deposit the ACCEPTED VERSION of the peer-reviewed article on their personal websites, their institution's repository or any non-commercial repository such as PMC, arXiv after **12 MONTHS of publication** on the journal website. In addition, an acknowledgement must be given to the original source of publication and a link should be inserted to the published article on the journal's/publisher's website.
3. If the research is funded by NIH, Wellcome Trust or any other Open Access Mandate, authors are allowed the archiving of published version of manuscripts in an institutional repository after the mandatory embargo period. Authors should first contact the Editorial Office of the journal for information about depositing a copy of the manuscript to a repository. Consistent with the copyright agreement, Bentham Science does not allow archiving of FINAL PUBLISHED VERSION of manuscripts.
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5. There is no embargo on the archiving of articles published under the OPEN ACCESS PLUS category. Authors are allowed deposition of such articles on institutional, non-commercial repositories and personal websites immediately after publication on the journal website.

D21 PLAGIARISM PREVENTION

Bentham Science Publishers uses the iThenticate software to detect instances of overlapping and similar text in submitted manuscripts. iThenticate software checks content against a database of periodicals, the Internet, and a comprehensive article database. It generates a similarity report, highlighting the percentage overlap between the uploaded article and the published material. Any instance of content overlap is further scrutinized for suspected plagiarism according to the publisher's Editorial Policies. *Bentham Science* allows an overall similarity of 20% for a manuscript to be considered for publication. The similarity percentage is further checked keeping the following important points in view:

D21.1 Low Text Similarity

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The text of every submitted manuscript is checked using the Content Tracking mode in iThenticate. The Content Tracking mode ensures that manuscripts with an overall low percentage similarity (but which may have a higher similarity from a single source) are not overlooked. The acceptable limit for similarity of text from a single source is 5%. If the similarity level is above 5%, the manuscript is returned to the author for paraphrasing the text and citing the original source of the copied material.

It is important to mention that the text taken from different sources with an overall low similarity percentage will be considered as a plagiarized content if the majority of the article is a combination of copied material.

D21.2 High Text Similarity

There may be some manuscripts with an overall low similarity percentage, but a higher percentage from a single source. A manuscript may have less than 20% overall similarity but there may be 15 % similar text taken from a single article. The similarity index in such cases is higher than the approved limit for a single source. Authors are advised to thoroughly rephrase the similar text and properly cite the original source to avoid plagiarism and copyright violation.

D21.3 Types of Plagiarism

We all know that scholarly manuscripts are written after thorough review of previously published articles. It is therefore not easy to draw a clear boundary between legitimate representation and plagiarism. However, the following important features can assist in identifying different kinds of plagiarized content. These are:

- Reproduction of others words, sentences, ideas or findings as one's own without proper acknowledgement.
- Text recycling, also known as self-plagiarism. It is an author's use of a previous publication in another paper without proper citation and acknowledgement of the original source.

- Paraphrasing poorly: Copying complete paragraphs and modifying a few words without changing the structure of original sentences or changing the sentence structure but not the words.
- Verbatim copying of text without putting quotation marks and not acknowledging the work of the original author.
- Properly citing a work but poorly paraphrasing the original text is considered as unintentional plagiarism. Similarly, manuscripts with language somewhere between paraphrasing and quoting are not acceptable. Authors should either paraphrase properly or quote and in both cases, cite the original source.
- Higher similarity in the abstract, introduction, materials and methods, and discussion and conclusion sections indicates that the manuscript may contain plagiarized text. Authors can easily explain these parts of the manuscript in many ways. However, technical terms and sometimes standard procedures cannot be rephrased; therefore Editors must review these sections carefully before making a decision.

D21.4 Plagiarism in Published Manuscripts

Published manuscripts which are found to contain plagiarized text are retracted from the journal website after careful investigation and approval by the Editor-in-Chief of the journal. A 'Retraction Note' as well as a link to the original article is published on the electronic version of the plagiarized manuscript and an addendum with retraction notification in the journal concerned.

D22 E-PUB AHEAD OF SCHEDULE

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D22.1 Disclaimer

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Articles appearing in E-Pub Ahead-of-Schedule sections have been peer-reviewed and accepted for publication in this journal and posted online before scheduled publication. Articles appearing here may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, ***Bentham Science Publishers***, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in articles of the E-Pub Ahead-of-Schedule.

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Current Drug Delivery: Guide for Authors

D1. ONLINE MANUSCRIPT SUBMISSION

An online submission and tracking service *via* Internet facilitates a speedy and cost-effective submission of manuscripts. The full manuscript has to be submitted online *via* Bentham's Content Management System (CMS) at bsp-cms.eurekaselect.com / **View Submission Instructions**

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It is imperative that before submission, authors should carefully proofread the files for special characters, mathematical symbols, Greek letters,equations, tables, references and images, to ensure that they appear in proper format.

References, figures, tables, chemical structures etc. should be referred to in the text at the appropriate place where they have been first discussed. Figure legends/captions should also be provided.

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A successful electronic submission of a manuscript will be followed by a system-generated acknowledgement to the principal/corresponding author. Any queries therein should be addressed to manuscript@benthamscience.org

D1.1 Editorial Policies

The editorial policies of *Bentham Science* Publishers on publication ethics, peer-review, plagiarism, copyrights/ licenses,errata/corrections and article retraction/ withdrawal can be viewed at Editorial Policy

D2 MANUSCRIPTS PUBLISHED

The Journal publishes peer-reviewed mini-reviews, letters and research papers written in English. Single topic/thematic issues may also be considered for publication.

D2.1 Single Topic Issues

These special issues are peer-reviewed and may contain invited or uninvited review/mini-review articles. A Single Topic Issue Editor will offer a short perspective and co-ordinate the solicitation of manuscripts (at least 10) for full-length thematic issues from leading scientists. Authors interested in editing a single topic issue in an emerging topic of outstanding developments in all important aspects of protein and peptide research, including structural studies, advances in recombinant expression, function, synthesis, enzymology, immunology, molecular modeling, drug design medicinal chemistry or rational drug design may submit their proposal to the Editor-in-Chief at ppl@benthamscience.org for consideration.

D2.2 Conference Proceedings

For proposals to publish conference proceedings in this journal, please contact us at proceedings@benthamscience.org for consideration.

D3 MANUSCRIPT LENGTH

D3.1 Mini-Reviews/ Research Articles/ Letters

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All manuscript types should be between 3000 and 8000 words excluding figures, structures,photographs, schemes and tables.

There is no restriction on the number of figures, tables or additional files e.g. video clips, animation and datasets, that can be included with each article online. Authors should include all relevant supporting data with each article (Refer to Supplementary Material section).

D4 MANUSCRIPT PREPARATION

The manuscript should be written in English in a clear, direct and active style. All pages must be numbered sequentially, facilitating in the reviewing and editing of the manuscript.

D5 MICROSOFT WORD TEMPLATE

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D6 MANUSCRIPT SECTIONS FOR PAPERS

Manuscripts may be divided into the following sections:

- Copyright Letter
- Title
- Title Page
- Structured Abstract
- Graphical Abstract
- Keywords
- Text Organization
- Conclusion
- List of Abbreviations (if any)
- Conflict of Interest

Appendix E

- Acknowledgements
- References
- Appendices
- Figures/Illustrations (if any)
- Chemical Structures (if any)
- Tables (if any)
- Supportive/Supplementary Material (if any)

D6.1 Copyright Letter

It is mandatory that a signed copyright letter should also be submitted along with the manuscript by the author to whom correspondence is to be addressed, delineating the scope of the submitted article declaring the potential competing interests, acknowledging contributions from authors and funding agencies, and certifying that the paper is prepared according to the ***'Instructions for Authors'***. All inconsistencies in the text and in the reference section and any typographical errors must be carefully checked and corrected before the submission of the manuscript. The article should not contain any such material or information that may be unlawful, defamatory, fabricated, plagiarized, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the copyright agreement. The authors acknowledge that the publishers have the legal right to take appropriate action against the authors for any such violation of the terms and conditions as laid down in the copyright agreement. Download the Copyright letter

D6.2 Title

The title of the article should be precise and brief and must not be more than 120 characters. Authors should avoid the use of non-standard abbreviations. The title must be written in title case except for articles, conjunctions and prepositions.

Authors should also provide a short 'running title'. Title, running title, byline, correspondent, footnote and key words should be written as presented in original manuscripts.

D6.3 Title Page

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Title page should include paper title, author(s) full name and affiliation, corresponding author(s) names complete affiliation/address, along with phone, fax and email.

D6.4 Structured Abstract

The abstract of an article should be its clear, concise and accurate summary, having no more than 250 words, and including the explicit sub-headings (as in-line or run-in headings in bold). Use of abbreviations should be avoided and the references should not be cited in the abstract. Ideally, each abstract should include the following sub-headings, but these may vary according to requirements of the article.

- Background
- Objective
- Method
- Results
- Conclusion

D6.5 Graphical Abstract

A graphic must be included with each manuscript for use in the Table of Contents (TOC). This must be submitted separately as an electronic file (preferred file types are EPS, PDF, TIFF, Microsoft Word, PowerPoint and CDX *etc*). A graphical abstract, not exceeding 30 words along with the illustration, helps to summarize the contents of the manuscript in a concise pictorial form. It is meant as an aid for the rapid viewing of the journals' contents and to help capture the readers' attention. The graphical abstract may feature a key structure, reaction, equation, *etc*. that the manuscript elucidates upon. It will be listed along with the manuscript title, authors' names and affiliations in the contents page, typeset within an area of 5 cm by 17 cm, but it will not appear in the article PDF file or in print.

Graphical Abstracts should be submitted as a separate file (must clearly mention graphical abstract within the file) online via Bentham's Content Management System by selecting the option “supplementary material”.

D6.6 Keywords

6 to 8 keywords must be provided.

D6.7 Text Organization

The main text should begin on a separate page and should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the Acknowledgements and Reference sections. For Letters/Research Articles, the manuscript should begin with the title page and abstract followed by the main text, which must be structured into separate sections as ***Introduction, Materials and Methods, Results, Discussion, Conclusion, List of abbreviations (if any), Conflict of Interest, Acknowledgements and References***. The Review Article should mention any previous important recent and old reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review. The authors are advised to present and discuss their observations in brief. Crystallization reports are no longer routinely published by the journal. Only reports on the first crystallization of proteins of substantial interest or reports on new crystallization techniques of wide application will be considered. Crystallization reports should include data describing the source of the protein, composition, pH and concentrations of precipitants and buffers etc., the method employed, crystal size and morphology, and time of growth etc. Unit cell dimensions, space-group assignments, resolution limit, specific volume and asymmetric unit contents must also be provided. The manuscript style must be uniform throughout the text and 10 pt Times New Roman fonts should be used. The full term for an abbreviation should precede its first appearance in the text unless it is a standard unit of measurement. The reference numbers should be given in square brackets in the text. Italics should be used for Binomial names of organisms (Genus and Species), for emphasis and for unfamiliar words or phrases. Non-assimilated words from Latin or other languages should also be italicized e.g. *per se, et al. etc*.

D6.8 Standard Protocol on Approvals, Registrations, Patient Consents & Animal Protection

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All clinical investigations must be conducted according to the Declaration of Helsinki principles. For all manuscripts reporting data from studies involving human participants, formal review and approval by an appropriate institutional review board or ethics committee is required. For research involving animals, the authors should indicate whether the procedures followed were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdf; published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

A specific declaration of such approval must be made in the copyright letter and in a stand-alone paragraph at the end of the Methods section especially in the case of human studies where inclusion of a statement regarding obtaining the written informed consent from each subject or subject's guardian is a must. The original should be retained by the guarantor or corresponding author. Editors may request to provide the original forms by fax or email.

D6.9 Authentication of Cell Lines

The NIH acknowledges the misidentification and/or cross-contamination of cell cultures e.g. HeLa cells being used in a research study as a serious problem. In order to ensure the validation of the work and proper utilization of resources, it is a prerequisite that correct reagents be used in studies dealing with established human (tumor) cell lines that have been cultured for more than 4 years up to the date of submission of the manuscript. Cell lines such as short-term cultures of human tumors, murine cell lines (as a catalog of DNA profiles is not yet available) and tumor cell lines established in the course of the study that is being submitted, are presently exempt from this rule. To minimize the risk of working with misidentified and/or contaminated cell lines, tests such as isoenzyme analysis, karyotyping/cytogenetic analysis and, more recently, molecular techniques of DNA profiling may be carried out to authenticate cell cultures. These tests may help confirm or establish the identity profile for a cell line. *Bentham Science* recommends that all cell lines be authenticated prior to submitting a paper for review. Authors are therefore required to provide authentication of the origin and identity of the cells by performing cell profiling either in their own laboratory or by outsourcing an approved laboratory or cell bank. Authentication is required when a new line is established or acquired, before freezing a cell line, if the

performance of the line is not consistent or results are unexpected, if using more than one cell line, and before publication of the study.

The cell lines profile should be cross-checked with the profile of the donor tissue of other continuous cell lines such as provided by the authentic data bank such as www.dsmz.de/fp/cgi-bin/str.html, ATCC® etc.

D6.10 Greek Symbols and Special Characters

Greek symbols and special characters often undergo formatting changes and get corrupted or lost during preparation of manuscript for publication. To ensure that all special characters used are embedded in the text, these special characters should be inserted as a symbol but should not be a result of any format styling (*Symbol* font face) otherwise they will be lost during conversion to PDF/XML.

Authors are encouraged to consult reporting guidelines. These guidelines provide a set of recommendations comprising a list of items relevant to their specific research design. Chemical equations, chemical names, mathematical usage, unit of measurements, chemical and physical quantity and units must conform to SI and Chemical Abstracts or IUPAC.

All kinds of measurements should be reported only in International System of Units (SI).

D6.11 Conclusion

A small paragraph summarizing the contents of the article, presenting the final outcome of the research or proposing further study on the subject, may be given at the end of the article under the Conclusion section.

D6.12 List of Abbreviations

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

D6.13 Conflict of Interest

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Financial contributions and any potential conflict of interest must be clearly acknowledged under the heading ‘Conflict of Interest’. Authors must list the source(s) of funding for the study. This should be done for each author.

D6.14 Acknowledgements

All individuals listed as authors must have contributed substantially to the design, performance, analysis, or reporting of the work and are required to indicate their specific contribution. Anyone (individual/company/institution) who has substantially contributed to the study for important intellectual content, or who was involved in the articles drafting the manuscript or revising must also be acknowledged. Guest or honorary authorship based solely on position (e.g. research supervisor, departmental head) is discouraged.

The specific requirements for authorship have been defined by the International Committee of Medical Journal Editors (ICMJE; www.icmje.org). Examples of authors' contributions are: 'designed research/study', 'performed research/study', 'contributed important reagents', 'collected data', 'analyzed data', 'wrote paper' etc. This information must be included in the submitted manuscript as a separate paragraph under the heading Acknowledgements. The corresponding author is responsible for obtaining permission from all co-authors for the submission of any version of the manuscript and for any changes in the authorship.

D6.15 References

References must be listed in the ACS Style only. All references should be numbered sequentially [in square brackets] in the text and listed in the same numerical order in the reference section. The reference numbers must be finalized and the bibliography must be fully formatted before submission.

See below few examples of references listed in the ACS Style:

D6.15.1 Journal Reference

- [1] Zheng, X. Q.; Li, C.; Wang, J. An information-theoretic approach to the prediction of protein structural class. *J. Comput. Chem.*, **2010**, *31*, 1201-1206.

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- [2] Mohabatkar, H.; Mohammad Beigi, M.; Esmaeili, A. Prediction of GABA(A) receptor proteins using the concept of Chou's pseudo-amino acid composition and support vector machine. *J. Theor. Biol.*, **2011**, *281*, 18-23.

D6.15.2 Book Reference

- [3] Crabtree, R.H. The Organometallic Chemistry of the Transition Metals, 3rd ed.; Wiley and Sons: New York, **2001**.

D6.15.3 Book Chapter Reference

- [4] Wheeler, D.M.S.; Wheeler, M.M. In: *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier Science B. V: Amsterdam, **1994**; Vol. *14*, pp. 3-46.

D6.15.4 Conference Proceedings

- [5] Jakeman, D.L.; Withers, S.G.E. In: *Carbohydrate Bioengineering: Interdisciplinary Approaches*, Proceedings of the 4th Carbohydrate Bioengineering Meeting, Stockholm, Sweden, June 10-13, 2001; Teeri, T.T.; Svensson, B.; Gilbert, H.J.; Feizi, T., Eds.; Royal Society of Chemistry: Cambridge, UK, **2002**; pp. 3-8.

D6.15.5 URL (WebPage)

- [6] National Library of Medicine. Specialized Information Services: Toxicology and Environmental Health. sis.nlm.nih.gov/Tox/ToxMain.html (Accessed May 23, **2004**).

D6.15.6 Patent

- [7] Hoch, J.A.; Huang, S. Screening methods for the identification of novel antibiotics. U.S. Patent 6,043,045, March 28, 2000.

D6.15.7 Thesis

- [8] Mackel, H. *Capturing the Spectra of Silicon Solar Cells*. PhD Thesis, The Australian National University: Canberra, December **2004**.

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D6.15.8 E-citations

- [9] Citations for articles/material published exclusively online or in open access (free-to-view), must contain the accurate Web addresses (URLs) at the end of the reference(s), except those posted on an author's Web site (unless editorially essential), e.g. 'Reference: Available from: URL'.

Some important points to remember:

- All references must be complete and accurate.
- All authors must be cited and there should be no use of the phrase *et al*.
- Date of access should be provided for online citations.
- Journal names should be abbreviated according to the Index Medicus/MEDLINE.
- Punctuation should be properly applied as mentioned in the examples given above.
- Superscript in the in-text citations and reference section should be avoided.
- Abstracts, unpublished data and personal communications (which can only be included if prior permission has been obtained) should not be given in the references section. The details may however appear in the footnotes.
- The authors are encouraged to use a recent version of EndNote (version 5 and above) or Reference Manager (version 10) when formatting their reference list, as this allows references to be automatically extracted.

D6.16 Appendices

In case there is a need to present lengthy, but essential methodological details, appendices must be used, which can be a part of the article. An appendix must not exceed three pages (Times New Roman, 12 point fonts, 900 max. words per page). The information should be provided in a condensed form, ruling out the need of full sentences. A single appendix should be titled APPENDIX, while more than one can be titled APPENDIX A, APPENDIX B, and so on.

D6.17 Figures/Illustrations

Appendix E

All authors must strictly follow the guidelines below for preparing illustrations for publication in ***Protein & Peptide Letters***. If the figures are found to be sub-standard, then the manuscripts will be rejected and the authors offered the option of figure improvement professionally by **Eureka Science**. The costs for such improvement will be charged to the authors.

Illustrations should be provided as separate files, embedded in the text file, and must be numbered consecutively in the order of their appearance. Each figure should include only a single illustration which should be cropped to minimize the amount of space occupied by the illustration.

If a figure is in separate parts, all parts of the figure must be provided in a single composite illustration file.

Photographs should be provided with a scale bar if appropriate, as well as high-resolution component files.

D6.18 Scaling/Resolution

Line Art image type is normally an image based on lines and text. It does not contain tonal or shaded areas. The preferred file format should be TIFF or EPS, with the color mode being Monochrome 1-bit or RGB, in a resolution of 900-1200 dpi.

Halftone image type is a continuous tone photograph containing no text. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 300 dpi.

Combination image type is an image containing halftone , text or line art elements. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 500-900 dpi.

D6.19 Formats

Illustrations may be submitted in the following file formats:

- **Illustrator**
- **EPS** (preferred format for diagrams)
- **PDF** (also especially suitable for diagrams)
- **PNG** (preferred format for photos or images)
- **Microsoft Word** (version 5 and above; figures must be a single page)
- **PowerPoint** (figures must be a single page)
- **TIFF**
- **JPEG** (conversion should be done using the original file)
- **BMP**
- **CDX** (ChemDraw)
- **TGF** (ISISDraw)

Bentham Science does not process figures submitted in GIF format.

For TIFF or EPS figures with considerably large file size restricting the file size in online submissions is advisable. Authors may therefore convert to JPEG format before submission as this results in significantly reduced file size and upload time, while retaining acceptable quality. JPEG is a 'lossy' format. However, in order to maintain acceptable image quality, it is recommended that JPEG files are saved at High or Maximum quality.

Zipit or Stuffit tools should not be used to compress files prior to submission as the resulting compression through these tools is always negligible.

Please refrain from supplying:

1. Graphics embedded in word processor (spreadsheet, presentation) document.
2. Optimized files optimized for screen use (like GIF, BMP, PICT, WPG) because of the low resolution.
3. Files with too low a resolution.
4. Graphics that are disproportionately large for the content.

D6.20 Image Conversion Tools

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There are many software packages, many of them freeware or shareware, capable of converting to and from different graphics formats, including PNG.

General tools for image conversion include Graphic Converter on the Macintosh, Paint Shop Pro, for Windows, and ImageMagick, available on Macintosh, Windows and UNIX platforms.

Bitmap images (e.g. screenshots) should not be converted to EPS as they result in a much larger file size than the equivalent JPEG, TIFF, PNG or BMP, and poor quality. EPS should only be used for images produced by vector-drawing applications such as Adobe Illustrator or CorelDraw. Most vector-drawing applications can be saved in, or exported as, EPS format. If the images were originally prepared in an Office application, such as Word or PowerPoint, original Office files should be directly uploaded to the site, instead of being converted to JPEG or another format of low quality.

D6.21 Color Figures/Illustrations

- **Color figures publication in the journal**: The cost for each individual page of color figures is **US$ 995**.
- Color figures should be supplied in CMYK and not RGB colors.

D6.22 Chemical Structures

Chemical structures must be prepared in ChemDraw (CDX) file and provided as separate file.

D6.23 Structure Drawing Preferences:

[As according to the ACS style sheet]

<u>Drawing Settings:</u>

Chain angle	120°
Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)

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Line width	0.6 pt (0.021cm, 0.0084in)
Margin width	1.6 pt (0.096cm)
Hash spacing	2.5 pt (0.088cm, 0.0347in)
Text settings:	
Font	Times New Roman
Size	8 pt
Under the Preference Choose:	
Units	points
Tolerances	3 pixels
Under Page Setup Use:	
Paper	US letter
Scale	100%

D6.24 Tables

- Data Tables should be submitted in Microsoft Word table format.
- Each table should include a title/caption being explanatory in itself with respect to the details discussed in the table. Detailed legends may then follow.
- Table number in bold font *i.e*. Table **1**, should follow a title. The title should be in small case with the first letter in caps. A full stop should be placed at the end of the title.
- Tables should be embedded in the text exactly according to their appropriate placement in the submitted manuscript.
- Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell are displayed as black lines.
- Tables should be numbered in Arabic numerals sequentially in order of their citation in the body of the text.
- If a reference is cited in both the table and text, please insert a lettered footnote in the table to refer to the numbered reference in the text.
- Tabular data provided as additional files can be submitted as an Excel spreadsheet.

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D6.25 Supportive/Supplementary Material

We do encourage to append supportive material, for example a PowerPoint file containing a talk about the study, a PowerPoint file containing additional screenshots, a Word, RTF, or PDF document showing the original instrument(s) used, a video, or the original data (SAS/SPSS files, Excel files, Access Db files etc.) provided it is inevitable or endorsed by the journal's Editor.

Supportive/Supplementary material intended for publication must be numbered and referred to in the manuscript but should not be a part of the submitted paper. In-text citations as well as a section with the heading "Supportive/Supplementary Material" before the "References" section should be provided. Here, list all Supportive/Supplementary Material and include a brief caption line for each file describing its contents.

Any additional files will be linked to the final published article in the form supplied by the author, but will not be displayed within the paper. They will be made available in exactly the same form as originally provided only on our Web site. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet). Supportive/ Supplementary material must be provided in a single zipped file not larger than 4 MB.

Authors must clearly indicate if these files are not for publication but meant for the reviewers'/editors' perusal only.

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D8 AUTHORS AND INSTITUTIONAL AFFILIATIONS

The author will be required to provide their full names, the institutional affiliations and the location, with an asterisk in front of the name of the principal/corresponding author. The corresponding author(s) should be designated and their complete address, business telephone and fax numbers and e-mail address must be stated to receive correspondence and galley proofs.

D9 PAGE CHARGES

No page charges will be levied to authors for the publication of their article.

D10 LANGUAGE AND EDITING

Manuscripts submitted containing language inconsistencies will not be published. Authors must seek professional assistance for correction of grammatical, scientific and typographical errors. Professional team available at Eureka Science may assist you in the English language editing of your article. Please contactEureka Science for a language editing quote at e-mail: info@eureka-science.com stating the total number of words of the article to be edited.

D11 언어 및 편집

영문 오타가 많은 원고는 출판되지 않을 것입니다. 영문 오타를 없애겠다는 조건으로 받은 원고는 영어 편집 전문회사인 유럽 공동 기술개발 기구로부터 가격 견적서가 보내 질 것입니다. 영어 작문에 어려움이 있는 비영어권 국가의 저자들은 원고를

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학술지에 제출하기 전에 영어 편집회사와 접촉할 것을 권합니다. 영어 편집 견적서를 받기 위해서 교정될 원고의 단어수를 적은 메일을 유럽 공동 기술개발 기구 메일인 info@eureka-science.com 로 보내시기 바랍니다.

D12 语言和编辑

含有很多英文印刷错误的提交稿将不予发表。接受发表的稿件其英文写作应是正确的；专业的语言编辑公司（尤里卡科学，可对稿件的英文润色提供报价。建议非英语国家、且英文写作欠佳的作者在投稿前先与语言编辑公司联系。请与尤里卡科学联系 info@eureka-science.com.

D13 EDITION ET LANGUE

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Authors will receive page proofs of their accepted paper before publications. To avoid delays in publication, proofs should be checked immediately for typographical errors and returned within **48 hours**. Major changes are not acceptable at the proof stage. If unable to send corrections within **48 hours** due to some reason, the author(s) must at least send an acknowledgement on receiving the galley proofs or the article will be published exactly as received and the publishers will not be responsible for any error occurring in the published manuscript in this regard.

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The corresponding author will be solely responsible for ensuring that the revised version of the manuscript incorporating all the submitted corrections receives the approval of all the co-authors of the manuscript.

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D16 OPEN ACCESS PLUS

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D18 REVIEWING AND PROMPTNESS OF PUBLICATION

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D21.1 Low Text Similarity

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The text of every submitted manuscript is checked using the Content Tracking mode in iThenticate. The Content Tracking mode ensures that manuscripts with an overall low percentage similarity (but which may have a higher similarity from a single source) are not overlooked. The acceptable limit for similarity of text from a single source is 5%. If the similarity level is above 5%, the manuscript is returned to the author for paraphrasing the text and citing the original source of the copied material.

It is important to mention that the text taken from different sources with an overall low similarity percentage will be considered as a plagiarized content if the majority of the article is a combination of copied material.

D21.2 High Text Similarity

There may be some manuscripts with an overall low similarity percentage, but a higher percentage from a single source. A manuscript may have less than 20% overall similarity but there may be 15 % similar text taken from a single article. The similarity index in such cases is higher than the approved limit for a single source. Authors are advised to thoroughly rephrase the similar text and properly cite the original source to avoid plagiarism and copyright violation.

D21.3 Types of Plagiarism

We all know that scholarly manuscripts are written after thorough review of previously published articles. It is therefore not easy to draw a clear boundary between legitimate representation and plagiarism. However, the following important features can assist in identifying different kinds of plagiarized content. These are:

- Reproduction of others words, sentences, ideas or findings as one's own without proper acknowledgement.
- Text recycling, also known as self-plagiarism. It is an author's use of a previous publication in another paper without proper citation and acknowledgement of the original source.

- Paraphrasing poorly: Copying complete paragraphs and modifying a few words without changing the structure of original sentences or changing the sentence structure but not the words.
- Verbatim copying of text without putting quotation marks and not acknowledging the work of the original author.
- Properly citing a work but poorly paraphrasing the original text is considered as unintentional plagiarism. Similarly, manuscripts with language somewhere between paraphrasing and quoting are not acceptable. Authors should either paraphrase properly or quote and in both cases, cite the original source.
- Higher similarity in the abstract, introduction, materials and methods, and discussion and conclusion sections indicates that the manuscript may contain plagiarized text. Authors can easily explain these parts of the manuscript in many ways. However, technical terms and sometimes standard procedures cannot be rephrased; therefore Editors must review these sections carefully before making a decision.

D21.4 Plagiarism in Published Manuscripts

Published manuscripts which are found to contain plagiarized text are retracted from the journal website after careful investigation and approval by the Editor-in-Chief of the journal. A 'Retraction Note' as well as a link to the original article is published on the electronic version of the plagiarized manuscript and an addendum with retraction notification in the journal concerned.

D22 E-PUB AHEAD OF SCHEDULE

Bentham Science Publishers are pleased to offer electronic publication of accepted papers prior to scheduled publication. These peer-reviewed papers can be cited using the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue. Articles ahead of schedule may be ordered by pay-per-view from the relevant links given by each article stated *via* the **E-Pub Ahead of Schedule**

D22.1 Disclaimer

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Articles appearing in E-Pub Ahead-of-Schedule sections have been peer-reviewed and accepted for publication in this journal and posted online before scheduled publication. Articles appearing here may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, ***Bentham Science Publishers***, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in articles of the E-Pub Ahead-of-Schedule.

Appendix F

Ethics committee application and approval: In vivo study

Faculty of Health Sciences
Animal Ethics Committee (AEC)
Cover Page

For office use only:	
AEC Application number	014/040

Instructions

- Researchers must ensure that they use the current version of the application which is available on http://www.health.uct.ac.za/research/animalethics/forms
- **Please do not include this cover page with your submission.**

New Protocol Application

- Researchers submitting a new application to conduct animal research must complete a New Protocol Application Form **(FHS003)**.
- This form has to go through a scientific review by a Departmental Research Committee (DRC), before it can be submitted to the Animal Research Ethics Committee (AEC) for review.
- Upon receiving the approval from the DRC, **ONE** copy of the application form with the DRC approval signature /letter, can then be scanned or hand-delivered to nosi.tsama@uct.ac.za or lamees.emjedi@uct.ac.za. An additional electronic copy of your original application which has been converted from a Word document to a PDF format (not an image file) is to be mailed to nosi.tsama@uct.ac.za or lamees.emjedi@uct.ac.za

Post Submission Guidance.

- Upon receipt by the Research Office, the application form will be forwarded to the AEC Exco who will send it out to two to three AEC members for pre-meeting review.
- Reviewers' queries, comments and suggestions will be forwarded to the principal investigator (PI) to allow the application to be amended before it goes for the full committee review.

Responding to the Committee's Feedback

- On a cover letter, copy each question or concern of the AEC, and provide a detailed and thoughtful response. Incomplete responses are likely to trigger a repeat query from the reviewer, which may slow down the process.
- Address all points and queries, using references where necessary.
- If a reviewer's feedback is unclear or ambiguous, contact the AEC Exco and request clarification. If you disagree with a comment or recommended change, provide your rationale.
- Submit 19 copies of a signed revised protocol with **all changes highlighted in bold or italics** on the paper copy so the reviewer can immediately determine where and what changes have been made. These signed revised copies must be submitted to the **Research Ethics office** within the time frame given.
- Proofread the final versions for grammatical, typographical and formatting errors. (The AEC studies applications closely. To avoid delays, the completed form should be proofread by someone other than the researcher who wrote it, and preferably by someone from another department who is thus independent. This person should also be asked to **draw attention to inconsistencies between answers given to the various sections of the application**)
- After the Committee meeting, feedback will be sent to the PI.
- Once the study is formally authorised, the researcher needs to submit a final signed copy of the application with all the changes as required.
- A formal authorisation letter will then be issued.

Annual Progress Reports (Continuing Review of On-going Research)

The PI is responsible for submitting an annual progress report to the AEC in a timely manner before the approval period for the study expires. The AEC will remind you one month before the deadline of submission of your report.

- The AEC has the authority to suspend or terminate research that does not comply with annual reporting requirements. The Annual Progress report form is available on the web page: http://www.health.uct.ac.za/research/animalethics/forms
- Annual Progress /Final Report

Faculty of Health Sciences
Animal Ethics Committee (AEC)

- Current forms to be downloaded from the Administrative Forms web page at http://www.health.uct.ac.za/research/animalethics/forms
- All changes to the current application based on feedback by the AEC will be reviewed again. Commencement of the study on animals can only be done after authorisation has been given. Any subsequent changes to the authorised application **MUST** be submitted as an amendment (FH005) and reviewed by the AEC before implementing.
- Please print double-sided where possible.
- **Important:**
  All protocols submitted to FHS AEC for review must first be approved by the DRC (Departmental Research Committee)

This application must be typed and one signed completed form submitted to:	**For office use only**	
Mrs Nosi Tsama Animal Research Ethics Committee E 52 Room 25, Old Main Building, Groote Schuur Hospital, Observatory, 7700 Telephone: +27 21 404 7682 Fax: +27 21 406 6411 An electronic copy of the original application (Word format which is saved as a PDF file) is to be forwarded to nosi.tsama@uct.ac.za or lamees.emjedi@uct.ac.za	Application No:	
	Species:	
	Total number of animals:	
	Severity category:	
	Date received:	
	Date authorised	

Category (select one)

This is a first submission	☒		
This is a resubmission	☐	Previous application number	

1. TITLE OF APPLICATION	Pharmacokinetic interactions between *Aloe vera* gel polysaccharides and indinavir **in rats**

2. DETAILS OF APPLICANT	
Title (e.g. Prof, Dr, Mr, Ms)	Dr
Forenames & Surname	Lubbe Wiesner
Qualifications (e.g. PhD)	PhD
Position or appointment	Senior Research Officer
If applicant is a **student,** please provide **name of supervisor**	

3. CONTACT DETAILS	
Address for correspondence:	University of Cape Town, Medical School OMB H55, Groote Schuur Hospital, Observatory, 7925, South Africa
Telephone number, extension	406 6476
Cell phone number	083 451 7260
Fax number:	448 1989
E-mail address:	lubbe.wiesner@uct.ac.za
Telephone number of Supervisor (if applicable)	

E-mail address of Supervisor (if applicable)	

4. DEPARTMENTAL RESEARCH COMMITTEE REVIEW

I declare that this research protocol has been **peer-reviewed** by the Research Committee of the Department of …… and has been judged to be relevant, **necessitates the use of animals to achieve its aims,** designed in accordance with accepted scientific practices and norms and is in the opinion of the reviewers to be likely to be successful in achieving its aim/s.

Signature		Print name		Date	

5. ANIMALS REQUIRED

Species/ Strain	UCT strain number	Sex (male, female, either, or both)	Age	Mass	Total numbers	Source
Sprague Dawley rats	Not applicable	Male	8 weeks	250 - 300g	48 (6 rats x 8 experimental groups)	SAVC

6. DURATION OF APPLICATION

Period for which the application is required (must not exceed three years)		Years	0	Months	4
Start date	**01 November 2014**	End date		**28 February 2015**	

7. PURPOSE (select category)

Research	x	Teaching/training		Other (specify)	

8. RESEARCH PARTICIPANTS

Include **all participants** involved (principal investigator, associate and assistant research participants) who will perform any **procedures/treatments** on, and who will **monitor the welfare** of the animals. **Note:** All individuals who perform procedures or services of Laboratory Animal Technologists must be registered or authorised by the SA Veterinary Council (SAVC) to do so. **It is imperative that you indicate which scientific study member can be contacted for after-hours emergencies, and provide their after-hours contact telephone number and email.**

If the applicant is a student then the PI (project supervisor) must be included in this section and take overall responsibility. The student then ticks all relevant boxes.

• Name • Position • Dept. • Signature	Contact details (Telephone; E-mail)	**Duties/Procedures to be performed on the animal. Tick appropriate box/boxes below**									State appropriate training and experience in such procedures and duties	Registration with / authorisation by *SAVC, HPCSA or NSCSA? Please supply registration / authorisation number*	UCT AEC Accredited Yes/ No *See below table for definitions
		Overall responsibility	Welfare monitoring of animals	Blood collection from live animals	Administration of Injections / inoculations / oral gavage (specify)	Administration of general anaesthesia	Performing surgical procedures (specify type)	Harvesting of tissue from anaesthetised animals	Other procedures (specify)	Killing animals / assistance with killing			
Dr Lubbe Wiesner Research Officer Dept Medicine	X6472 Lubbe.wiesner@uct.ac.za 0834517260	X									Dr Wiesner has 10 years' experience in all areas specified	Yes HPCSA MW 0009296	Y
Ms Lonette Wallis PhD student	(018) 299 2858								Compound preparation		Ms Wallis will only prepare test formulations		N
Mr Trevor Finch Scientific officer	X6496 Trevor.finch@uct.ac.za **0797302915**		X	Blood from tail tip	Oral gavage	Isoflurane admin				X	Mr Finch has 30 years' experience in all areas specified	Yes SAVC Authorisation ART 03/6011	Y
Dr Liezl Gibhard Post-doc Dept Medicine	X6152 Liezl.gibhard@uct.ac.za								Compound preparation		Dr Gibhard has 4 years' experience in all areas specified		Y

* UCT AEC Accredited: Persons who have successfully completed the UCT FHS AEC-accredited Animal Ethics certification course; or persons who have successfully completed similar certification courses at other institutions that are also accredited by the UCT FHS AEC

9. STUDY INFORMATION: Background

Provide a brief introductory statement **in non-technical terms** that explains what problems, questions, needs, observations, or new ideas have led to the planning of this study. (**Note**: Must be understandable to lay people outside of the field). **Maximum word count: 300 words**

Underlying medical issues/problem/scientific question that have led to this study

In Sub-Saharan Africa, an increasing number of patients are using traditional herbal medicines for the management of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) (Nagata *et al*., 2011). Because of potential pharmacokinetic and pharmacodynamic interactions between the traditional herbal medicines and anti-retroviral drugs (ARV's), there are serious concerns amongst health practitioners for the safety of these patients (Nagata *et al.,* 2011). Some herbal remedies such as St. John's wort (*Hypericum perforatum*) and garlic (*Allium sativum*) have been proven to reduce the plasma concentrations of certain anti-retroviral drugs including indinavir and saquinavir, which could lead to possible drug resistance and therefore reduced efficacy of the drug (Pal & Mitra, 2006).

Many of the medicinal effects of aloe leaf extracts have been attributed to the polysaccharides found in the inner leaf tissue or gel (Ni *et al*., 2004). *In vitro* studies have shown that acemannan, considered as one of the main polysaccharides in *A. vera* gel material, has potential synergistic effects when used in combination with certain nucleoside reverse-transcriptase inhibitors in human U1 cells infected with herpes simplex virus (HSV-1) and HIV-1. Juice prepared from the *A. vera* plant has promoted the expression of various enzymes including cytochrome P450 1A2 (CYP1A2) and cytochrome P450 3A4 (CYP3A4) as well as multidrug resistant protein 1 (MDR1). *A. vera* gel extract did not show a significant effect on the active transport of digoxin from the basolateral to apical direction across epithelial cells (i.e. P-glycoprotein related efflux) (Djuv & Nilson, 2008; Cordier & Steenkamp., 2011). However, *A. vera* juice has been reported to increase the bioavailability of both vitamins C and E in humans (Vinson, Al Kharrat & Andreoli, 2005).

Since the *A. vera* plant has traditionally been used for medicinal purposes over centuries and aloe health drinks become more popular, it is important to determine the potential pharmacokinetic interactions that this traditional herbal medicinal plant may have when combined with Western medicines such as ARV's. Information regarding these interactions is needed to prevent potential therapeutic failure, but may also be used to the benefit of the patient if it is able to improve the drug's bioavailability and this may be used to reduce the dose needed to reach optimum efficacy.

Supporting evidence: e.g. preliminary data from pilot studies /previous studies/in vitro studies

In vitro studies have shown that *A. vera* gel and whole leaf extracts increase the transport of different drugs across human epithelial cell monolayers (i.e. Caco-2 cell line) as well as across excised rat intestinal tissue. The mechanism of action of the *A. vera* gel to increase drug absorption across the intestinal tissue was found to be the opening of tight junctions between adjacent epithelial cells, while precipitated polysaccharides from the *A. vera* leaf gel inhibited efflux transport of a drug that is a substrate for efflux transporters (Chen *et al*., 2009; Beneke *et al*., 2012; Beneke *et al*., 2013; Lebitsa *et al*., 2012).

Rationale for using animals

Although herb-drug pharmacokinetic interactions between *A. vera* gel and Western drugs have been shown to exist by *in vitro* studies, it is important to establish to what extent these pharmacokinetic interactions occur when administered to whole animals *in vivo*. Pharmacokinetic data from *in vivo* studies in animals will indicate if significant pharmacokinetic interactions exist between *A. vera* gel and ARV drugs (e.g. indinavir) with all physiological functions intact. This information can then be used to develop innovative drug delivery systems that may provide optimum plasma levels at lower doses or to prevent unwanted side-effects in patients that use aloe herbal products in an uncontrolled manner with ARV therapy.

List of publications supporting the above:

BENEKE C., VILJOEN A., HAMMAN J. 2012. *In vitro* drug absorption enhancement effects of *Aloe vera* and *Aloe ferox. Scientiae Pharmaceutica,* 80:475-486.

BENEKE C., VILJOEN A., HAMMAN J. 2013. Modulation of drug efflux by Aloe materials: An *in vitro* investigation across rat intestinal tissue. *Pharmacognosy Magazine,* 9:S44-S48.

CHEN, W., LU, Z., VILJOEN, A. & HAMMAN, J. 2008. Intestinal drug transport enhancement by Aloe Vera. *Planta Medica*, 75:587-595.

CORDIER, W. & STEENKAMP, V. 2011. Drug interactions in African herbal remedies. *Drug Metabolism and Drug Interactions*, 26:53-63.

DJUV, A. & NILSEN, O.G. Caco-2 Cell Methodology and Inhibition of the P-glycoprotein Transport of Digoxin by *Aloe vera* Juice. *Phytotherapy Research*, 22:1623-1628.

LEBITSA T., VILJOEN A., LU Z., HAMMAN J. 2012. *In vitro* drug permeation enhancement potential of Aloe gel materials. *Current Drug Delivery*, 9:297-304.

NAGATA, J.M., JEWC, A.R., KIMEUD, J.M., SALMENA, C.R., BUKUSIE, E.A. & COHEN, C.R. 2011. Medical pluralism on Mfangano Island: Use of medicinal plants among persons living with HIV/AIDS in Sub District, Kenya. *Journal of Ethnopharmacology,* 135:501-509.

NI, Y.; TURNER, D.; YATES, K.M.; TIZARD, I. 2004. Isolation and characterisation of structural components of *Aloe vera* L. leaf pulp. *International Immunopharmacology,* 4:1745-1755.

PAL, D. & MITRA, A.K. 2006. MDR- and CYP3A4-mediated drug–herbal interactions. *Life Sciences*, 78:2131–2145.

VINSON, J.A., Al KHARRAT, H. and ANDREOLI, L. 2005. Effect of *Aloe vera* preparations on the human bioavailability of vitamins C and E. *Phytomedicine*, 12:760-765.

10. AIMS OF THE STUDY

Please be brief and succinct.

To determine if different *A. vera* gel polysaccharide components have significant effects on the *in vivo* bioavailability of an ARV drug (i.e. indinavir) in rats.

11. HYPOTHESIS

If a hypothesis is being tested (explanatory research) please state what it is.

N/A

12. POTENTIAL BENEFITS OF THE RESEARCH FINDINGS

These are required to aid the reviewing committee in performing a harms/benefit assessment of the proposal. The total cumulative harm to the animals should be weighed up against the likely real benefits to humans or animals. (Guidelines to determine cost benefit analysis can be found in Chapter 4 of the APC Review of Cost Benefit Assessment in the use of Animals in research (June 2003) (summaries are in boxes 4, 5 and 6 in this chapter) at http://www.health.uct.ac.za/research/animalethics/policies/

List references supporting your analysis.

The knowledge generated by this *in vivo* study can be used to potentially prevent treatment failure or adverse effects in HIV/AIDS patients that ingest *A. vera* juice or leaf extracts simultaneously with indinavir treatment. Clinical significance of herb-drug interactions can only be effectively determined by means of *in vivo* studies (Tarirai *et al*., 2010). Furthermore, the data will indicate if *A. vera* gel polysaccharides are suitable permeation enhancers for the development of advanced drug delivery systems with increased bioavailability of drug compounds, which will allow administration of lower doses of drugs such as indinavir to obtain optimum therapeutic levels (Whitehead *et al*., 2008).

References:

TARIRAI, C., VILJOEN, A.M. & HAMMAN, J.H. 2010. Herb-drug pharmacokinetic interactions reviewed. *Expert Opinion in Drug Metabolism and Toxicity*, 6(12):1-24.

WHITEHEAD, K., KARR, N, MITRAGOTRI, S. 2008. Discovery of synergistic permeation enhancers for oral drug delivery. *Journal of Controlled Release*, 128:128-133.

13. FOCUS ON REPLACEMENT: JUSTIFICATION FOR THE USE OF SENTIENT ANIMALS

Please provide justification for the use of an animal model rather than an in-vitro or other alternative model by answering the following questions.

Circle the correct option		*Provide reasons to support your Yes or No answer*
Are there any in vitro/other non-animal alternatives that could be used to address the aims of this study? If so, which models?	Yes/**No**/not sure/ Not applicable	*In vitro* tests have been completed (Chen *et al*., 2009; Lebitsa *et al*., 2012; Beneke *et al*., 2012; Beneke *et al*., 2013), but these *in vitro* models do

		not provide clinical relevant data needed for patient advice and for dosage form development.
Have you considered using alternatives to animals for this study?	Yes/**no**/not applicable	*In vitro* tests on transport and metabolism interactions between the *A. vera* polysaccharides and indinavir form part of this research project, which allows refinement and reduction of animal use in the *in vivo* part of the study.
Are the animal species and genetic strain appropriate models for this study? Motivate why.	**Yes**/no/not sure	Male Sprague Dawley rats are a well-known *in vivo* model for drug pharmacokinetic studies (Steyn *et al*., 2011) including for herb-antiretroviral drug pharmacokinetic interactions (Ho *et al*., 2009). It is essential to establish the extent and potential hazards of the pharmacokinetic interactions in small animals before continuation to larger animals, primates and/or human subjects.

List of references supporting the above:

BENEKE C., VILJOEN A., HAMMAN J. 2012. *In vitro* drug absorption enhancement effects of *Aloe vera* and *Aloe ferox. Scientiae Pharmaceutica,* 80:475-486.

BENEKE C., VILJOEN A., HAMMAN J. 2013. Modulation of drug efflux by Aloe materials: An *in vitro* investigation across rat intestinal tissue*. Pharmacognosy Magazine,* 9:S44-S48.

CHEN, W., LU, Z., VILJOEN, A. & HAMMAN, J. 2008. Intestinal drug transport enhancement by *Aloe vera*. *Planta Medica*, 75:587-595.

HO, Y.F., HUANG D.K., HSUEH W.C., LAI M.Y., YU H.Y., TSAI T.H. 2009. Effects of St. John's wort extract on indinavir pharmacokinetics in rats: Differentiation of intestinal and hepatic impacts. *Life Sciences*, 85:296-302.

LEBITSA T., VILJOEN A., LU Z., HAMMAN J. 2012. *In vitro* drug permeation enhancement potential of Aloe gel materials. *Current Drug Delivery*, 9:297-304.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W-C., HAYNES, R.K., KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of PheroidTM technology. *International Journal of Pharmaceutics*, 414, 260–266.

14. FOCUS ON REDUCTION AND REFINEMENT
Please provide justification for:

(a) The number of animals required.

Please justify the sample size in relation to the expected magnitude of treatment effects (i.e. differences in measured values between treatment and control groups), the expected variability/variance of measured values, statistical power, and the chosen significance threshold. Provide data from the literature or from your previous work on this model where available. (Animal researchers are asked to please comment on their chosen "threshold").

If applicants require assistance with determining the statistical power and sample size, the faculty biostatistician, Mr Henri Carrara can be consulted: henri.carrara@uct.ac.za

Sample size per group	Six (6)

Provide supporting information that justifies the above group size:

Note: This section requires a Power analysis to be performed to determine the number of animals required for the study. Data from previous experiments which may have similar protocols can also substantiate the number of animals requested. One needs to establish the number of animals per group and the percentage power (i.e. 95%) that will detect a difference between means that will provide the chose significance level (e.g. $p < 0.05$)

A. Expected effect-size	50%
B. Expected variability/variance of measured values	10%

C. Statistical power selected	90%
D. Significance threshold (p value) selected	0.05

(b) Sex of animals required.

If a single sex is required, please justify

Male/Female/either: Male, to prevent potential influence of sex related factors on pharmacokinetics of the model drug (Ofotokun *et al.,* 2007).

List of References supporting sections a) and b):

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W-C., HAYNES, R.K., KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of PheroidTM technology. *International Journal of Pharmaceutics*, 414, 260–266.

OFOTOKUN, I., CHUCK S.K., HITTI E.T. 2007. Antiretroviral pharmacokinetic profile: A review of sex differences. *Gender Medicine*, 4:106-119.

15. STATISTICAL ANALYSIS

State the proposed statistical analysis on the data from this study, indicate whether a statistician was consulted, and state who will be doing the analysis.

a) Stats Analyses:

It was proven in previous *in vivo* pharmacokinetic studies that 6 animals per group are sufficient in order to provide meaningful statistical data for drug absorption enhancement type pharmacokinetic studies (Steyn *et al.,* 2011).

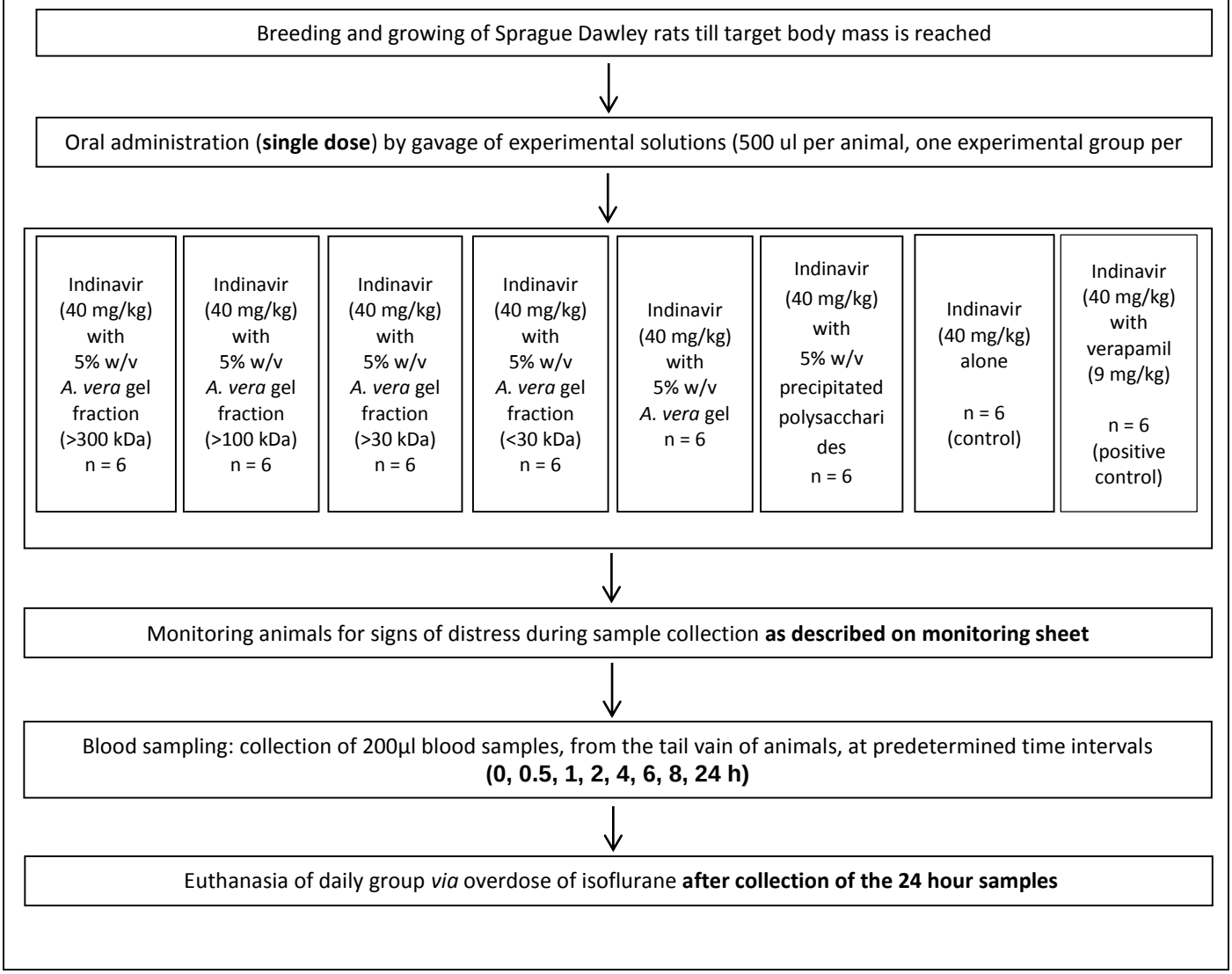
STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W-C., HAYNES, R.K., KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of PheroidTM technology. *International Journal of Pharmaceutics*, 414, 260–266.

b) Consulting Statistician: (even if none consulted then justify the reasons for the above analysis):

Consulted with Dr Paolo Denti, who is the division of pharmacology's pharmacometrician.

16. OVERVIEW OF EXPERIMENTAL DESIGN

In a flow diagram, summarise how the animals will be allocated to experimental and control groups, indicating the schedule for treatment, sampling, and clearly indicating the pre-determined endpoint for the animals. Include the parameters to be measured/final tissue analyses to be performed in order to achieve the aims of the study. For all control groups, include the rationale for including the specific control group/s (i.e. sham controls, method controls etc.)



17. DETAILS OF EXPERIMENTAL PROCEDURES AND STATEMENT OF COMPETENCE

Provide a detailed description **(in the order in which the procedures will be performed)** of all procedures, treatments, tissue sampling that the animals will undergo, and the method of killing, if applicable. Indicate in Section 8 who will perform each procedure, and their appropriate competence for the tasks.

Ensure that the following are included where applicable:

- Anaesthesia: (drug, route of administration, anaesthetic monitoring, expected duration of anaesthesia).
- Surgery: (incision site, surgical procedure, wound closure)
- Inoculations: (substance/organism, inoculation site/route of administration, volume, frequency)
- Analgesia (Substance, dosage, route of administration, frequency)
- Sampling from live animals: (e.g. blood, method of sampling, volume taken, frequency, application of local anaesthetic)
- Non-surgical –based experiments: (details of how the animals will be handled and what conditions they will be subjected to)
- Killing: (Method, time point after start of experiment/procedure)

1. Animal selection

The test subjects will be randomly selected by means of systematic random sampling, where N = 48, n = 6. For the first group, a starting point between numbers 1-7 will be randomly selected and every 7th numbered individual thereafter will be taken. For the second group a starting point between the first 6 remaining numbers will be randomly selected and every 7th numbered individual thereafter will be taken. This will be repeated for groups 3 to 7. Group 8 will consist of the remaining 6 rats.

2. Administration of solutions to rats

Solutions of the different weight fractions of the *A. vera* gel polysaccharides and indinavir will be administered orally to the rats with the aid of an oral gavage tube at a volume of 500 µl per animal. Indinavir will be administered at a concentration of 40 mg/kg as previously used in a herb-drug pharmacokinetic study in rats (Ho *et al.*, 2009) and the aloe gel and polysaccharides will be administered at a concentration of 5 % w/v previously shown to be effective for drug absorption enhancement in the Caco-2 cell model (Chen *et al*., 2009). Indinavir administered alone serves as a normal control group, while verapamil will serve as a positive control for efflux inhibition of indinavir since precipitated polysaccharides showed the ability to inhibit efflux across excised rat intestinal tissue (Beneke *et al*., 2013). Eight experimental groups each consisting of six animals will be used. The experimental groups are: 4 x *Aloe vera* polysaccharide weight fractions with indinavir, 1 x *Aloe vera* whole leaf gel with indinavir, 1 x precipitated *Aloe vera* gel polysaccharides with indinavir, 1 x indinavir alone, 1 x verapamil with indinavir. The total population size is therefore 48 rats.

Composition of the experimental solutions for administration to the rats:

Group 1: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* polysaccharide fraction >300 KDa in size.

Group 2: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* polysaccharide fraction >100 KDa in size

Group 3: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* polysaccharide fraction >30 KDa in size

Group 4: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* polysaccharide fraction <30 KDa in size

Group 5: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* gel material.

Group 6: receive a solution consisting of indinavir at a concentration of 40 mg/kg with 5 % w/v *A. vera* precipitated polysaccharides

Group 7: receive a solution consisting of indinavir at a concentration of 40 mg/kg

Group 8: receive a solution consisting of indinavir at a concentration of 40 mg/kg with verapamil in water (9 mg/kg) (positive control for P-gp efflux inhibition)

3. Blood sampling

Blood samples (200 µl) will be collected at specified time intervals (i.e. 0, 0.5, 1, 2, 4, 6, 8, 24 h) from the animals' tail and plasma will be recovered from each sample.

4. Analysis of blood samples and pharmacokinetic (PK) modelling

A liquid chromatography tandem mass spectrometery (LC/MS/MS) method for the analysis of indinavir and its active metabolite, M6 will be developed and used to analyse the plasma samples. Bioavailability profiles will be constructed using WinNonlin software to obtain the relevant pharmacokinetic parameters, such as peak plasma concentration (C_{max}), time of peak plasma concentration (T_{max}) and area under the curve (AUC) for purpose of comparison between the groups. The blood plasma samples will all be stored at -80°C before LC-MS analysis.

5. Euthanization of animals

After treatment and blood collection, all animals will be euthanasised *via* overdose of isoflurane.

List of publications/supportive information for the above procedures:

BENEKE C., VILJOEN A., HAMMAN J. 2013. Modulation of drug efflux by Aloe materials: An *in vitro* investigation across rat intestinal tissue. *Pharmacognosy Magazine,* 9:S44-S48.

CHEN, W., LU, Z., VILJOEN, A. & HAMMAN, J. 2008. Intestinal drug transport enhancement by *Aloe vera*. *Planta Medica*, 75:587-595.

HO, Y.F., HUANG D.K., HSUEH W.C., LAI M.Y., YU H.Y., TSAI T.H. 2009. Effects of St. John's wort extract on indinavir pharmacokinetics in rats: Differentiation of intestinal and hepatic impacts. *Life Sciences*, 85:296-302.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W-C., HAYNES, R.K., KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of PheroidTM technology. *International Journal of Pharmaceutics*, 414, 260–266

18. REPETITION

Is this study a repetition of previous work performed by yourself or others?

- If **Yes**, explain why it is being repeated. i.e. are you addressing novel questions.
- If **No,** is it part of on-going research being conducted by your group.

No

19. PUBLICATIONS

List not more than ten recent publications in which you were a participant/author/co-author involving the use of the same species of animal/s and in research fields similar to that in the current application.

J. Dewald Steyn, Lubbe Wiesner, Lissinda H. du Plessis, Anne F. Grobler, Peter J. Smith, Wing-Chi Chan, Richard K. Haynes, Awie F. Kotzé, Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of PheroidTM technology, International Journal of Pharmaceutics, 414, 2011, 260–266

Makoah N. Aminake, Aman Mahajan, Vipan Kumar, Lubbe Wiesner, Dale Taylor, Carmen de Kock, Anne Grobler, Peter J. Smith, Marc Kirschner, Axel Rethwilm, Gabriele Pradel and Kelly Chibale, Synthesis and evaluation of hybrid drugs for a potential HIV/AIDS-Malaria combination therapy, Bioorganic & Medicinal Chemistry 20 (2012) 5277 - 5289

Marli C Lombard, David D N'Da, Jaco C Breytenbach, Natasha Kolesnikova, Christophe T Van Ba, Sharon Wein, Jennifer Norman, Paolo Denti, Henri Vial and Lubbe Wiesner, Antimalarial and Anticancer Activities of Artemisinin-quinoline Hybrid-Dimers and Pharmacokinetic Properties in Mice, European Journal of Pharmaceutical Sciences, 47/5 (2012), 834 – 841

Marli C Lombard, David D N'Da, Christophe Tran Van Ba, Sharon Wein, Jennifer Norman, Lubbe Wiesner and Henri Vial, Potent in vivo anti-malarial activity and representative snapshot pharmacokinetic evaluation of artemisinin-quinoline hybrids, Malaria Journal, 2013, 12:7

Tukulula, Matshawandile; Njoroge, Mathew; Abay, Efrem; Mugumbate, Grace; Wiesner, Lubbe; Taylor, Dale; Gibhard, Liezl; Norman, Jennifer; Swart, Kenneth; Gut, Jiri; Rosenthal, Philip; Barteau, Samuel; Streckfuss, Judith; Kameni-Tcheudji, Jacques; Chibale, Kelly, Synthesis, in vitro and in vivo pharmacological evaluation of new 4-aminoquinoline-based compounds (2013) CS Medicinal Chemistry Letters Manuscript ID: ml-2013-00311r.R1

Lissinda H. du Plessis, Chrizaan Slabbert, Este van Huysteen, Lubbe Wiesner and Awie F. Kotzé, Formulation and evaluation of Pheroid® vesicles containing mefloquine for the treatment of malaria, Journal of Pharmacy and Pharmacology (2013)

20. ANIMAL WELLBEING

Note: All PI's **MUST** have discussed aspects relating to all subsections of this section with the Faculty Veterinarian or registered laboratory animal technologist and refer to the AEC guidelines prior to this submission. (Details for the above can be found at AEC web address http://www.health.uct.ac.za/research/animalethics/policies/.)

20.1. Location: After animals have been issued to the study, indicate the following: (Tick the appropriate box for each column).

(For both of the above, if not at the UCT Research Animal Facility (RAF), explain why not).

Location of housing

	Location of animals prior to start of experiment (Pre-	**Location where experiments will be**	**Location of animals after**

	treatment housing)	performed	treatment/ procedures
RAF: BSL1			
RAF: BSL2			
RAF: BSL3			
Pre-Clinical Pharmacology	X	X	X
Hatter Institute Level 4			
Bioclones lab			
HUB Basement			
HUB Behaviour room level 3			
HUB Cardiovasc Physiol lab level 3			
HUB Neuroscience lab level 4			
HUB Neuroscience lab level 5			
HUB Cell Biology lab level 6			
Other: Specify			

If Pre, intra and post procedures are not done at the RAF, then justify.

20.2. Caging, social requirements and environmental enrichment

Indicate how the animals will be housed and what provisions have been made for their physical and psychological wellbeing i.e. comforts socialisation, species-specific behavioural needs and enrichment of their cage environment. If standard environmental enrichment will not be given to the animals, justify this by relevant references.

The guidelines for all species in the SOP by the AEC must be strictly followed. Details are at http://www.health.uct.ac.za/research/animalethics/policies/

If deviating from the SOP, then please provide reason for deviation (and possible supportive information – e.g. publications)

Animals will be caged in conventional cages in groups of 3 before and during the experimental period. The necessary rat houses and bedding will be supplied

20.3. Measures to be taken to minimise the detrimental effects of experimental procedures on the animals

a) Describe the anticipated negative effects of the experiment on the animals and when these are likely to manifest: (e.g. discomfort, pain, anxiety, fear, or subtle and overt signs of suffering, lasting harm).

Animals will be handled gently by experienced technical staff.

The animals will be euthanized if adverse events occur, which cause pain or distress.

b) Describe the expected clinical course of the condition/disease model being studied (if known).

Clinical course	When the signs are expected to occur	List Expected signs
1st clinical signs	The study will only be conducted over a period of 24 hours per study group and no clinical signs are expected	N/A
Physical deterioration	N/A	N/A
Distress	Unlikely to occur because exposure of rats to the drug is limited to 24 h, but signs may be present any time after administration of the compound.	Potential neurological symptoms. The highest dose of indinavir tested in rats was 640 mg/kg/day and at this dose an increased incidence of thyroid adenomas was seen in male rats.
Death	Unlikely.	N/A

c) Name/list the measures to be taken to minimise the effects listed in a) during the pre-procedure, intra procedure and post procedure & during transport (if applicable). Include details of pain killers and anaesthetics. If no painkillers are to be administered or if only for a short duration, fully justify this by relevant references.

Note: As a manifestation of pain in animals is not always easily recognised, the internationally accepted principle is that any procedure which is liable to cause pain in humans will cause at least a similar level of pain in animals. A reasoned scientific justification for the decision to withhold the use of anaesthetics and analgesics will be required, if any potentially painful procedure is to be performed.

The animals will be monitored for changes in appearance and natural behaviour after administration of the solutions. Animals will specifically be observed for signs of depression, hyperactivity, pilo-erect fur, response to induced stimuli and dehydration (skin pliability).

The animals will be euthanized if adverse toxic events occur.

All animals will be euthanized after completion of the pharmacokinetic study.

20.4. Secondary adverse effects of the experimental procedures which may arise in the animals

Researchers should be aware that unexpected effects could occur.

State any adverse events that could arise secondary to the experimental procedures/treatments (e.g. localised infection, septicaemia, toxicity, or model-specific complications), and indicate which specific **signs** (physical and behavioural e.g. not eating) will be monitored for as an indication of the development of such adverse events.

Adverse effects	**Describe the expected Clinical sign that indicates this effect**
Adverse effects from oral gavage; gastric rupture, inadvertent tracheal administration, aspiration pneumonia, oesophageal perforation or oesophageal impaction	Arched back if intestinal discomfort is present.

20.5. Experiments that, by design, unavoidably induce progressive pain, or impact on wellbeing.

For experimental procedures/treatments that, by design, will cause loss of function, progressive illness, pain, suffering, distress or lasting harm to the animals, clearly indicate at which pre-determined endpoint the objectives of the study will be reached.

If progression to death (or close to death) is the pre-determined endpoint, provide additional and detailed justification for this requirement, explaining what important useful information this will add to the study, beyond that already gained by the other experimental measurements in the study; also indicate which other measurements of disease progression will be measured in this study, in order to prevent having to use death (or close to death) again as a pre-determined endpoint in future studies of this nature.

In the case where a toxic event may become apparent i.e. convulsion, collapse, lack of responsiveness to stimuli, the animal will be euthanized immediately. The animals will be monitored for changes in appearance and natural behaviour. Throughout the experiment, the animals will be observed for signs of normal activity, abnormal activity, inactivity, condition of fur coat, grooming and response to external stimulus.

20.6. Euthanasia (Humane endpoint)
Provide a clear indication of the circumstances, if any, under which euthanasia will be performed for welfare reasons, **prior to the pre-determined end-point** as defined in Section 16 above. Specify the specific signs that will be monitored for, to identify animals that must be euthanized prior to the study's endpoint. **Important:** Only this section of the protocol will be used by the AEC to evaluate the defined humane endpoints.
The animals will be monitored closely during the experimental procedure. The findings will be recorded on a chart (see last page of this application), and will be humanely killed if they show signs of onset of distress or pain.

21. STRESS AND DISCOMFORT SEVERITY (Please tick where appropriate)		
Category A (**none**)	Experiments on sentient animals that are expected to produce no discomfort, pain, suffering, distress or lasting harm.	
Category B **(mild)**	Experiments on sentient animal species expected to produce only mild discomfort, e.g. single blood sampling, injections, anaesthetics, or procedures on anaesthetised animals that do not regain consciousness.	
Category C **(moderate)**	Experiments that involve some discomfort to sentient animals, e.g. surgical procedures under anaesthesia, or repeated dosing/injections/sampling over time.	X
Category D **(severe)**	Experiments that involve significant stress or discomfort to sentient animals.	

22.1. WELFARE MONITORING OF ANIMALS (also complete Section 30).

How often will each of the animal subjects in this study be monitored (pre-, intra- **and** post-procedure/treatment), to ensure their continuous comfort, wellbeing and humane treatment? Please note that pain is not easily recognised in many laboratory animal species.

Indicate who will perform welfare monitoring in each stage of the study, and also record this in Section 8 (including their appropriate competence for this task).

Which specific signs will be monitored for, to measure the welfare of the animals (Sections 20.3, 20.4 and 20.6)? Adapt the Animal Monitoring Sheet for this study (Section 30) to include these signs, and also submit this with the application.

Important: Only this section of the protocol will be used by the AEC to evaluate the welfare-monitoring schedule.

	Monitoring frequency – refer to welfare monitoring SOP (see link below)	Name of person performing monitoring and statement of competency
Pre-treatment/procedure	Once daily	Mr Trevor Finch
Intra-treatment/procedure e.g. during surgical procedure	Monitoring at sample collection time points.	Mr Trevor Finch
Post-treatment/procedure	Monitoring for clinical signs of stress.	Mr Trevor Finch
Welfare Monitoring SOP: AEC001: Monitoring the Welfare of Experimental Animals **http://www.health.uct.ac.za/research/animalethics/policies/**		

22.2. DAILY ANIMAL HUSBANDRY

In cases where the routine animal husbandry staff (e.g. animal attendants) for the facility where the research is being conducted, will not be performing routine daily animal husbandry, indicate here who will do so. Routine daily animal husbandry includes cage cleaning, feeding and providing water, supplying environmental enrichment and bedding, monitoring room temperature, light intensity and cycles, ventilation and general environmental conditions (including over weekends and on holidays).

Mr Trevor Finch

23. FATE OF THE ANIMALS AND METHOD OF DISPOSAL

Will the animals be killed at the end of the experiment? If so, please explain why, if it is not obvious from the experimental design. If the animals are **NOT** going to be killed, then explain what the fate of the animals will be once the study is completed.

The animals will be euthanized using 5% isoflurane and cervical dislocation performed to ensure death. The carcases will be bagged, frozen and incinerated via the established medical waste disposable system used by the Faculty of Health Sciences (Sanumed).

24.1. METHOD OF KILLING (Please tick category, and give details in section 17)

Inhalation (specify agent and system used in section 25 below)		Exsanguination under general anaesthesia (specify who will ensure death in section 17 above)	
Fatal dose of anaesthetic agent (specify agent and dose in section 25)	X	Lethal injection whilst under anaesthesia (specify agent and dose in section 25)	
Other (specify here and in section 25 as required, and justify in section 17 above)			

24.2. METHOD OF CONFIRMING DEATH (Death must be confirmed in all animals, prior to disposal)			
Decapitation (e.g. guillotine; sharp scissors in neonates)		Dislocation of the neck (i.e. manual breaking of the upper neck)	X
Exsanguination (i.e. bleeding out, e.g. by cardiac puncture; eye enucleation)		Permanent cessation of the circulation (e.g. removal of the heart; transect aorta)	
Destruction of the brain (e.g. crush cranium with strong artery forceps in neonates)		Confirmation of the onset of *rigor mortis* (i.e. generalised muscle stiffness) with blue mucous membranes. This may require waiting for 10-60 minutes after the euthanasia procedure has been completed.	
Other			

25: DRUGS AND SUBSTANCES TO BE ADMINISTERED

List **all substances** administered to the animals, as well as **all scheduled substances used in this protocol**. Indicate the route of administration and dosage per body mass for each substance. Include all drugs including sedatives, anaesthetics, analgesics and killing agents.

Substance	Route/Site	Dose	Frequency	Name and signature of all persons administering the substance
Isoflurane	Air infusion	5%	Once at point of euthanasia	Trevor Finch
Indinavir sulphate	Oral gavage	40 mg/kg	Once at onset of study groups 1-8	Trevor Finch
Aloe vera weight fraction (>300 kDa) with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 1	Trevor Finch
Aloe vera weight fraction (>100 kDa) with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 2	Trevor Finch
Aloe vera weight fraction (>30 kDa) with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 3	Trevor Finch
Aloe vera weight fraction (<30 kDa) with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 4	Trevor Finch

Aloe vera gel material with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 5	Trevor Finch
Precipitated *Aloe vera* polysaccharide with indinavir	Oral gavage	5% w/v 40 mg/kg	Once at onset of study group 6	Trevor Finch
Verapamil with indinavir	Oral gavage	9 mg/kg 40 mg/kg	Once at onset of study group 7	Trevor Finch

Person legally authorised to prescribe and direct the administration of the schedule 2-6 drugs listed above (i.e. veterinarian; or appropriately authorised medical doctor / pharmacist / dentist):

Name	Dr Bert Mohr		
Capacity (e.g. veterinarian)		SAVC / HPCSA registration / authorisation number	D97/3983
Signature		Date	

Person taking primary legal responsibility for the safekeeping of and maintaining t he drug registers of the schedule 2-6 drugs listed above: PI or officially appointed person:

Name	Mr. Wynand Smith		
Signature		Date	

26. BIOHAZARD STATEMENT

Important: All participants must be aware of and familiar with the MDSS Safety Sheets for each of the compounds/organisms used in this study.

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Important: All participants must be aware of and familiar with the MDSS Safety Sheets for each of the compounds/organisms used in this study.

a) Is this a requirement for the project? Tick YES [x] NO [ ]

If yes, please append proof of approval (copy of certificate) from the Faculty Biosafety Committee/ GMO committee

b) List all infectious organisms (genus & species) and all of their strains that will be included in this study.

N/A

c) List all chemical/biological compounds (e.g. drugs, toxin, hormone, growth factors) that will be given to the animals used in this study.

Aloe vera leaf gel extract and isolated polysaccharides

Indinavir

Verapamil

Isoflurane

d) Will animals be subjected to radiation? Tick YES [ ] NO [x]

If yes, please append proof of approval for the authorised SOP from the Radiation Protection Committee

27. DECLARATION

1. I, ……, as **Principal Investigator** in this application hereby declare that I am familiar with the ideas, principles and responsibilities as outlined in section 29, and will ***personally*** undertake to see that these are upheld in the conduct of this study, should it be authorised.

2. I understand that I am **legally responsible** for all aspects of the study.

3. I understand that the UCT-appointed Inspection Veterinarian has the right to examine the animals and the implementation of the protocol unannounced at any time

4. In my opinion, all persons named and working under my supervision have the **appropriate training and skills required** to carry out their responsibilities as indicated.

5. I undertake to ensure I have **sufficient human resources** to perform the required welfare monitoring of all animals in this and all other animal studies of which I am principal investigator that may be running concurrently with this study.

6. I undertake not to unreasonably deviate from the authorised protocol, and to report such deviations to the HSF AEC within 24 hours (using the Minor Amendments Form FHS005).

7. In the event that any animal dies prior to the declared endpoint, I will ensure that the death is reported to the UCT Research Animal Facility and that a post-mortem will be performed in the presence of HSF veterinarian or registered laboratory animal technologist, and a formal report submitted to the HSF AEC.

8. I undertake to furnish the HSF AEC with a final **report** on the outcome of the study at its conclusion (using FHS004), including a reference to any publication arising from this study.

9. Should the study period be for more than 1 year, I undertake to submit **annual progress reports** to the HSF AEC (using FHS004). These will be submitted 12 monthly from the month of initial approval by the Committee.

10. If a **biohazard** declaration is necessary, this has been submitted to the Faculty Biosafety Committee, and proof of their approval of the safety practices stipulated in this application appended. I as PI will ensure that all participants and animal facility staff are fully informed of the hazards and that appropriate Biohazard labels are attached to each animal cage.

11. I declare that I have read the current FHS AEC Welfare Monitoring SOP AEC001: Monitoring the Welfare of Experimental Animals available at http://www.health.uct.ac.za/research/animalethics/policies/ and that I will abide by the SOP's requirements.

Signature (PI)	Print name	Date

Support from Head of Department

Note: Your attention is drawn to the responsibilities of the Head of Department as specified in the University's "Code of Ethics and Procedures for the Use of Animals in Teaching and Research" at http://www.health.uct.ac.za/research/animalethics/policies/

In my opinion, the Principal Investigator is competent to perform and oversee the experiment and I support this application.

Signature of HOD	Print name	Date

28. ADDITIONAL DECLARATION FOR RESEARCH INVOLVING NON-HUMAN PRIMATES

1. I, ……, as Principal Investigator in this application hereby declare that I have read the UCT non-human primate policy. (http://www.researchoffice.uct.ac.za/research_information/policies/animals/)

2. I understand that the UCT-appointed Inspection Veterinarian has the right to examine the animals and the implementation of the protocol unannounced at any time

3. I undertake to communicate any authorised amendments to the protocol to the UCT-appointed Inspection Veterinarian.

4. In the event that any animal dies prior to the declared endpoint, I will ensure that a post-mortem is performed by a veterinarian, and a formal report submitted to the HSF AEC.

5. I recommend that the non-human primates used in my study be made available for other studies (to be followed-up by the HSF AEC).

YES		**NO**	

Signature (PI)		**Date**	

29. PRINCIPLES

Animal Welfare

Ethical review of proposed experiments on sentient animals is necessary because such animals have moral standing. Accordingly, there are restrictions on how such animals may be treated. Although there is disagreement on whether animals may be used *at all* in experiments that cause deprivation, fear, discomfort, distress, pain or death, there is now widespread agreement that animals used in scientific experiments:

- May not be used in studies that are methodologically unsound, frivolous, trivial, or which unnecessarily duplicate earlier studies.
- May not be subjected to **unnecessary** hunger, thirst, disease, parasitism, injury, discomfort, pain, fear, suffering, and distress, lasting harm or social deprivation.

Therefore, animals held for, and used in, scientific experiments:

- Must be kept comfortable under conditions that are suitable to their species, enabling them to express normal behaviour. These include adequate space, company of conspecifics, and adequate stimulation/environmental enrichment.
- Must be monitored for ill-health and distress, and, where necessary, enjoy rapid diagnosis and either treatment or other alleviation of their condition (including euthanasia).
- Must be handled and treated in ways that either avoid or at least minimize distress, pain and suffering.

The Three 'R's

Proposals for research on sentient animal subjects must satisfy three principles:
Replacement: Sentient animals may not be used if they can be replaced by non-animal alternatives.
Reduction: The number of animals used must be reduced to the minimum that will allow the objectives of the study to be attained.
Refinement: Experimental methodology and procedures should be refined in a way that minimizes the causation of pain, suffering, distress, discomfort, lasting harm, fear and social deprivation to the experimental animal.

Responsibility

Everyone using animals, whether for experimentation, teaching, testing or provision of tissues or body fluids is responsible in their personal capacity for assuring that the animals are afforded the highest levels of welfare and protection from abuse.

Responsibilities of the Principal Investigator:

a. To read and comply with the University's *Code of Ethics and Procedures for the Use of Animals in Teaching and Research.*(http://www.health.uct.ac.za/research/animalethics/policies/)
b. To maintain detailed records of all procedures performed on the animals.
c. To ensure that all animals used are clearly identified with the allocated AEC authorisation number by means of labels on cages, pens or rooms.
d. To ensure that all the designated associate and assistant personnel are qualified and competent to perform the allocated procedures and that no other personnel will be allowed to perform any procedures without written authorisation from the Animal Ethics Committee.
e. To ensure that there will be no deviation from any of the procedures or study design as specified in the application without the prior written approval of the Animal Ethics Committee.
f. To ensure that, in the event of a situation arising during the course of the experiment whereby an animal is found to be suffering from severe pain or distress, a veterinarian will be consulted for advice. In the event that the pain or distress cannot be alleviated, the experiment on this animal will be terminated, the animal immediately euthanized and the Animal Ethics Committee informed. If the welfare endpoint has been reached, the animal should be euthanized immediately, without first calling the veterinarian. Thereafter, the researcher must immediately inform the staff of the RAF and the AEC.
g. All animals that are found dead must be promptly removed from their cage and the death reported to the UCT-RAF without delay, and a post-mortem examination performed by a laboratory animal technologist or veterinarian. In studies where animals are often found dead, closer and more frequent welfare monitoring must be performed.

(Example only - adapt the monitoring sheet for each specific study as required)

30. WELFARE MONITORING SHEET (One sheet per animal. Each animal to be monitored at least once daily*, including weekends)

FHS AEC number		**Short title**	Pharmacokinetic interactions between *Aloe vera* gel polysaccharides and indinavir
Name of monitor(s)	**Trevor Finch**	**Monitoring frequency**	**Once daily**

	Date		**Date**		**Date**		**Date**		**Date**		**Date**		**Date**	
Animal Number:														
	AM	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**
Weight Baseline weight: …… grams														
Humane endpoint: ….. grams														
Measurement frequency:														
No Discomfort or Stress														
Discomfort or Stress Score 1 (**mild**) or 2 (**moderate**) If **moderate** (2), monitor twice daily														
Physical Deterioration														
Distress (euthanize immediately)														
Time of death														
Euthanized (E) or **Found dead** (FD)														
Signature of monitor														

Add additional study-specific signs to the lists below:

- No discomfort or stress: Normally active; inquisitive; hair-coat clean; normal appetite.
- Discomfort or stress: Hair-coat ruffled (hair erect); decreased or increased activity; avoidance behaviour; isolation from the group; depressed; decreased appetite.
- Physical deterioration: Discharge from eyes; loss of general condition; weight loss ≧ 15%; dehydration (tented skin); weakness; decreased motility.
- Distress: Very weak; unresponsive to touch; unconscious; convulsing; difficulty breathing.

*** Additional action to take**

If more than **mild Discomfort or Stress**, score as 2 on the sheet and **monitor twice daily.**

If the animal shows signs of becoming **Distressed**, immediately inform the Principal Investigator. If the animal is not likely to recover and no action can be taken to alleviate its distress, the animal **must immediately be euthanized.**

If an animal is kept alive while it is in a state of distress, this will constitute a violation of the principles of the humane care of animals.

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences Animal Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
e-mail: nosi.tsama@uct.ac.za
http://www.health.uct.ac.za/fhs/research/animalethics/

06 November 2014

Dr L Wiesner
Pharmacology
K-Floor
Old Main Building

Dear Dr Wiesner

PROTOCOL TITLE: PHARMACOKINETIC INTERACTIONS BETWEEN ALOE VERA GEL POLYSACCHARIDES AND INDINAVIR IN RATS.

FHS AEC REF NO: 014/040

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **authorised** your protocol, which will terminate on **06 November 2017.**

Number of animals & species: 48 Rats

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the authorisation of this protocol imposes the following obligations on the (PI) principal investigator:

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **30 November 2015**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **30th November 2017**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as authorised, or as amended.
4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).

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5. Ensuring that you as the PI (principal investigator) immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
6. Ensuring that you as the PI (principal investigator) alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
7. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
8. If the principal investigator or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
9. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
10. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

pp

PROF PJ COMMERFORD
CHAIR, FHS AEC

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