

Enhancement of nasal drug delivery by means of beneficial pharmacokinetic interactions

W Gerber

 **orcid.org / 0000-0002-8185-3552**

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Supervisor:	Prof JH Hamman
Co-supervisor:	Prof JD Steyn
Assistant supervisor:	Prof AF Kotzé

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LIST OF ABBREVIATIONS

A

ABC	-	ATP-binding cassette(s)
AFG	-	<i>Aloe ferox</i> gel
AFG	-	<i>Aloe ferox</i> whole leaf extract
ALI	-	Air-liquid interface
AMMG	-	<i>Aloe muth-muth</i> gel
AMMWL	-	<i>Aloe muth-muth</i> whole leaf extract
ATCC	-	American Type Culture Collection
ATP	-	Adenosine tri-phosphate
AUC	-	Area under the curve
AVG	-	<i>Aloe vera</i> gel
AVWL	-	<i>Aloe vera</i> whole leaf extract

B

BCRP	-	Breast cancer resistance protein
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C

Cap	-	Capsaicin
CFTR	-	Cystic fibrosis transmembrane conductance regulator
CL	-	Clearance
CLDN	-	Claudin-1
C _{max}	-	Maximum plasma concentration
CYP(x)	-	Cytochrome P450 (with particular iso-enzyme)

D

DAG	-	Dehydroandrographolide
DAPI	-	4',6-diamidino-2-phenylindole
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DOX	-	Doxorubicin
E		
ER	-	Efflux ratio
ES	-	Estrone sulphate
F		
F	-	Bioavailability
FBS	-	Fetal bovine serum
FD4	-	FITC-dextran 4400
FDA	-	Food and Drug Administration
FFCLB	-	Delocalized lipophilic cation FF covalently bound to chlorambucil
H		
HIV	-	Human immunodeficiency virus
¹ H-NMR	-	Hydrogen-1 nuclear magnetic resonance
HPLC	-	High-pressure liquid chromatography
HREC	-	Human research ethics committee
K		
KRB	-	Krebs-Ringer bicarbonate buffer
L		
LCC	-	Liquid-covered culture

LY - Lucifer Yellow

M

MATE - Multi-drug and toxin extrusion transporter

MDR - Multidrug resistance protein

MEM - Minimum essential media

MPP⁺ - [³H]N-methyl-4-phenyl pyridinium iodide

MRP - Multidrug resistance-associated protein

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NEAA - Non-essential amino acids

NWU - North-West University

O

OAT - Organic anion transporter(s)

OCT - Organic cation transporter(s)

P

PAH - *p*-Aminohippurate

P_{app} - Apparent permeability coefficient

PBS - Phosphate-buffered saline

P-gp - P-glycoprotein(s)

Pip - Piperine

PTP - Polyethylene terephthalate

R

R123 - Rhodamine 123

RSD - Relative standard deviation

S

SEDDS - Self-emulsifying drug delivery system

SLC - Solute-carrier(s)

T

$T_{1/2}$ - Half-life time

TAP - Transporter associated with antigen processing

TEER - Trans-epithelial electrical resistance

TJ - Tight junction(s)

TKI - Tyrosine kinase inhibitor

T_{max} - Time to maximum plasma concentration

TRPV1 - Transient receptor potential vanilloid 1

U

UGT - Uridine 5'-diphospho-glucuronosyltransferase

Z

ZO-1 - Zonula occluden 1

ABSTRACT

Drug products and dosage forms are seldom taken in complete isolation. The possibility exist to ingest drugs together with foods, herbs, dietary supplements and/or other drugs, which may result in pharmacokinetic or pharmacodynamic interactions between the compounds. Such interactions have predominantly been associated with negative outcomes such as inefficacy, adverse effects and toxicity. However, the same interactions may be used in a strategic manner to potentially be beneficial to the patient. Beneficial pharmacokinetic interactions include enhancing absorption, inhibiting metabolism and/or decreasing excretion, resulting in improved bioavailability and prolongation of a drug's plasma levels, respectively. Beneficial pharmacodynamic interactions include additive or synergistic effects at drug receptor level. Enhancement of absorption during beneficial pharmacokinetic interactions can mechanistically be attributed to increased membrane permeation through tight junction opening or structural changes in membrane conformation, modulation of carrier proteins through inhibition of efflux proteins or stimulation of uptake transporters.

The nasal cavity has been identified as a potential alternative drug delivery route for large molecular weight compounds, compounds that are extensively metabolised or compounds that possesses unfavourable physico-chemical properties for oral delivery. The reason for this is because the nasal cavity's epithelial layer has a relatively high permeability, large surface area and drug delivery that occur directly into the systemic circulation.

The aim of this study was to identify and investigate the potential beneficial pharmacokinetic interactions (i.e. enhanced nasal drug delivery) of selected natural compounds (i.e. different plant extracts and materials) with different model compounds in two different *in vitro* nasal models.

A literature review article was published in the journal "Pharmaceutics", which discussed potential beneficial pharmacokinetic interactions that selected foods, herbs, dietary supplements and drugs may have on concomitantly administered drugs that can be exploited in the future to the benefit of the patient. Based on the literature review that pharmacokinetic interactions may be used to the benefit of the patient a variety of natural compounds were investigated for possible nasal drug delivery enhancement. The natural compounds that were selected for this investigation included two pepper extracts, namely capsaicin and piperine, as well as the gel and whole leaf extract of three aloe species (*Aloe vera*, *A. ferox* and a newly cultivated hybrid *A. muth-muth*). These plant extracts and materials have not been studied for nasal drug delivery enhancement before.

Various pepper fruits have traditionally been used for seasoning of food, but also as perfuming agents and as herbal medicines. Furthermore, phytochemicals isolated from different pepper species have been found to alter the pharmacokinetic profile of orally administered drugs with regards to transporter modulation and metabolism inhibition. In the current contribution, it was

shown that capsaicin inhibited p-glycoprotein (P-gp) efflux of rhodamine 123 (R123) to a larger extent as piperine in nasal epithelial cells. However, piperine modulated a higher increase in FITC-dextran 4400 (FD4) permeation across the nasal epithelium via mechanisms that still need to be confirmed in future studies. Cytotoxicity studies on the RPMI 2650 cell line indicated that capsaicin was non-cytotoxic up to a concentration of 200 μM and piperine up to a concentration of 500 μM (based on cell viability above 80%). The histological analysis of the excised nasal tissue and cultured RPMI 2650 cell layers was in coherence with each other, indicating that some damage occurred after treatment with 200 μM capsaicin, but no changes were observed for piperine up to a concentration of 50 μM . With regards to the selected aloe species, multiple medicinal applications for different extracts and materials from these plants have been known for centuries. However, beneficial pharmacokinetic interactions through opening of tight junctions and inhibition of efflux were only recently discovered. It has been hypothesized that these pharmacokinetic interactions may be used as a tool to improve bioavailability of poorly absorbed drugs, especially peptide molecules and other large molecular weight drugs, which are not sufficiently delivered via the oral route of administration. It was concluded from this study that the gel and whole leaf extract materials from all three aloe species could enhance permeation of FD4 across the nasal epithelium, albeit to different extents and rates. Similar permeation enhancement trends were found in the RPMI 2650 cell model and the excised sheep nasal tissue model. Histological analysis showed that some of the leaf materials had detrimental effects on the nasal epithelium, except those from *A. ferox* and *A. muth-muth*, which caused relatively low toxicity in both nasal epithelial models.

Furthermore, two nasal delivery models (i.e. the RPMI 2650 cell line and excised sheep nasal mucosa) were compared with regards to epithelial thickness, selected tight junction protein expression, extent of P-gp related efflux and paracellular permeation of marker compounds. It was found that the epithelial thickness of the RPMI 2650 cell line compared well with that of the excised sheep nasal tissue ($53.3 \pm 8.1 \mu\text{m}$ and $52.9 \pm 4.4 \mu\text{m}$, respectively). Although the location and distribution of tight junction proteins and F-actin differed between RPMI 2650 cell layers grown at the air-liquid interface (ALI) from those in the excised sheep nasal epithelial tissue, the extent of paracellular permeation of FD4 was similar in these two models ($P_{\text{app}} = 0.48 \times 10^{-6} \text{ cm.s}^{-1}$ and $0.46 \times 10^{-6} \text{ cm.s}^{-1}$). Similarly, a P-gp substrate (R123) presented with an identical efflux ratio value of 2.33 in both models. It was concluded that the two models can be deployed interchangeably and will yield similar results in nasal membrane permeation studies.

In conclusion, this research showed for the first time the possibility of the selected natural compounds to be used as functional excipients in nasal drug delivery, specifically to increase their epithelial permeation and subsequently bioavailability with relatively low toxicity.

Key words: Aloe; bio-enhancers; capsaicin; efflux inhibition; excised sheep nasal mucosa; nasal delivery; pharmacokinetics; piperine; RPMI 2650; tight junction modulation.

UITTREKSEL

Geneesmiddelprodukte en dosseervorme word selde heeltemaal geïsoleerd geneem. Die moontlikheid bestaan dat geneesmiddels saam met voedsel, kruie, aanvullings en/of ander geneesmiddels ingeneem word, wat moontlik kan lei tot farmakokinetiese of farmakodinamiese interaksies tussen die verbindings. Sulke interaksies is oorwegend geassosieer met negatiewe uitkomstes soos ondoeltreffendheid, nuwe-effekte en toksisiteit. Alhoewel, dieselfde interaksies kan op 'n strategiese wyse potensieel gebruik word tot voordeel van die pasiënt. Voordelige farmakokinetiese interaksies sluit bevordering van absorpsie, inhibering van metabolisme en/of verlaging van ekskresie in wat verhoogde biobeskikbaarheid en verlenging van die geneesmiddel se plasmavlakke tot gevolg het. Voordelige farmakodinamiese interaksies sluit toevoeglike of sinergistiese effekte op geneesmiddel-reseptor vlak in. Bevordering van absorpsie gedurende voordelige farmakokinetiese interaksies kan meganismes toegeskryf word aan verhoogde membraandoorlaaibaarheid deur die opening van hegte bindings, strukturele veranderinge aan membraan bouvorm, modulering van draer-proteïene deur effluks inhibisie of deur stimulerings van opname-transporters.

Die nasale holte is reeds geïdentifiseer as 'n potensieële alternatiewe geneesmiddeltoedieningsroete vir groot-molekulêre verbindings, verbindings wat breedvoerig gemetaboliseer word of verbindings wat onvanpaste fisies-chemiese eienskappe besit vir orale toediening. Die rede hiervoor kan toegeskryf word aan die nasale holte se relatiewe poreuse epiteellaag, groot oppervlaksarea en direkte aflewering tot in die sistemiese sirkulasie.

Die doel van hierdie studie was om potensieële voordelige farmakokinetiese interaksies te identifiseer en te ondersoek met die doel om nasale geneesmiddelaflewering te bevorder deur van natuurlike verbindings (d.i. verskillende plantekstrakte en materiale) gebruik te maak in twee verskillende *in vitro* nasale modelle.

'n Literatuuroorsigartikel was in die joernaal "Pharmaceutics" gepubliseer wat die potensieel voordelige farmakokinetiese interaksies van voedsel, kruie, aanvullings en geneesmiddels met ander gelyktydig toegediende geneesmiddels bespreek en hoe dit toegepas kan word in die toekoms tot die voordeel van die pasiënt. Gebaseer op die oorsig dat farmakokinetiese interaksies gebruik kan word tot die voordeel van die pasiënt, is verskeie natuurlike verbindings geselekteer om moontlike nasale afleweringsbevordering te bestudeer. Die natuurlike verbindings wat geselekteer was vir ondersoek in hierdie studie, was twee peper ekstrakte, naamlik kapsisien en piperien, asook die jel en heelblaarekstrak van drie aloë spesies (*Aloe vera*, *A. ferox* 'n nuut gekultiveerde hibried *A. muth-muth*). Hierdie plantekstrakte en materiale was nog nooit van tevore in die verhoging van nasale geneesmiddelaflewering bestudeer nie.

Verskeie vrugte van pepers is tradisioneel aangewend as reuk- en geurmiddels vir voedsel, maar ook as kruie-medisyn. Bykomend is gevind dat fitochemikalieë wat van verskillende pepers geïsoleer is, die farmakokinetiese profiel van oraal toegeniende geneesmiddels kan affekteer met betrekking tot transportermodulasie en metabolisme-inhibisie. In die huidige bydrae is vasgestel dat kapsisien p-glikoproteïen (P-gp) effluks van rodamien 123 (R123) geïnhibeer het tot 'n groter mate as piperien. Desnieteenstaande, piperien het 'n groter verhoging in FITC-dekstraan 4400 (FD4) deurlaatbaarheid getoon deur middel van meganismes wat verdere verfyning en bepaling benodig. Sitotoksiteit studies op die RPMI 2650 sellyn het getoon dat kapsisien non-toksies was tot 'n konsentrasie van 200 μM en piperien tot 'n konsentrasie van 500 μM (gebaseer op 'n sellewensvatbaarheid bo 80%). Histologiese analise van die uitgesnyde nasale weefsel asook die RPMI 2650 sellyn was in ooreenstemming met mekaar, waar geringe skade aan die selle aangebring is met behandeling met 200 μM kapsisien, maar geen veranderinge is waargeneem vir piperien by 'n konsentrasie van 50 μM nie. Met betrekking tot die geselekteerde aloë spesies is meervoudige medisinale toepassings al vir eeue bekend vir verskillende materiale en ekstrakte van hierdie plante. Die voordelige farmakokinetiese interaksies deur opening van hegte bindings en inhibisie van effluks is egter eers relatief onlangs ontdek. Daar word voorgestel dat bogenoemde farmakokinetiese interaksie as 'n instrument gebruik kan word om die biobeskikbaarheid van swak geabsorbeerde geneesmiddel te verbeter, vernameam peptiedmolekules en hoë molekulêre massa geneesmiddels wat nie gepas is vir orale toediening nie. Daar was bevind in heirdie studie dat die gel en heelblaarekstrakte van al die geselekteerde aloë spesies deurlaatbaarheid van FD4 verbeter het, elk tot verskillende mate en koerse. Soortgelyke deurlaatbaarheid tendense is ook gesien vir beide die RPMI 2650 sellyn en uitgesnyde skaap nasale weefsel. Histologiese analise het gewys dat sekere blaargedeeltes 'n negatiewe effek op die nasale epiteel gehad het met uitsondering van *A. ferox* en *A. muth-muth* wat relatiewe lae toksisiteit teweeg gebring het in beide modelle.

Verder is twee nasale afleweringsmodelle, naamlik die RPMI 2650 sellyn en uitgesnyde skaap nasale mukosa modelle met mekaar vergelyk na gelang van epiteeldikte, gekose hegtebindingsproteïenuitdrukking, mate van P-gp verwante effluks en parasellulêre deurlaatbaarheid van merker-molekules. Daar was gevind dat die epiteeldikte van die RPMI 2650 sellyn goed ooreenstem met dié van die uitgesnyde skaap nasale mukosa ($53.3 \pm 8.1 \mu\text{m}$ en $52.9 \pm 4.4 \mu\text{m}$, onderskeidelik). Alhoewel die plasing en verspreiding van hegtebindingsproteïene en F-aktien verskil het tussen die RPMI 2650 sellyn wat by die lug-water tussenvlak (LWT) gegroei is en die uitgesnyde skaap nasale epiteelweefsel, was die mate van parasellulêre transport van FD4 ook ooreenstemmend tussen die twee modelle ($P_{\text{app}} = 0.48 \times 10^{-6} \text{ cm.s}^{-1}$ en $0.46 \times 10^{-6} \text{ cm.s}^{-1}$, onderskeidelik). Soortgelyk was die effluksverhouding van 2.33 vir 'n P-gp substraat, genaamd R123 identies tussen die twee modelle. Daar is tot die gevolgtrekking gekom dat die twee modelle

uitruilbaar gebruik kan word en sal ooreenstemmende resultate lewer tydens nasale membraandeurlaatbaarheidstudies.

Ter samevatting het hierdie navorsing vir die eerste keer die moontlikheid aangedui dat die gekose natuurlike stowwe gebruik kan word as funksionele hulpstowwe in nasale dosseervorme met die oog om membraandeurlaatbaarheid te verhoog en gevolglik die biobeskikbaarheid te verhoog met gelyktydige relatiewe lae toksisiteit.

Sleutelwoorde: Aloe; bio-bevorderaars; kapsisien; effluks inhibisie; gedissekteerde skaap nasale mukosa; nasale aflewering; farmakokinetika; piperien; RPMI 2650; hegte binding modulerings.

PREFACE

As the title of this thesis suggests, the current study aims to indicate that beneficial pharmacokinetic interactions caused by pepper extracts, namely piperine and capsaicin, as well as leaf materials from *Aloe vera*, *A. ferox* and *A. muth-muth* can be used to enhance nasal delivery of drugs. The *in vitro* RPMI 2650 cell line and an *ex vivo* excised sheep nasal mucosa model were compared and used to determine mechanisms involved that lead to model compound permeation enhancement.

This thesis is compiled in article format according to the guidelines set by the North-West University, South Africa. It consists of an introductory chapter, followed by a review article that was published in the peer-reviewed journal "*Pharmaceutics*" (DOI: 10.3390/pharmaceutics10030106). Chapter 3 contains a research article that was submitted to the "*Journal of Drug Delivery Science and Technology*" for consideration for publication. This is followed by a research article that was published in the peer-reviewed journal "*Planta Medica*" (DOI: 10.1055/a-0978-5172), and another one that was submitted to the "*Journal of Herbmед Pharmacology*" for consideration for publication. Chapter 6 concludes the thesis and provides future recommendations. Author guidelines for each of the journals in which article were published or submitted for publication are provided in Appendices A, B, D and E, with the certificates of submission in Appendix C and F, respectively. Additional information such as ethical approval, certificates of analysis and experimental data can be found in Appendices G to M.

Author contributions

I, Werner Gerber, am the main researcher who was co-responsible for the planning and solely responsible for execution of experiments, along with interpretation and presentation of initial data yields. In addition, I was responsible for planning, layout and writing of all first drafts of the articles presented below as well as the subsequent compilation of the final thesis. The co-authors of the articles presented are acknowledged for their contributions and is listed below in alphabetical order.

Prof. J.H. Hamman

Main supervisor and co-author responsible for conceptual design, funding acquisition, intellectual and expert inputs, and critical evaluation of research outputs and draft manuscripts of all four articles.

Prof. A.F. Kotzé

Assistant supervisor and co-author responsible for funding acquisition and critical evaluation of research outputs in all four articles.

Prof. J.D. Steyn

Co-supervisor and co-author responsible for conceptual design, funding acquisition and critical evaluation of research outputs in all four articles.

Dr. H. Svitina

Co-author responsible for technical assistance, expert advice and critical evaluation of research outputs on histology, toxicity and statistics in the method article and both research articles.

Prof. C. Weldon

Co-author responsible for provision of equipment, expert advice and critical evaluation of research outputs on histology and toxicity in the method article and both research articles.

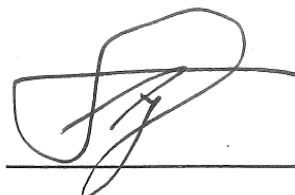
Statement by co-authors

I hereby confirm that I approve the submission of the aforementioned manuscripts that form part of the thesis "*Enhancement of nasal drug delivery by means of beneficial pharmacokinetic interactions*" submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmaceutics at the North-West University of South Africa. I herewith give consent to the main author, Werner Gerber, to include the manuscripts as part of his thesis.



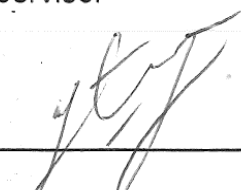
Prof. J.H. Hamman

Supervisor



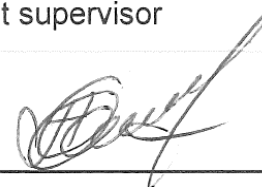
Prof. A.F. Kotzé

Assistant supervisor



Prof. J.D. Steyn

Co-supervisor



Dr. H. Svitina

Co-author



Prof. C. Weldon

Co-author

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1 Introduction

1.1 Nasal drug delivery

The nose in humans primarily functions as a tool for olfaction and respiration. Furthermore, it is also responsible for cleaning, heating and humidifying inspired air, but also for mucociliary clearance of mucus and other foreign objects and immunological activities. In healthy individuals, the nasal cavity consists of two symmetrical halves divided by the septum. Each half is further anatomically sub-divided into the vestibules, with an adjacent atrium, respiratory region and the olfactory region. Turbinates, or conchae, together with ciliated columnar cells, increase the total effective surface area to approximately 150 cm² in humans. The total volume of the nasal cavity ranges between 15 and 20 ml (Gonçalves *et al.*, 2016; Kim, 2008; Lungare *et al.*, 2016).

The nasal cavity provides a suitable platform as an alternative drug delivery route. Firstly, the nasal cavity presents with a relatively large surface area and high blood supply because of extensive vascularisation of the nasal mucosa. This, together with the relatively leaky epithelium and bypassing of the first pass (i.e. hepatic) metabolism, contribute to delivering drug molecules at a sufficient rate and concentration into the systemic circulation (Kim, 2008; Kreft *et al.*, 2015; Wengst & Reichl, 2010). Furthermore, because the olfactory and trigeminal nerves are in direct contact with the nasal cavity, it is also possible to deliver drugs directly to the brain by bypassing the blood-brain barrier. The nasal route of drug administration can therefore be used for local treatment (i.e. nasal decongestants and antihistamines), systemic delivery of drugs that are extensively enzymatically degraded or poorly absorbed in the gastrointestinal tract (i.e. protein and peptide drugs) and/or for direct nose to brain delivery (Gonçalves *et al.*, 2016; Naik & Nair, 2014).

However, the nasal drug administration route is also associated with some challenges. For example, mucociliary clearance reduces residence time in the nasal cavity to 10 – 20 min. Furthermore, the volume for liquid dosage form administration is restricted to between 25 and 200 µl. The nasal cavity also exhibits some enzymatic activity, albeit to a much smaller extent than the different areas of the gastrointestinal tract (Gonçalves *et al.*, 2016; Lungare *et al.*, 2016; Naik & Nair, 2014).

1.2 Models to evaluate nasal drug delivery

Several models, developed from different species, have been characterized to evaluate nasal drug delivery. An important role of all physiological membranes is to maintain a barrier function to prevent free passage of harmful substances. This barrier function in the epithelium mediated by tight junctions can easily be quantified by measuring the trans-epithelial electrical resistance (TEER) across the epithelium. Dolberg & Reichl (2016) stated that the TEER for the human nasal epithelium is between 75 and 100 Ω/cm^2 and this value may serve as a valuable yardstick for comparative purposes with other models, which are intended to mimic the characteristics of human nasal epithelia.

1.2.1 Primary cell lines

One form of *in vitro* studies is the cultivation of primary cells, where cells are harvested directly from a specific organ in a living subject. Examples include cells obtained from respiratory mucosa explants from patients undergoing turbinectomies for nasal obstruction, discomfort or chronic sinusitis. Although primary cell cultures have similar advantages as that of continuously proliferating cell lines, they do unfortunately also have some drawbacks. These disadvantages include the high expenses of the cell culturing technique, difficult isolations and maintenance of the tissues, ethical concerns and uncertain reproducibility because of naturally initiated senescence (Dolberg & Reichl, 2016; Gonçalves *et al.*, 2016; Kreft *et al.*, 2015).

1.2.2 Immortalised cell lines

Indefinite proliferating cell line models are often used for the following reasons: they present with genetic homogeneity, they do not entail ethical concerns that are commonly associated with animal studies, they deliver reproducible results and are easily maintained under the correct culture conditions (Gonçalves *et al.*, 2016; Kreft *et al.*, 2015). As mentioned, these cells proliferate indefinitely if cultured and maintained in the correct manner and can also be standardised with ease to decrease inter-laboratory variations (Kreft *et al.*, 2015; Reichl & Becker, 2012). The RPMI 2650 cell line is currently the only commercially available human nasal epithelial cell line. It was isolated in the early 1960's from a nasal septum squamous cell carcinoma and is increasingly being studied for use as an *in vitro* model to investigate human nasal drug delivery, pharmacokinetics and toxicity (Dolberg & Reichl, 2016; Reichl & Becker, 2012; Wengst & Reichl, 2010). The RPMI 2650 cell line was found to present with TEER values ranging from 60 – 80 Ω/cm^2 when grown at the air-liquid interface (ALI). Furthermore, during *in vitro* culturing this cell line was also found to have an enzymatic

metabolic barrier, which is similar to that found in humans. *In vitro*, these cells also form intercellular tight junctions and thus represent permeability values similar to excised human nasal mucosa (Kreft *et al.*, 2015; Reichl & Becker, 2012; Wengst & Reichl, 2010).

1.2.3 Excised animal tissues

Ex vivo models using excised animal nasal tissues have been studied extensively (Dolberg & Reichl, 2016; Kim, 2008; Schmidt *et al.*, 1998; Schmidt *et al.*, 2000). They are especially advantageous, because of the ease of access, the relatively low costs and the presence of intact cells and enzymatic cascades. Tissues from animal species such as rabbit, dog, pig, sheep and cattle have been used to study transport and metabolism in the epithelium of the nasal cavity (Kim, 2008; Schmidt *et al.*, 1998). Some drawbacks related to *ex vivo* models, however, include the ethical concerns with regards to using animal tissue (if specifically bred for research purposes, but tissues can also be obtained from animals slaughtered for meat purposes) and the different protein expression and barrier properties of animals compared to humans (i.e. inter-species differences). For example, Dolberg & Reichl (2016) showed that the TEER values for rabbit, cattle and pig nasal epithelia were 52 Ω/cm^2 , 40 to 200 Ω/cm^2 and 70 Ω/cm^2 , respectively. These differences in epithelial characteristics make it difficult to accurately compare transport across animal tissue models with that across human nasal tissue.

1.3 Pharmacokinetic drug interactions

Drugs may be taken together with food, herbs, dietary supplements and/or other drugs. The interactions between co-administered substances and drugs may lead to pharmacokinetic interactions, which in broad terms entail modulation of drug absorption, distribution, metabolism and/or excretion. These interactions are generally difficult to pre-anticipate and quantify and have until relatively recently been described as undesirable (Edwards, 2012; Mouly *et al.*, 2017). However, strategic deployment of substances for inhibiting efflux proteins (i.e. P-gp) and inhibiting metabolic enzymes can lead to increased plasma concentrations of drugs, therefore using pharmacokinetic interactions as an advantage to the patient since lower doses can provide therapeutic plasma levels (Jia *et al.*, 2016; Li *et al.*, 2014). On the other hand, pro-drugs or drugs with pharmaceutically active metabolites may show more favourable pharmacokinetic profiles when administered with an enzyme inducer (Edwards, 2012, Renukuntla *et al.*, 2013).

1.4 Aloe plant materials

Aloe leaves consist of three distinct layers, all of which can be used for therapeutic or pharmacokinetic effects. The green outer layer, known as the rind, is a thick, photosynthesising protective layer that synthesises carbohydrates, proteins and other constituents. The innermost gelatinous part of the leaf is known as the aloe pulp, consisting of parenchyma cells containing aloe gel. In between the rind and the pulp, the aloe latex is found. The latex is a bitter, yellow exudate substance, which is regulated by the United States of America's Food and Drug Administration (FDA) as a laxative (Akaberi *et al.*, 2016; Boudreau & Beland, 2006; Chen *et al.*, 2009; Sahu *et al.*, 2013).

Aloe plant material consists of approximately 75 phytochemical constituents. These include anthrones, alkaloids, flavonoids, glycoproteins, naphthalenes, while the bioactivity may be attributed to compounds like aloin, aloesin, aloenin, aloeresin, aloe-emodin and polysaccharides. One such polysaccharide active compound is aloverose (also known as acetylated polymannose or acemannan). Aloverose has a large β -(1,4)-linked galactomannan backbone with acetylated mannose residues. It is composed of mannose, glucose and galactose monomers at a ratio of 31:1:1 and has been studied extensively for its medicinal properties (Akaberi *et al.*, 2016; Sahu *et al.*, 2013; Sierra-García *et al.*, 2014).

1.4.1 *Aloe vera*

Aloe vera (L.) Burm. f. (= *Aloe barbadensis* Miller) is a perennial plant that has been used for centuries for its medicinal and healing properties. Specific effects have not been correlated with each individual phytochemical and polysaccharide and it was proposed that a synergistic action of more than one chemical ingredient is possible (Akaberi *et al.*, 2016; Sahu *et al.*, 2013; Sánchez-Machado *et al.*, 2017).

1.4.2 *Aloe ferox*

Aloe ferox Mill. (= *Aloe candelabrum* A. Berger) is an aloe species indigenous to the South and South-Western coastal regions of South Africa. The main traditional use of the plant as a laxative can be attributed to the bitter yellow latex found just beneath the outer rind. HPLC analyses of *A. ferox* leaf exudate indicated that aloesin, aloeresin A & C, aloin A & B, aloinoside A & B as well as 5-hydroxyaloin were present in the yellow latex. Three major active compounds that made out 70 to 97% of total dry latex weight were aloeresin A:aloesin:aloin in a 4:3:2 ratio (Chen *et al.*, 2012; O'Brien *et al.*, 2011).

1.4.3 *Aloe muth-muth*

Aloe muth-muth is a new species of aloe that was cultivated through forced pollination between *Aloe vera* and *Aloe ferox*. *A. muth-muth* leaves were provided by Mr. Jaap and Hannes Viljoen from Rooiklip Cycad Nursery, Swellendam, South Africa and this plant name is in the process of being registered. A herbarium voucher was deposited at the Mildred van der Merwe herbarium, North-West University, South Africa with accession number PUC0014886 (Collector: JNJ Viljoen 1).

1.4.4 Medicinal uses of aloe plant materials

Some of the most prominent and extensively described therapeutic effects of aloe include wound healing and anti-inflammatory effects. Wound healing can most probably be attributed to aloverose. It is believed that aloverose interacts with growth factor receptors of fibroblasts leading to an increase in collagen production, which can lead to faster tissue regeneration. As for the anti-inflammatory effects, a compound named C-glucosyl chromone inhibits prostaglandin production via the arachidonic acid pathway. Furthermore, different aloe species have also presented with hepatoprotective and anti-diabetic actions. Phytosterols, like lophenol and cycloartanol have shown anti-diabetic properties in type-2 diabetic rats. However, the method of action is still somewhat unclear. Two different reviews, from Sahu *et al.* (2013) and Sánchez-Machado *et al.* (2017) indicated that polysaccharides increased insulin levels in the blood and phytosterols causes upregulation of fatty acid oxidation in the liver, respectively. Polysaccharides in aloe can further be used as an antibiotic, anticancer, cosmetic and mood stabilising agent, but it is still widely accepted that further studies are required to evaluate the mechanisms involved (Akaberi *et al.*, 2016; Sierra-Garcá *et al.*, 2014; Sahu *et al.*, 2013; Sánchez-Machado *et al.*, 2017).

1.4.5 Pharmacokinetic effects of aloe plant materials

In addition to the therapeutic effects of the different parts of the aloe plant, it was observed in an *in vivo* study that *A. vera* gel and whole leaf health drinks could enhance the bioavailability of vitamin C and E in humans (Vinson *et al.*, 2005). Furthermore, the ever-evolving search for transport enhancing agents had eventually led to experiments with naturally occurring compounds including different aloe species. Transport enhancing agents are especially required where delivery of drugs is challenging due to unfavourable physico-chemical properties (e.g. poor solubility and membrane permeability) and/or a high degree of first pass metabolism (Beneke *et al.*, 2012; Chen *et al.*, 2009; Ojewole *et al.*, 2012).

Increased drug transport of poorly permeable compounds in the presence of aloe leaf materials (i.e. gel and whole leaf) has been shown via the buccal, gastro-intestinal and transdermal routes of administration. A study by Ojewole *et al.* (2012) found that the transport of didanosine, a drug that is transported paracellularly, was increased across buccal mucosa as much as 11-fold, indicating that *A. vera* gel is capable of potentially opening intercellular tight junctions. Similarly, a study conducted on gastro-intestinal models, indicated an increase in insulin transport across Caco-2 cells, while simultaneously decreasing the TEER (Chen *et al.*, 2009; Beneke *et al.*, 2012, Lebetsa *et al.*, 2012). The results from a recent study on Caco-2 cell monolayers indicated that modulation of the paracellular pathway by *A. vera* gel and whole leaf materials had occurred as a result of the opening of tight junctions between adjacent epithelial cells (Haasbroek *et al.*, 2019). In addition, a study conducted to evaluate the potential enhancement of drug transport across skin by aloe leaf materials indicated that both *A. vera* and *A. marlothii* could increase transdermal drug delivery. Interestingly, *A. ferox* whole leaf also increased transdermal transport, but not *A. ferox* gel (Fox *et al.*, 2014). However, no studies have yet been conducted regarding the potential nasal permeation enhancement effects by aloe materials, neither has *Aloe muth-muth* ever been deployed for pharmacokinetic studies.

1.5 Pepper extracts

The production of peppers (family *Solanacea*) is economically one of the most significant agriculture products in warm climate regions around the globe. Therefore, the routine use of peppers in both fresh and dry forms is enormous with approximately 38 million tonnes produced annually (Baenas *et al.*, 2019). Usually deployed as a pungent spice and flavouring to numerous cuisines from different cultures, the medicinal effects of peppers have been noticed from as early as the development of Mayan pharmacopoeia in Mesoamerica. There it was indicated for multiple ailments, which included bowel distress, earaches and for wound healing (Omolo *et al.*, 2014; Zhai *et al.*, 2013). With modern findings indicating certain pharmacokinetic modulation effects by pepper extracts, piperine and capsaicin, this study aimed to evaluate the effects thereof on nasal drug delivery for the first time.

1.5.1 Piperine

Piperine is a naturally occurring alkaloid, which is extracted from *Piper nigrum* Linn. (black pepper) and *Piper longum* Linn. (long pepper). Piperine has recently been investigated as a bioenhancer in order to improve drug/herb bioavailability. The bioenhancing effects of piperine can be attributed to one or more of the following mechanisms: inhibition of efflux

proteins, inhibition of enzymes responsible for metabolism of compounds, changes in the lipid dynamics of cell membranes and by increasing thermogenesis. Furthermore, piperine has also been indicated to have anti-inflammatory, anti-neoplastic, immunomodulatory and analgesic effects (Ajazuddin *et al.*, 2014; Bhardwaj *et al.*, 2002; Khajuria *et al.*, 2002; Li *et al.*, 2016; Shoba *et al.*, 1998).

Piperine is known to be a potent inhibitor of the P-glycoprotein (P-gp) efflux transporter in human intestinal epithelial cells (i.e. Caco-2 cell line). A study conducted on the transport of two known P-gp substrates, digoxin and cyclosporin, showed a dose dependent decrease in secretory transport of digoxin, while simultaneously increasing transport in the absorptive direction. The results suggested that efflux had decreased due to the presence of piperine, which was able to inhibit P-gp related efflux (Ajazuddin *et al.*, 2014; Bhardwaj *et al.*, 2002).

The second bioenhancing attribute of piperine is the possible inhibition of metabolising enzymes, such as CYP3A4. Studies indicated that the AUC and C_{max} of emodin, an active component in Chinese herbs, were increased with a percentage of 221% and 258%, respectively in rats when administered together with piperine (Di *et al.*, 2015). To ensure that it was CYP3A4 that was being inhibited, Bhardwaj *et al.* (2002) conducted a study that found an inhibition of up to 80% of D-617 formation in the presence of piperine. D-617 is a metabolite, which forms after verapamil has been metabolised by CYP3A4. The reduction in metabolite formation from verapamil indicated that piperine was able to substantially inhibit CYP3A4. Furthermore, similar improvements in pharmacokinetic parameters were found with the co-administration of selected antibiotics with piperine, which are known to exhibit poor bioavailability (Ajazuddin *et al.*, 2014).

1.5.2 Capsaicin

Capsaicin, the benchmark for the Scoville Heat Scale, is the primary alkaloid present in pepper fruits, which grants peppers like chillies the familiar hot taste (Baenas *et al.*, 2019; Kanda *et al.*, 2018). Although it is known that capsaicin has been part of traditional herbal remedies for centuries, it is only in recent modern medicine developments that capsaicin was proven to have modulatory effect on absorption and metabolism.

Capsaicin was proven to inhibit both P-gp efflux as well as CYP3A metabolism by causing a plasma concentration increase of a substrate (cyclosporin A) for both pathways in rats (Zhai *et al.*, 2013). The same effect was earlier found on the P-gp inhibitory effect of capsaicin when the efflux ratio of [3 H]-digoxin was reduced four-fold across Caco-2 intestinal cells (Han *et al.*, 2006).

One of the most intensively studied aspects of capsaicin on pharmacokinetics is the effect it exerts on tight junctions. Numerous studies proved that capsaicin can increase paracellular transport of macromolecular markers by means of tight junction modulation. This is achieved by a unique ability of capsaicin to cause Ca^{2+} influx into cells, leading to cofilin dephosphorylation and the resulting opening of tight junctions by decreasing occludin and re-localisation of actin chains (Kanda *et al.*, 2018; Nagumo *et al.*, 2007; Shiobara *et al.*, 2013).

2 Research problem

Macromolecular therapeutics (e.g. protein and peptide drugs) are notoriously difficult to deliver systemically via the oral route of drug administration and are therefore mainly administered by means of injections. The physico-chemical properties of these drugs make it especially susceptible to enzymatic degradation, while the large size of these molecules prevents any transcellular or paracellular transport across epithelial surfaces. The nasal route of drug administration provides an alternative, convenient and less invasive way to deliver macromolecular drugs systemically. This is possible due to its relatively leakier epithelia, increased vascularity and its avoidance of the first-pass metabolic effect. Despite these advantages, the delivery of large molecular weight drugs may still be relatively low from the nasal route of administration, which means drug absorption enhancers need to be co-administered. The main research problem therefore was to investigate the beneficial pharmacokinetic effects of different aloe plant materials on nasal epithelial drug transport that have already been described across buccal, gastro-intestinal and transdermal tissues, but to the author's knowledge, no drug absorption enhancing effects of aloe plant materials have been investigated across nasal epithelial tissues to date. The same can be said for pepper extracts; piperine and capsaicin. Piperine has been proven to inhibit intestinal P-gp-mediated efflux, while it was found that capsaicin mediates a cascade of reactions, which led to the opening of intestinal tight junction proteins. However, neither of these pepper extracts have been investigated for possible absorption enhancement effects of nasal drug delivery.

A secondary research problem to be investigated was the characterisation and comparison of different *in vitro* models for screening of nasal drug delivery. Although the RPMI 2650 human nasal epithelial cell line has been used for drug transport studies, it is much more expensive and time consuming than excised tissues from animals (especially nasal epithelial tissue obtained from animals slaughtered for meat production purposes). It was therefore important to compare these two models in terms of drug transport, histology and tight junction protein expression to determine if the excised tissue model could provide similar results to that of the *in vitro* cell model.

3 Aims and objectives

3.1 General aim

The aim of this study is to investigate the effects of selected plant materials on permeation of a macromolecular model compound and an efflux transporter substrate model compound across *in vitro* and *ex vivo* nasal models. Specifically, this aim entails determination of the effects of the gel and whole leaf materials of three different aloe species (i.e. *Aloe vera*, *Aloe ferox* and *Aloe muth-muth*) on the transport of FITC-dextran 4400 (FD4) as well as pepper extracts (i.e. piperine and capsaicin) on the transport of both FD4 and rhodamine 123 (R123) across RPMI 2650 cell multilayers and excised sheep nasal tissues. Additionally, a secondary aim of this study include the comparison of the two nasal permeation models with each other with regards to the histology, thickness of the epithelial layers, tight junction protein expression and permeation of different model compounds as well as to determine the toxicity of the selected plant materials on the RPMI 2650 cell line.

3.2 Specific objectives

- To verify a fluorescence spectrometry analytical method for analyses of a macromolecular model compound (FD4) (Nakamura *et al.*, 2002; Wengst & Reichl, 2010) and an efflux transporter model compound (R123) (Wang *et al.*, 2009; Zhao *et al.*, 2016).
- ¹H-NMR analysis of *Aloe muth-muth* gel and whole leaf material to identify as well as quantify marker molecules, which represent a chemical fingerprint of the aloe materials investigated in the study.
- Validate pepper extracts purity with regards to a reference standard using HPLC analysis.
- To grow and characterise RPMI 2650 nasal epithelial cell multilayers on ThinCert™ polyethylene terephthalate membrane inserts by means of the air-liquid interface (ALI) technique.
- To compare the RPMI 2650 cell line and excised sheep nasal tissue with regards to whole histology, tight junction protein expression and permeation of model compounds (FD4 and R123).
- To conduct transport studies on FD4 in the absorptive direction across the RPMI 2650 cell monolayers as well as excised sheep nasal mucosa to determine the

effects of the selected aloe plant materials and pepper extracts on macromolecular permeation.

- To conduct bi-directional transport experiments on R123 across the RPMI 2650 cell monolayers as well as excised sheep nasal mucosa to determine the effects of the pepper extracts on P-gp related efflux.
- To conduct cytotoxicity studies on the selected aloe materials and pepper extracts using the RPMI 2650 cell line.

4 Outsourcing and consultants

Chemical characterisation of *Aloe muth-muth* plant materials was outsourced to Spectral Services in Germany. Fluorescent immunocytochemistry by confocal laser scanning microscopy was outsourced to the Department of Biochemistry at the University of Pretoria, South Africa with the assistance of Dr Hanna Svitina (post-doctoral fellow at North-West University).

5 Ethics

Sheep nasal tissues were obtained from animals slaughtered at Potchefstroom abattoir for meat production purposes and not for research purposes. The aspects which require ethical consideration include control regarding the site of tissue collection (e.g. authorised abattoir) and the correct disposal of the nasal tissue after completion of the transport studies. A category 0 ethics application was submitted to the AnimCare ethics committee of the North-West University (NWU), which was approved (approval certificate nr: NWU-00285-17-A5). The use of RPMI 2650 cells have been successfully registered with the HREC ethics committee of the NWU.

6 Outline of thesis

This thesis is submitted in article format according to North-West University guidelines. After the introductory Chapter 1, Chapter 2 is a review article published in "*Pharmaceutics*" (DOI: 10.3390/pharmaceutics10030106) which focuses on beneficial pharmacokinetic interactions with drugs. Chapter 3 is presented as a method development article that presents a comparison between the RPMI 2650 cell line and excised sheep nasal tissue. This was submitted for publication in "*Journal of Drug Delivery Science and Technology*". Two full research articles are presented as Chapter 4 and 5, respectively. Chapter 4 discusses beneficial pharmacokinetic interactions of pepper extracts to improve nasal drug delivery and

was published in "*Planta Medica*" (DOI: 10.1055/a-0978-5172). The second research article is presented in Chapter 5 where the beneficial pharmacokinetic interactions of three different aloe species on nasal drug delivery are discussed. This work was submitted for publication in "*Journal of Herbmed Pharmacology*". The final chapter concludes the findings in the thesis and recommends future studies to further develop and define the work. All author guidelines as well as different data sets can be found in the appendices at the end of the thesis.

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CHAPTER 2

REVIEW ARTICLE: “BENEFICIAL PHARMACOKINETIC DRUG INTERACTIONS: A TOOL TO IMPROVE THE BIOAVAILABILITY OF POORLY PERMEABLE DRUGS”

Chapter 2 is presented in form of a review article entitled “*Beneficial pharmacokinetic drug interactions: A tool to improve the bioavailability of poorly permeable drugs*” which was published in the peer-reviewed journal “*Pharmaceutics*” in 2018 (volume 10, issue 106; DOI: 10.3390/pharmaceutics10030106). The author guidelines of the journal can be found in Appendix A.

As part of this study’s endeavours to deploy pharmacokinetic interactions to enhance nasal drug delivery, a review was written that gives an overview of existing knowledge of how interactions between drugs and simultaneously ingested foods, herbs, dietary supplements and other drugs can be used to our benefit.

Review

Beneficial Pharmacokinetic Drug Interactions: A Tool to Improve the Bioavailability of Poorly Permeable Drugs

Werner Gerber, Johan D. Steyn, Awie F. Kotzé and Josias H. Hamman *

Centre of Excellence for Pharmaceutical Sciences, North-West University, 2520 Potchefstroom, South Africa; wgerber99@gmail.com (W.G.); dewald.steyn@nwu.ac.za (J.D.S.); awie.kotze@nwu.ac.za (A.F.K.)

* Correspondence: sias.hamman@nwu.ac.za; Tel.: +27-18-299-4035

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Abstract: Simultaneous oral intake of herbs, supplements, foods and drugs with other drug(s) may result in pharmacokinetic or pharmacodynamic interactions with the latter. Although these interactions are often associated with unwanted effects such as adverse events or inefficacy, they can also produce effects that are potentially beneficial to the patient. Beneficial pharmacokinetic interactions include the improvement of the bioavailability of a drug (i.e., by enhancing absorption and/or inhibiting metabolism) or prolongation of a drug's plasma level within its therapeutic window (i.e., by decreasing excretion), whereas beneficial pharmacodynamic interactions include additive or synergistic effects. Mechanisms by which pharmacokinetic interactions can cause beneficial effects include enhancement of membrane permeation (e.g., structural changes in the epithelial cell membranes or opening of tight junctions), modulation of carrier proteins (e.g., inhibition of efflux transporters and stimulation of uptake transporters) and inhibition of metabolic enzymes. In the current review, selected pharmacokinetic interactions between drugs and various compounds from different sources including food, herb, dietary supplements and selected drugs are discussed. These interactions may be exploited in the future to the benefit of the patient, for example, by delivering drugs that are poorly bioavailable in therapeutic levels via alternative routes of administration than parenteral injection.

Keywords: bioavailability; efflux inhibition; pharmacokinetic interactions; tight junction modulation; enzyme inhibition

1. Introduction

Many patients are taking food, herbs, supplements and/or over-the-counter health products together with their prescribed medications [1,2]. Concomitant use of these products may lead to food-drug, herb-drug, supplement-drug and/or drug-drug interactions [3,4]. Although these interactions are usually complicated and unpredictable, it is known that they can indeed influence the pharmacodynamics and/or pharmacokinetics of some co-administered drugs [1–5]. Pharmacodynamic interactions mainly involve diverse reactions at receptor sites resulting in antagonistic or synergistic effects as well as causing changes in physiological environments, while pharmacokinetic interactions affect the absorption, distribution, metabolism and excretion of the co-administered agent (e.g., food component, herb, supplement, health product ingredient) and/or co-administered drug [1,2,4,5]. These interactions have been associated with negative effects, which include either adverse effects/toxicity or insufficient plasma concentrations of the co-administered drug(s). However, it is possible that these interactions can improve the pharmacodynamics and/or pharmacokinetics of a co-administered drug [1,3,4].

Figure 1 depicts a schematic illustration of the major areas where pharmacokinetic interactions may occur after oral drug administration.

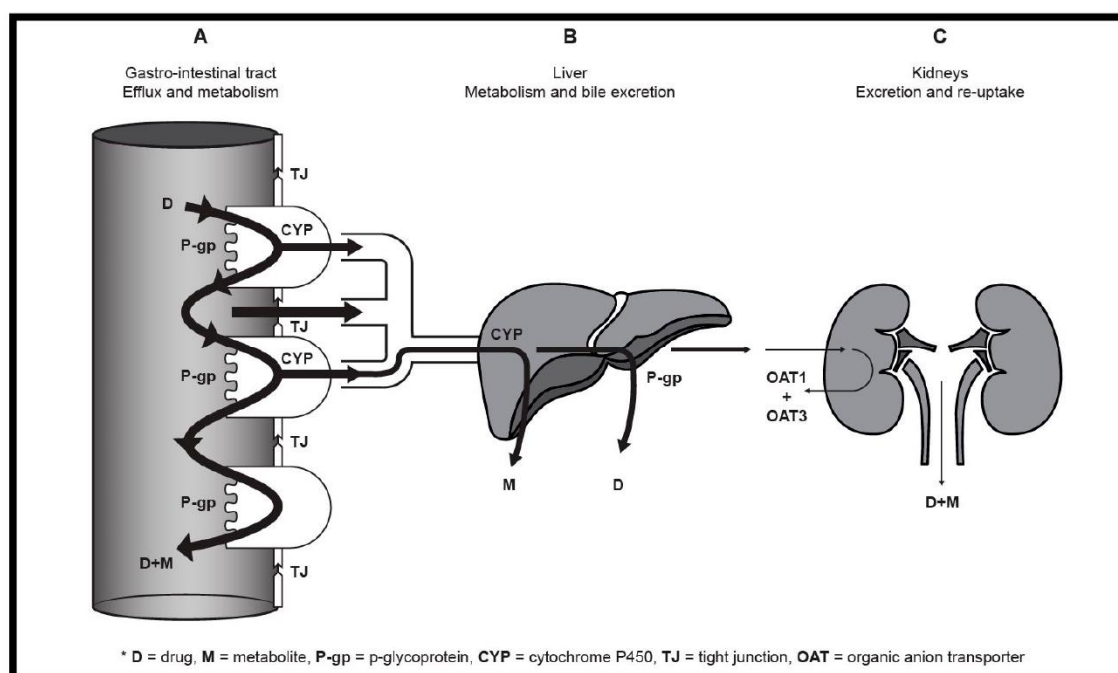


Figure 1. Schematic illustration of the major sites where pharmacokinetic drug interactions can occur after oral administration including the (A) gastro-intestinal tract, (B) liver and (C) kidneys.

The most prominent reasons why drugs present with poor oral bioavailability include low solubility, extensive pre-systemic metabolism, poor membrane permeability and active efflux transportation (Figure 1A,B). All these characteristics cause a reduction in the total amount of drug that will become available in the plasma [2,4,6]. Deliberate inhibition of pre-systemic metabolism or efflux can achieve improved bioavailability and prolonged half-lives of poorly bioavailable drugs with consequently less frequent dosing and a lower total required dose (i.e., inhibition of P-glycoprotein [P-gp], cytochrome P450 [CYP450] and organic anion transporters [OAT] 1 and 3) [1,4,6]. This will result in cost savings and improved patient compliance [1]. Figure 1 indirectly also illustrates different possible areas that may be targeted for beneficial pharmacokinetic interactions that can lead to increased drug bioavailability. The mechanisms of these interactions as well as examples of such interactions are discussed in more detail in the sections below.

2. Mechanisms of Pharmacokinetic Drug Interactions

2.1. Carrier-Mediated Transporters

Carrier-mediated transporters refer to a collection of proteins that are found on cellular membranes throughout the body which are mainly responsible for processes that mediate cellular uptake and transcellular permeation. These processes may either be via active transport or passive facilitated transport. Active transport refers to adenosine tri-phosphate (ATP)-dependent processes where a permeant is transported against its own concentration gradient, whereas passive facilitated transport occurs in the absence of ATP and with the concentration gradient of the permeant. Both types of transporters mainly allow the passage of solutes into cells and organelles, but due to the fact that each transporter can only carry one molecule at a time, the processes are saturable [7–9].

Active transport can occur via either primary-active or secondary-active transporters. Primary-active transporters hydrolyze ATP directly in order to be able to transport the permeant across a membrane. Members of the ATP-binding cassette (ABC) superfamily are examples of primary-active transporters. Secondary-active transporters are mainly ion pumps. Examples include the superfamily

of transporters encoded by the solute-carrier (SLC) gene such as organic anion transporters (OATs). The indirect dependence on ATP is why they are referred to as secondary-active transporters [7–9].

The ABC superfamily of transporters is responsible for a broad range of ATP-dependent efflux transport of compounds with diverse chemical properties. The most abundantly distributed ABC transporter is P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1), encoded by the *ABCB1* gene, which is found in the liver, kidneys, testes, on the intestinal brush border membrane and the blood-brain barrier [10–12]. Other ABC transporters include cystic fibrosis transmembrane conductance regulator (CFTR), transporter associated with antigen processing (TAP), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 1 (MRP1) of which the latter two are often found in cancerous tissues [7,10,11]. The principle function of these transporters is to efflux any xenobiotic compounds out of the cell, thus keeping the host safe of a wide range of toxic compounds. Many drug molecules also present with a high affinity to one or more of these transporters, thus leading to reduced bioavailability and/or pharmacological response (e.g., anti-cancer drugs are pumped out of tumor cells). The reason for decreased bioavailability after oral administration due to efflux is because the drug molecules are actively transported back towards the apical side of the intestinal epithelial cell membranes and into the lumen of the gastrointestinal tract [10,11]. By inhibiting ABC efflux transporters, it is therefore possible to increase the bioavailability of efflux transporter substrates [11,12].

One family of the SLC superfamily with clinical relevance is the OAT uptake transporters expressed mainly in the renal proximal tubules encoded specifically by four of the *SLCO* gene families [7,13–15]. OAT1 and OAT3 are situated on the basolateral side of the membrane and are responsible for excretion of compounds by tubular secretion of metabolites and/or other xenobiotics from the blood to the urine (Figure 1C). In contrast, OAT4 is situated on the apical side of the membrane where it is responsible for re-absorbing anionic compounds from the urine to the blood [13,15]. Since certain drugs are mainly excreted via the kidneys, the OATs play a role in the reduction of plasma levels of such drugs via excretion.

2.2. Metabolism

Pre-systemic metabolism is one of the first hurdles any orally ingested compound faces as it occurs in the gastro-intestinal tract and the liver (Figure 1A,B) before the compound reaches the systemic circulation. Inhibition of pre-systemic metabolic enzymes may result in more drug molecules reaching the site of absorption unchanged and unaltered leading to increased bioavailability [6]. Metabolism in the liver occurs in two main phases; phase 1 is largely dictated by oxidation reactions, whereas phase 2 consists of conjugation processes [16,17]. CYP450 is the most abundant metabolic enzyme system in the human body and this superfamily of enzymes are responsible for over 60% of all drugs' metabolism by means of phase 1 reactions (i.e., oxidation, reduction and hydrolysis) [1,4,16,18]. The quantity of sub-families of CYP450 differs in different tissues. Most notably, CYP3A4/5 consists of approximately 82% of CYP450 in the small intestines, but represents only 39% of CYP450 in the liver. On the other hand, where CYP2C9 is only represented by 13% in the small intestine, 25% of this enzyme is found in the liver. Furthermore, CYP2B6, CYP2D6, CYP2A6, CYP2E1 and CYP1A2 make up 1%, 2%, 6%, 9% and 18% of enzymes present in the liver, respectively [4]. Other enzyme families active during phase 1 metabolism include flavin-containing monooxygenases and epoxide hydrolases [17]. CYP450 is responsible for decreasing the bioavailability of drugs, except in the limited cases of pro-drugs where metabolites are the pharmacologically active compounds. Many compounds have been identified to be the inhibitors of CYP450, thus actively increasing the bioavailability of drugs that are substrates of CYP450 enzymes. CYP450 inhibition may therefore have the risk of increasing the toxicity of certain drugs, but have the potential to be deployed to benefit poorly bioavailable drugs that are susceptible to extensive pre-systemic metabolism [1,4,16,18].

Only after phase 1 metabolism introduced functional groups to molecules being metabolised, can the conjugating processes of phase 2 begin, by which water solubility of the conjugate is

greatly increased. Phase 2 metabolism may occur through glucuronidation mediated by uridine 5'-diphospho-glucuronosyltransferase (UGT), sulfation mediated by sulfotransferases, methylation mediated by methyltransferases, N-acetylation mediated by N-acetyltransferases and glutathione conjugation by glutathione-S-transferases [17]. One of the most studied phase 2 metabolic enzymes remains UGT, which is a superfamily of enzyme that plays a major role in the conjugation of drugs in the liver by means of glucuronidation. By mediating such glucuronidation processes, UGT also detoxifies endogenous toxins like bilirubin, bile acids and fat-soluble vitamins [19–22]. It has been previously reported that many drugs are substrates for UGT metabolism, but also that numerous phytochemicals are glucuronidated in the body by UGT [23]. The possibility for drug interactions exist by means of competitive inhibition of UGT, which may lead to increased bioavailability of drugs that are substrates for this enzyme.

3. Potential Beneficial Pharmacokinetic Interactions between Drugs and Other Substances

As mentioned before, pharmacokinetic drug interactions involving modulation of certain active transporters and metabolic pathways by co-administered substances may lead to unwanted outcomes. When efflux transporters and metabolic enzymes are induced, it may lead to achievement of sub-therapeutic drug plasma levels that are associated with pharmacological ineffectiveness. On the other hand, when efflux transporters and metabolic enzymes are inhibited, it may lead to improved bioavailability and more effective therapeutic outcomes if the drug plasma level is within the therapeutic window. However, achievement of supra-therapeutic drug plasma levels are associated with adverse effects and possibly also toxicity [24,25]. Although the outcomes of arbitrary drug interactions are often unpredictable, unwanted and complicated, which in some cases may be serious and have life-threatening consequences [4], it may be used in a controlled manner to the therapeutic benefit of patients. It is therefore not surprising to note that herb-herb and herb-drug combinations are prescribed for this reason [1,3,18].

The focus in this paper is on beneficial pharmacokinetic interactions and specifically on more recent discoveries in this field, whilst well-known interactions such as those caused by grapefruit, green tea, probenecid and St. John's wort are also mentioned. The discussions in this paper are based on recently reported (most studies within 5 years) scientific studies that were obtained from an electronic literature search using ScienceDirect, GoogleScholar and Scopus as search engine. The studies were selected to represent additive agents from different broad categories including food, herbs, dietary supplements and drugs that were investigated for pharmacokinetic interactions with a drug/marker molecule in different models including ex vivo, in vitro and/or in vivo. Only studies that reported on potential beneficial pharmacokinetic interactions (i.e., improved drug bioavailability or increased membrane permeation) were selected and are summarised after discussion in Table 2 (Section 4).

3.1. Food-Drug Interactions

The term “food” can be defined as materials that consist of different chemical components such as proteins, carbohydrates and fat that can be used in an organism as nutrients to sustain growth, repair and maintain vital processes as well as furnish energy [26]. It is also known that the peroral route of drug delivery is by far the most used and preferred route of drug administration due to its safety, ease of administration and fixed dosage administration. Orally ingested drugs may come into contact with ingested food. Food can affect drug absorption via different mechanisms such as changing gastric emptying time, the pH and residence time of the drug in the gastrointestinal tract, the hepatic blood flow and by modulating efflux transporters as well as pre-systemic metabolism [4]. One of the classical pharmacokinetic interactions of food components with drugs is that of grapefruit juice with nifedipine [27]. Certain recently discovered pharmacokinetic interactions of selected food components that may enhance drug bioavailability to the benefit of the patient are discussed in the sections below.

3.1.1. Black Pepper

Piperine (1-piperoylpiperidine) is a major bioactive alkaloid constituent of long pepper (*Piper longum* L.) and black pepper (*Piper nigrum* L.) [28–30]. Piperine is believed to have anti-inflammatory, anti-hypertensive and hepatic protective properties [29,30]. It is often combined with other herbal medicines such as curcumin and was found to enhance the absorption of these co-administered herbs and drugs [31].

Mechanistic studies indicated that piperine is able to inhibit the active efflux protein transporter, P-gp, as well as the CYP3A4 enzyme [28,29]. These studies were conducted using in vitro models, such as the human colon carcinoma cell line (Caco-2) [28], and it was only relatively recently evaluated by using in vivo animal models [29]. Peroral administration in a male Sprague-Dawley rat model was mainly used due to the obvious ingestion route of pepper but also due to the high concentrations of P-gp transporters and CYP3A4 enzymes present in the enterocytes, thus presenting a large area for possible interactions [29]. Piperine was also investigated as a means to increase the bioavailability of another bioactive herbal compound, emodin. Piperine increased the area under the plasma concentration time curve (AUC), which indicates an increase in the total amount of drug absorbed, and maximum plasma concentration (C_{max}) of emodin by 221% and 258%, respectively. Simultaneously, the AUC and C_{max} of emodin glucuronide, a metabolite of emodin, were reduced by 267% and 369%, respectively. These results indicate that piperine is able to inhibit glucuronidation of emodin significantly [29].

Another example is that of docetaxel, which is an anti-cancer drug, but has limited clinical application due to its low bioavailability. Li et al. [30] conducted a study to determine the pharmacokinetic profile of docetaxel in the presence of piperine. Combined use increased the initial concentration (C_0) of docetaxel three times and the AUC with 61.7%. Simultaneously, piperine's $t_{1/2}$ increased significantly while a moderate increase was observed in its C_{max} and AUC_{0-t} [30].

It is helpful to know that concentrations of piperine needed for significant pharmacokinetic interactions are non-toxic to intestinal epithelial tissues as indicated by TEER and [3H]inulin transport studies [28,29]. With this knowledge, Shao et al. [31] developed a self-emulsifying drug delivery system (SEDDS) to enhance bioavailability and solubility of piperine in order to develop a potentially useful system to clinically use piperine. In the formulation, the SEDDS increased the relative bioavailability of piperine by 625.7% compared to self-prepared capsules. It was also found that the formulation's C_{max} and AUC_{0-60h} values were approximately five times greater than that of the capsules.

3.1.2. Orange Juice

Some of the most abundant bioflavonoids in orange juice include hesperidin, nobiletin and tangeretin [32]. Furthermore, it has been reported to exhibit biological effects like anti-inflammatory, cholesterol lowering and neuroprotective activities [33]. Recently, studies showed that tangeretin and nobiletin increased intracellular concentrations of anti-cancer drugs in vitro due to inhibition of P-gp and BCRP [32,33]. Fleisher et al. [32] found that tangeretin and nobiletin significantly inhibited P-gp-mediated by increasing intracellular concentration of a small molecule tyrosine kinase inhibitor (TKI), dasatinib, by 314% and 476%, respectively when compared to the control of dasatinib alone [32]. Furthermore, the same study also reported a significant inhibition of BCRP-mediated transport. Intracellular concentration of dasatinib was increased by 845% and 949% for tangeretin and nobiletin, respectively when compared to the control of dasatinib alone [32]. The latter results are especially important because they are similar to results found with a potent known BCRP inhibitor, elacridar (5 μM), which increased intracellular concentrations of dasatinib by 782% [32].

Most TKIs are known to be substrates for both P-gp and BCRP and are also mainly delivered orally in a once daily dose. Therefore, the results show that potential beneficial interactions may arise by taking the TKIs with a specific diet. Orange juice contains an average concentration of 2.633 μM tangeretin and 6.345 μM nobiletin, therefore intestinal concentrations higher than their individual IC_{50} values for BCRP inhibition may be easily obtained [32].

Along with its cytotoxic effects, the effect that tangeretin had on P-gp was again confirmed in 2016 by efflux inhibition of rhodamine 123 (R123) and doxorubicin (DOX) across Caco-2 cells [33]. The study found that the efflux ratio values of both R123 and DOX were decreased to ≈ 1 in the presence of tangeretin, which is much lower than the control of ≈ 7 for both markers. Similar efflux inhibition of a known P-gp inhibitor, quinidine (20 μM), was achieved by tangeretin at a concentration of 2.51 μM . Accumulation of doxorubicin and flutax-2 in Caco-2 cells were also clearly portrayed in the presence of tangeretin by means of fluorescence microscopy [33]. This study also found that tangeretin is able to stimulate P-gp ATPase, while simultaneously inhibiting verapamil-stimulated ATPase activity. The authors postulated that tangeretin might competitively inhibit substrate binding sites on P-gp, which would explain the increase in ATPase activity with simultaneous decrease in transporting activity.

3.1.3. Resveratrol

Resveratrol is a polyphenolic phytoalexin found abundantly in berries, grape skins and red wine. It has proven anti-oxidant and anti-inflammatory effects, thus leading to a wide range of health benefits, which include cardiovascular protection [5,34,35]. Pharmacokinetic studies in human volunteers indicated that resveratrol is able to inhibit CYP2C9 and CYP2E1 in vivo [34,35]. For CYP2C9 inhibition studies, diclofenac was used as a substrate and taken orally after 10 days of daily oral resveratrol treatment. Pharmacokinetic parameters of C_{max} , AUC and half-life ($t_{1/2}$) was determined after a 10-day treatment and these parameters were each found to be 68.2%, 98.8% and 57.1% higher than in the control group whom received diclofenac alone [35]. These results showed significant inhibition of CYP2C9 metabolism, which indicated that lower doses of diclofenac were needed for pharmacological effects and may therefore reduce the common known side-effects of diclofenac. Furthermore, chlorzoxazone is often used as a probe for specific CYP2E1 metabolism studies. Resveratrol was found to not only increase certain pharmacokinetic parameters of chlorzoxazone after 10 days of oral pretreatment, but also decreased the plasma concentration of a known metabolite, 6-hydroxychlorzoxazone. The C_{max} , AUC and $t_{1/2}$ of chlorzoxazone were increased by 54.2%, 72.2% and 34.8%, respectively [34]. In both scenarios, the clearance parameters, K_{Cl} and CL/F , were also decreased [34,35].

Furthermore, results exist that indicate that resveratrol can also inhibit various transporter proteins [5]. A comprehensive study by Jia et al. [5] showed that resveratrol inhibited P-gp, MRP-2 and organic anion transporter (OAT) 1 and 3. Methotrexate, a known substrate of P-gp, presented with increased intestinal absorption in vivo and in vitro in the presence of resveratrol. The AUC and C_{max} parameters were increased by factor 1.1 and 1.5, respectively, while simultaneously shortening the T_{max} . Here it is accepted that intestinal P-gp- and MRP2-mediated transport was inhibited by resveratrol seeing that permeability studies using the rat everted sac method yielded similar results. However, after intravenous administration of methotrexate along with resveratrol in rats, the $t_{1/2}$ of methotrexate increased by 66% while clearance was decreased by 45%. The study further found that the urinary secretion of methotrexate statistically significantly decreased. Taking the aforementioned results into account, it is a good indication of the ability of resveratrol to inhibit OAT1 and OAT3 [5].

3.2. Herb-Drug Interactions

In the context of the current discussion, the term “herb” refers to a plant or plant part that is valued specifically for its medicinal, savory or aromatic qualities [36]. Herbal medicines are often used by patients in conjunction with their conventional medicines. This may happen without the prescribing physician knowing about it. Concurrent use of different remedies poses a potential risk for patients, especially with herb-drug interactions being capable of modulating pharmacodynamic and pharmacokinetic profiles of drugs [2,37,38]. Well known herb-drug interactions have been thoroughly described in the literature and includes interactions such as St John’s wort’s induction of P-gp expression and metabolism [2,30], UGT inhibition by milk thistle, saw palmetto and cranberry [23] and

Ginkgo biloba's pharmacodynamic brain protective properties [15]. Recently explored possible beneficial pharmacokinetic interactions regarding commonly used herbs are described in the following sections.

3.2.1. Aloe Leaf Materials

Aloe is a genus of perennial succulent plants consisting of more than 400 species. As a medicinally versatile plant, aloe latex and gel have for centuries been used for its laxative and wound healing properties. Other medicinal activities include anti-cancer, anti-oxidant, anti-inflammatory, anti-diabetic, anti-ulcer and immunomodulatory effects. Materials from different aloe species are being investigated for new and innovative medicinal properties and applications [39,40].

The in vitro drug absorption enhancing effects of *A. vera* gel and whole leaf materials were first shown across intestinal epithelial cell (Caco-2) monolayers [41], which was attributed to opening of tight junctions between adjacent epithelial cells as indicated by a reduction in trans-epithelial electrical resistance. Other publications also reported the ability of aloe materials to increase intestinal membrane permeation of various drugs in vitro [42,43], which was also achieved when the aloe materials were incorporated into solid oral dosage forms (i.e., dual phase spherical bead system) [44]. It was found that *Aloe vera* liquid preparations could increase the bioavailability of vitamins in humans when co-administered [45]. Ojewole et al. [46] found that *A. vera* gel could also increase the buccal absorption of the anti-retroviral drug, didanosine, by as much as 11-fold [46]. They postulated that the mechanism responsible for the absorption enhancing effects may be because of increased residence time due to muco-adhesive abilities of aloe gel or that the polysaccharides in aloe are able to weaken the epithelial barrier by dismantling intercellular junctions.

Three different species of aloe, namely *A. vera*, *A. ferox* and *A. marlothii* were deployed to study potential modulation of cimetidine efflux [37]. This in vitro study found that precipitated polysaccharides from *A. vera* gel inhibited the efflux of cimetidine (i.e., reduced the efflux ratio from 1.452 to 0.860), while the whole leaf and gel materials had no effect. Interestingly, *A. ferox* and *A. marlothii* showed the opposite effect with the gel and whole leaf materials inhibiting efflux, while the precipitated polysaccharides had seemingly induced efflux. The reason presented for this phenomenon was the different chemical composition of the three species [37].

A. vera and *A. marlothii* gel as well as *A. ferox* whole leaf materials were shown to improve ketoprofen diffusion across dermatomed skin [47]. Furthermore, different fractions of *A. vera* gel polysaccharides were found to inhibit indinavir metabolism in an in vitro LS180 cell model, while changing the pharmacokinetic parameters in vivo in male Sprague-Dawley rats after oral co-administration [38].

3.2.2. *Salvia Miltiorrhiza*

Salvia miltiorrhiza is a traditional medicinal herb used for hundreds of years in China for an assortment of cardiovascular related diseases. Locally referred to as Danshen, the plant is commonly known as red sage in the Western world [48–50]. Due to its clinical effectiveness, there are currently more than 900 commercial preparations available containing Danshen in China [50]. Interactions of *S. miltiorrhiza* with other cardio-active drugs have been studied, which have shown that this herb may have a profound effect on drug displacement from serum albumin [48–50].

Chemical isolation and characterisation of *S. miltiorrhiza* have shown more than 60 phytochemical compounds to be present. Included in these compounds are water-soluble phenolic acids, salvianolic acid B and rosmarinic acid. These two phenolic acids are the most abundant and are believed to be the most bioactive in *S. miltiorrhiza* [49,50].

Serum albumin is the most abundant protein in the circulatory system. It acts as a carrier, which directly determines the pharmacokinetic and pharmacodynamic properties of the drug. Two binding sites, subdomain IIA (a.k.a. Sudlow I) and subdomain IIIA (a.k.a. Sudlow II) are the two main areas of ligand binding [48,49]. Two studies conducted independently of one another have proven that salvianolic acid B can be bound to human serum albumin by interaction in the hydrophobic subdomain

IIA cavity [48,49]. More recently, it was found that salvianolic acid B had such a high affinity for human serum albumin that it was able to displace site-specific warfarin from subdomain IIA, releasing more warfarin molecules into the plasma, which may lead to increased bleeding [50].

This is of potential importance as many drugs bind to serum albumin once absorbed into the blood stream. The efficacy of drugs also largely depends on the extent of albumin complexes as only the unbound fraction of the drug is available for pharmacological action or exposed for metabolism [48,49]. In the future, it may be possible to increase the pharmacological activity of highly bound drugs at lower doses by concomitant administration of *S. miltiorrhiza*.

3.2.3. *Andrographis Paniculata*

Commonly referred to as green chiretta, *Andrographis paniculata* is an indigenous plant to South East Asia and has been deployed for centuries in traditional remedies to treat airway ailments, inflammation and more recently, acquired immunodeficiency syndrome (AIDS) [19,51]. In recent years, several different constituents of the plant have been identified and distinguished and consequently tested for any possible herb-drug interactions. A recent publication indicated the ability of an aqueous extract of *A. paniculata* to inhibit human liver glucuronidation activity [19]. This study found that metabolism of an UGT substrate, 4-methylumbelliferone, was significantly inhibited. Another extracted constituent of *A. paniculata*, named dehydroandrographolide (DAG), was found to inhibit P-gp mediated efflux of digoxin by as much as 70% in MDR1-MDCKII cells and 94% in Caco-2 cells [2]. The authors of the study postulated the mechanism for P-gp inhibition as competitive binding of DAG to transport sites, thus inhibiting the secretory transport of digoxin, while subsequently increasing the intracellular concentration of the drug. In the same study they also found that DAG significantly inhibited CYP3A4 [2].

Furthermore, arabinogalactan (an aqueous extract phytochemical component of *A. paniculata*) may also increase the plasma levels of co-administered drugs by displacing the drug molecules from serum albumin. It was found by fluorescent quenching that arabinogalactan bind to bovine serum albumin in a concentration dependent manner [51].

3.2.4. Emodin

Emodin is an active constituent of multiple medicinal plants such as *Cassia angustifolia* (senna), *Aloe barbadensis* (aloe) and *Reum officinale* (rhubarb). It has been deployed as traditional medicine to assist with obesity-related problems, as an anti-diabetic agent and as a laxative. Recently it has been discovered that emodin may inhibit migration of cancer cells, but may not reach clinically significant plasma concentrations due to its low bioavailability as a result of extensive glucuronidation [29]. Emodin proved to be a strong inhibitor of P-gp mediated efflux by reducing in vitro digoxin efflux by 94% in MDR1-MDCKII cells and 82% in Caco-2 cells. This correlated well with the 51% increase in the AUC_{total} parameter of digoxin in the presence of emodin [2]. Due to emodin's own possible anti-cancer effects it may hold great promise to administer emodin in conjunction with other anti-cancer therapeutics that are also substrates for efflux transporters.

3.3. Dietary Supplement-Drug Interactions

The term “dietary supplement” is specifically defined in this context as an orally ingested product that is intended to supplement a person's diet and is not necessarily considered food (e.g., vitamins, minerals, herbs, amino acids) [52]. Understanding dietary supplement-drug interactions is especially important as a relatively large number (about 25%) of individuals that make use of dietary supplements on a regular basis, take it with one or more prescription drugs [20]. Furthermore, it is already known that dietary supplements may inhibit or induce enzymes resulting in altered bioavailability of drugs and subsequent adverse effects and/or loss of efficacy [18,20].

3.3.1. Licorice (*Glycyrrhiza* Species)

Glycyrrhiza, a genus of plants commonly known as licorice, has been used in Asian countries since ancient times as a traditional medicine designated to treat allergies, virus infections and inflammation. Recently, glycyrrhizin was isolated from *Glycyrrhiza* plant species and has been deployed as sweetener and flavouring agent in prepared foods [15,16,18]. *Glycyrrhiza glabra*, *G. inflata* and *G. uralensis* are the three species of the plant that is mostly used in dietary supplements. Although the three species are often used interchangeably, they present with slightly different phytochemical constituents [18]. Studies identified six flavonoids extracted from *Glycyrrhiza* plants that presented with the capability of inhibiting CYP450 mediated metabolism. The extent of CYP450 inhibition by different phytochemicals from *Glycyrrhiza* species is shown in Table 1.

Table 1. Effects of selected chemical components of *Glycyrrhiza* species on CYP450 enzyme inhibition [16,18].

Extract	Cytochrome P450 iso-enzymes ¹								
	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4
18 β -Glycyrrhetinic acid	+	+	+	++	+	+	+	+	++
Glabridin	+	+	+	++	+	+	+	+	++
Glycoumarin	++	+	++	+++	+++	+++	++	+	+
Isoliquiritigen	+	+	+	+++	+++	+	+	++	++
Licochalcone A	++	+	+	+++	+++	+++	++	++	++
Licoricidin	+	+	+++	+++	+++	+++	++	++	++

¹ + = weak inhibition, ++ = moderate inhibition and +++ = strong inhibition.

Licochalcone A inhibited CYP450 in a time-dependent manner [16,18] and therefore the possibility exists that certain components of dietary supplements such as licorice may benefit the pharmacokinetic properties of extensively metabolized drugs. The variation in enzyme inhibition by different phytochemical components requires determination of the exact chemical compound in a given dietary supplement that causes the drug bioavailability enhancement effect.

A study conducted on the transporter inhibition potential of 18 β -glycyrrhetinic acid, showed significant reduction (56%) of *p*-aminohippurate (PAH) transport via the hOAT1 transporter. This indicates the ability to decrease renal secretion and stimulate re-absorption into the blood [15]. The same study also showed a significant stimulation of estrone sulfate (ES) uptake via hOAT4 transporters. As the hOAT4 transporter is mainly responsible for re-uptake into the blood from the urine, stimulation thereof may be used as a beneficial interaction to maintain therapeutic levels of renally excreted drugs.

3.3.2. Carotenoids

Carotenoids are yellow pigments found in many plant and animal species. A sub-group of these pigments, named xanthophylls, are widely marketed as dietary supplements to be used for cancer prevention as well as in cardiovascular and chronic inflammatory diseases due to their anti-oxidant effects [20,53].

More specifically, a single xanthophyll named β -carotene recently showed potential to play a role in dietary supplement-drug interactions by being able to inhibit efflux transporter proteins as well as phase 2 metabolic enzymes [20,53]. With regards to transporter proteins, it was found that β -carotene significantly inhibits P-gp function in a concentration-dependent manner. The mechanism of action was attributed to the capability of β -carotene to stimulate P-gp ATPase as well as binding to P-gp while slightly changing the protein conformation. Furthermore, it may also be important to note that β -carotene weakly inhibits BCRP, but to a much lesser extent than P-gp [53]. With regards to metabolism inhibition, it was shown that β -carotene has the ability to mildly inhibit UGT1A1, UGT1A3 and UGT 1A4, with the highest affinity being for UGT1A1 [20].

Both these interactions may potentially be used to improve the pharmacokinetics of drugs in patients, specifically with the concurrent use of anti-neoplastic drugs. When taking into account that

β -carotene possesses its own anti-cancer effects, the P-gp and metabolism inhibition may serve as beneficial interactions to improve drug levels in the cancer cells while providing potential synergistic effects.

3.3.3. Green Coffee Beans

Two components from green coffee bean extract namely caffeic acid and dicaffeoylquinic acid, were tested for interactions with transporter proteins. The results indicated potential modulation of pharmacokinetic parameters, which resulted in higher bioavailability of certain drugs [14,15].

Caffeic acid inhibited both hOAT1 and hOAT2 transporters [14]. PAH and ES were used as substrates for hOAT1 and hOAT2 and both their uptake was decreased in a dose dependent way. Complete inhibition of uptake transporters was observed at a caffeic acid concentration of 1 mM, which occurred by means of a competitive mechanism [14].

Dicaffeoylquinic acid was also tested for possible hOAT transporter modulation. Two enantiomers, 1,3- and 1,5-dicaffeoylquinic acid, both showed the ability to inhibit hOAT1 and 3, with the exception that 1,5-dicaffeoylquinic acid inhibited these transporter proteins to a much lower extent. Inhibition of PAH transport was found to be 64% and 22% for the 1,3- and 1,5-enantiomer, respectively, while the transport of ES was inhibited by 41% and 35% by the enantiomers, respectively [15]. These results indicated that dicaffeoylquinic acid can influence the re-absorption of drugs that are substrates of active transporters in the kidney resulting in higher blood plasma levels for longer periods of time. The effect of 1,3- and 1,5-dicaffeoylquinic acid on the hOAT4 uptake transporter was also tested and they significantly stimulated active transport of ES. This may further contribute to bioavailability enhancement due to stimulation of hOAT4-mediated drug transport [15].

3.4. Drug-Drug Interactions

Even though only two drugs that are taken simultaneously by a patient can already cause drug-drug interactions, poly-therapy increases the risk of clinically significant interactions to occur between drugs. One of the most common causes of adverse drug reactions is drug-drug type interactions, but these interactions may also be responsible for an increase in clinical efficacy [54].

3.4.1. Ivermectin

Ivermectin is an anthelmintic drug of the avermectin class. Drug interactions of ivermectin in the presence of efflux transporter substrates were studied and indicated the ability of ivermectin to inhibit P-gp- and BCRP-related efflux. The efflux ratio of R123, a P-gp substrate, was reduced by approximately 3-fold in an excised rat intestinal tissue model by ivermectin. The same study also found that the secretory transport of danofloxacin, a BCRP substrate, was decreased almost 4-fold by ivermectin [10]. The results of this study therefore indicated that ivermectin has the ability to inhibit the efflux transporters, P-gp and BCRP. Similar efflux transporter inhibitory results were observed in human and mouse neuroblastoma cells [55].

Ivermectin inhibited the secretory transport of lopinavir and saquinavir by approximately 2.5 fold, whilst increasing the absorptive transport of lopinavir almost 3-fold. Due to the overlapping of helminthic infection and HIV in certain areas, especially sub-Saharan Africa, these drug-drug interactions between the two treatment regimens can possibly be used to the benefit of patients [56].

3.4.2. Chlorambucil

Chlorambucil is an anti-neoplastic drug belonging to the DNA-alkylating class [57,58]. Drug-drug interactions with other anti-neoplastic drugs may lead to beneficial pharmacokinetic profiles, resulting in more efficient cancer treatment.

Initial studies with regards to potential pharmacokinetic interactions between chlorambucil and other drugs, demonstrated its capability to inhibit P-gp related efflux of a known substrate, 5-carboxyfluorescein, in leukemia and lymphoma cells. A concentration dependent trend was identified where 50, 100 and 200 $\mu\text{mol/L}$ of chlorambucil inhibited 5-carboxyfluorescein efflux to 88.9%, 40.9% and 5.2% of the control,

respectively [58]. These results were put into practice very recently when Liu et al. [11] synthesized a tumor-targeting anti-cancer agent by covalently binding delocalized lipophilic cation FF to chlorambucil (FFCLB). They found that FFCLB increased intracellular adriamycin concentrations dramatically, to an even larger extent than the known potent P-gp inhibitor, verapamil [11].

Furthermore, chlorambucil was demonstrated to inhibit the active transporter, OATP1B3, which is exclusively expressed in the liver and indirectly plays a role in the controlled release of gall bladder and pancreas secretions necessary for digestion. The inhibitory effect of chlorambucil may decrease hepatic metabolism of lipid molecules, but the effect on normal physiological processes by this inhibition is still unclear [13,57].

3.4.3. Telaprevir

Telaprevir is a relatively new direct acting anti-viral, used for the treatment of hepatitis C. The literature widely reports on the capability of telaprevir to inhibit P-gp transport by competitive inhibition [13,58–60].

A comprehensive study by Kunze et al. [13] identified telaprevir as an inhibitor of organic cation transporter 2 (OCT2) and multi-drug and toxin extrusion transporter 1 (MATE1). OCT2 is a renal transporter that mediates tubular secretion via basolateral uptake, while MATE mediates tubular secretion, but via efflux from the apical side [13]. The uptake of [³H]N-methyl-4-phenyl pyridinium iodide (MPP⁺) via OCT2 was reduced by half in the presence of telaprevir, while the efflux transport of metformin via MATE1 was reduced 8-fold [13].

4. Discussion

In this paper, a collection of pharmacokinetic interactions are discussed within the broad categories of food-drug, herb-drug, dietary supplement-drug and drug-drug interactions as summarised in Table 2.

Table 2. A summary of selected scientific pharmacokinetic interaction studies used in the discussions of this paper.

Type of Study	Additive Agent	Drug/Marker	Mechanism	Effect	Ref.
Ex vivo	Ivermectin	R123	P-gp	Inhibited	[10]
		Danofloxacin	BCRP		
	Selected aloe components	Cimetidine	P-gp	Inhibited	[37]
		Insulin	Tight junctions	Opened	[44]
		Didanosine	Buccal Absorption	Increased	[46]
In vitro		Ketoprofen	Transdermal delivery	Increased	[47]
	DAG	Digoxin	P-gp	Inhibited	[2]
	Emodin				
	Resveratrol	Methotrexate	P-gp, MRP2, OAT1 and OAT3	Inhibited	[5]
	Chlorambucil	Adriamycin	P-gp	Inhibited	[11]
	Telaprevir	MPP ⁺	OCT2	Inhibited	[13]
		Metformin	MATE1		
	Caffeic acid	PAH and ES	OAT1 and OAT3	Inhibited	[14]
	Dicaffeoylquinic acid	PAH and ES	OAT1 and OAT3	Inhibited	[15]
	18β-Glycyrrhethinic acid	PAH	OAT1	Inhibited	
		ES	OAT4	Induced	
	Selected <i>Glycyrrhiza</i> species	Selected isoform-selective markers	CYP450	Inhibited	[16,18]
	Aqueous <i>Andropogonis paniculata</i> extract	4-methylumbelliferone	UGTs	Inhibited	[19]
	β-Carotene	Selected isoform-selective markers	UGTs	Inhibited	[20]
		R123	P-gp	Inhibited	[53]
		Mitoxantrone	BCRP		

Table 2. Cont.

Type of Study	Additive Agent	Drug/Marker	Mechanism	Effect	Ref.
In vitro	Piperine	Digoxin and Cyclosporin A	P-gp	Inhibited	[28]
		Verapamil	CYP3A4		
	Tangeretin	Dasatinib	BCRP and P-gp	Inhibited	[32]
		Doxorubicin	P-gp	Inhibited	[33]
	Nobiletin	Dasatinib	BCRP and P-gp	Inhibited	[32]
		Indinavir	CYP	Inhibited	[38]
	Selected aloe leaf material	Insulin	Tight junctions	Opened	[41]
		FITC-dextran	Tight junctions	Opened	[43]
	Salvianolic acid B	Human serum albumin	Albumin	Competitive binding	[48,50]
		Bovine serum albumin			[49]
	Arabinogalactan	Bovine serum albumin	Albumin	Competitive binding	[51]
	Ivermectin	H33342 dye	P-gp and BCRP	Inhibited	[55]
		Lopinavir	P-gp posited	Inhibited	[56]
	Chlorambucil	[³ H]-Cholecystokinin octapeptide	OAT1B3	Inhibited	[57]
		5-Carboxyfluorescein	MRP1	Inhibited	[58]
In vivo	Telaprevir	[³ H]-Estrone 3-sulfate	P-gp	Inhibited	[59]
		Calcein assay			[60]
	DAG	Ketoconazole	CYP3A4/5	Inhibited	[2]
	Resveratrol	Methotrexate	P-gp, OAT1 and OAT3	Inhibited	[5]
		Chlorzoxazone	CYP2E1	Inhibited	[34]
		Diclofenac	CYP2C9	Inhibited	[35]
	Piperine	Emodin	UGT	Inhibited	[29]
		Docetaxel	P-gp and CYP3A4 posited	Inhibited	[30]
	Selected aloe leaf material	Indinavir	CYP	Inhibited	[38]
		Vitamins C and E	Intestinal absorption	Increased	[45]

Inhibition of efflux transporters and metabolism enzymes by co-administered compounds may lead to increased bioavailability of drugs with poor pharmacokinetic properties. Some of these interactions can potentially be coordinated by including functional excipients (i.e., compound that causes beneficial interaction) with the drug in a dosage form. This technique may even be used to lower the quantity of active drug required per administered dose due to increased bioavailability. A classic example of such an application is probenecid that is combined with certain antibiotics to prolong the circulation time of the antibiotics in the systemic circulation. However, such combinations to deliberately cause interactions must be used with caution as inhibition of metabolism and efflux may also lead to increased systemic concentrations of other chemical compounds. Furthermore, drug absorption enhancement by functional excipients has been associated with toxic effects and tissue damage, which should be avoided.

Although some potential drug bioavailability enhancers have been identified, further research is necessary to find appropriate modulators that can be safely administered with drugs to improve their pharmacokinetic properties and ultimately also their efficacy. Due to a relative shortage of information on the clinical significance of some pharmacokinetic interactions, it is important to mention here that a need exists for future studies to provide clinical data on this topic.

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CHAPTER 3

RESEARCH ARTICLE: “EVALUATION OF RPMI 2650 CELL LAYERS AND EXCISED SHEEP NASAL TISSUE AS MODELS FOR NASAL DRUG DELIVERY SCREENING”

Chapter 3 is in the form of a method article that was submitted to the “*Journal of Drug Delivery Science and Technology*” for peer-review and publishing in 2019. The author guidelines of the journal can be found in Appendix B with the certificate of submission in Appendix C. Ethical approval for the use of animal tissue along with the validation of analytical methods and permeation data are given in Appendix G, I and J, respectively.

The nasal delivery of drugs presents a potential alternative to deliver compounds directly into the systemic circulation. As the aim of the thesis entails the enhancement of nasal delivery through beneficial interactions, the need arises to compare, characterise and standardise different models for pre-clinical investigation of nasal drug delivery.

1 **Evaluation of RPMI 2650 cell layers and excised sheep nasal tissue as models for**
2 **nasal drug delivery screening**

3 Werner Gerber¹, Dewald Steyn¹, Awie Kotzé¹, Hanna Svitina¹, Ché Weldon², Josias
4 Hamman¹

5 ¹Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag
6 X6001, Potchefstroom, 2520, South Africa

7 ²School of Environmental Sciences and Development, North-West University, Private Bag
8 X6001, Potchefstroom 2520, South Africa

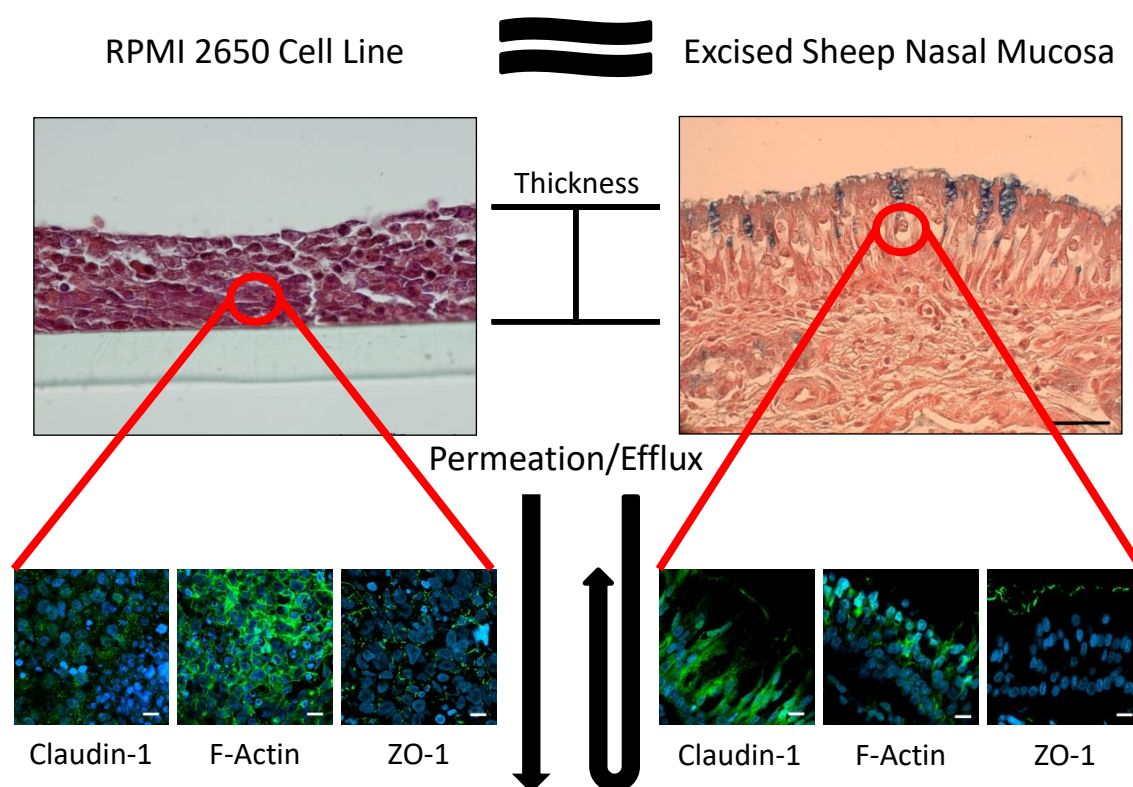
9 **Correspondence**

10 Prof. Josias Hamman (PhD), Centre of Excellence for Pharmaceutical Sciences, North-West
11 University, Private Bag X6001, Potchefstroom, 2520, South Africa. Email:
12 sias.hamman@nwu.ac.za Phone: +27 18 299 4035 Fax: +27 18 299 2248

Highlights

- The nasal delivery route presents an opportunity to deliver high molecular weight compounds directly into the systemic circulation.
- Models for pre-clinical investigation of nasal delivery of drugs need to be characterized and standardised.
- The RPMI 2650 cell layer grown at the air liquid interface and excised sheep nasal tissue models were found to be similar in epithelial thickness, tight junction expression and permeation of model compounds in terms of paracellular and efflux-mediated permeation.
- Both the RPMI 2650 cell layer and excised sheep nasal tissue models can be used for drug permeation studies across the nasal epithelium when tight junction and efflux protein modulation are involved.

Graphical abstract



Abstract

The nasal route of drug administration has been identified as a potential alternative for the oral route of drug administration, especially for systemic delivery of large molecular weight compounds. Major advantages of the nasal route of drug delivery include the high vascularity and relatively high permeability of the nasal epithelium as well as the fact that it circumvents first-pass hepatic metabolism. The RPMI 2650 cell line and *ex vivo* sheep nasal mucosa models have been evaluated in this study with respect to epithelial thickness, selected tight junction protein expression, extent of p-glycoprotein (P-gp) related efflux of a model compound and paracellular permeation of a marker compound. The RPMI 2650 cell layer grown under liquid cover conditions (LCC) was comparatively thinner ($23.9 \pm 4.1 \mu\text{m}$) than the epithelial layer of the excised sheep nasal mucosal tissue ($52.9 \pm 4.4 \mu\text{m}$), but the epithelial thickness of the RPMI 2650 cell layers grown under air liquid interface (ALI) conditions ($53.3 \pm 8.1 \mu\text{m}$) compared well with that of the excised mucosal model. Although the location and distribution of tight junction proteins and F-actin differed between the RPMI 2650 cell layers grown under ALI conditions from those in the excised sheep nasal epithelial tissue, the extent of paracellular permeation of FITC-dextran 4400 (FD4) was similar in these two models ($P_{\text{app}} = 0.48 \times 10^{-6} \text{ cm.s}^{-1}$ and $0.46 \times 10^{-6} \text{ cm.s}^{-1}$). The bi-directional permeation of rhodamine 123 (R123) yielded the same efflux ratio values ($\text{ER} = 2.33$) in both models. The RPMI 2650 cell model therefore yielded similar permeation results for different model compounds in comparison with the excised sheep nasal mucosa model.

Key words: RPMI 2650, sheep nasal mucosa, pharmacokinetics, tight junctions, efflux, permeation

1 Introduction

The nasal route of drug administration presents an opportunity for effective systemic delivery of drugs. The human nasal cavity has a relatively large surface area (150 cm²), a high level of vascularization and a relatively loosely-arranged epithelial layer with higher permeability potential as compared to other regions in the body. Drug transport across the nasal epithelium can occur via passive diffusion (both transcellularly and paracellularly), transcytosis as well as active and carrier-mediated transport mechanisms. When considering these factors it is apparent that the nasal route of drug delivery provides a unique opportunity to deliver drugs directly into the systemic circulation. The nasal cavity also presents an opportunity to potentially deliver protein and peptide type drugs systemically. This may lead to better patient compliance as nasal delivery is much less invasive and painful compared to parenteral administration such as injections [1-4].

Efficient models are needed during the pre-clinical drug development process to evaluate basic drug-like characteristics such as membrane permeation properties. There is growing compliance among the animal science community with the “Three Rs” principle that promotes reducing the use of *in vivo* animal models, refining these models and replacing animals with alternative models such as *in vitro* cell cultures [5,6].

The RPMI 2650 cell line is currently the only commercially available nasal epithelial model that can be used for *in vitro* drug transport studies to predict nasal drug absorption. The cells were initially harvested from an anaplastic squamous cell carcinoma of the human septum, and thus closely resemble the normal human diploid karyotype. However, early deployment of the cells as a permeation model yielded disappointing results with cells failing to form a homogeneous polarized monolayer. Later refinement of the technique discovered the importance of simulating physiological conditions by growing the cells at the air-liquid interface (ALI). When grown by means of the ALI technique, RPMI 2650 cells have been found to produce mucoid material, express tight junctions as well as transporter proteins while developing microvilli. Furthermore, the cells demonstrated similar transepithelial electrical resistance (TEER) and permeability values as well as producing enzymes comparable to human physiological conditions [3,4,7].

Excised sheep nasal mucosa can be deployed as an *ex vivo* model for evaluating nasal drug permeation and it has been reported to present similar nasal permeation trends as found in humans [8]. Furthermore, it has also been reported that sheep nasal mucosa present with similar TEER values, metabolism and transporter expression to human nasal mucosa [2,9].

82 The current study intensively evaluated the RPMI 2650 cell model as well as the sheep
83 nasal mucosa model with respect to key factors which are known to govern the extent and
84 efficiency of nasal drug delivery. The two selected nasal drug delivery models were
85 evaluated in terms of histology, model compound permeation (paracellular and active efflux
86 transport), tight junction protein expression as well as P-gp and MRP-1 protein expression.

2 Materials and methods

2.1 Materials

The RPMI 2650 cell line (Human nasal septum carcinoma) was purchased from American Type Culture Collection (ATCC, Cat. number: CCL-30™) and sheep nasal mucosa was collected directly after slaughter of sheep from a local abattoir in Potchefstroom, South Africa. Minimum essential media (MEM, Cat. number: 21090-055), 4',6-diamidino-2-phenylindole (DAPI, Cat. number: D1306), fetal bovine serum (FBS, Cat. number: 42Q8078K) and phosphate-buffered saline (PBS, Cat. number: AD18274264) were purchased from ThermoFischer Scientific. L-glutamine (Cat. number: BE17-605E), Pen-Strep (Cat. number: DE17-602E) and trypsin-EDTA (0.25% w/v) (Cat. number: SV30031.01) were purchased from Whitehead Scientific. Krebs-Ringer bicarbonate buffer (KRB) was prepared according to the product information sheet provided by Sigma-Aldrich [10]. Alcian Blue (Cat. number: B8438), Eosin Y (Cat. number: M7145), FITC-dextran 4400 (FD4, CAS number: 60842-46-8), hematoxylin (Cat. number: GHS132), Lucifer Yellow (LY, CAS number: 67769-47-5), non-essential amino acids (NEAA, Cat. number: M7145) and rhodamine 123 (R123, CAS number: 62669-70-9) were purchased from Sigma-Aldrich. ThinCert™ 12-well plates (Cat. number: 665110) and ThinCert™ polyethylene terephthalate-coated inserts (Cat. number: 665641) with 0.4 µm pore size, 2 x 10⁶ cm⁻² pore density and 1.131 cm² culture surface were purchased from Greiner Bio-One. ZO-1 antibodies (Cell Signalling Technology, Cat. number: 13663) were purchased from Anatech. Alexa Fluor 488 (Cat. number: 150077), Claudin-1 antibodies (Cat. number: ab129119), CytoPainter Phalloidin-iFluor 488 (Cat. number: ab176753) and Mounting media (Cat. number: ab104129) were all purchased from Biocom Ltd for Abcam.

2.2 Ethics

Approval of the use of excised sheep nasal mucosa tissue samples from animals that were already slaughtered for other purposes, e.g. meat production (i.e. low risk category) was obtained from the North-West University's animal ethics committee (NWU-AnimCare REC), with ethics approval certificate nr: NWU-00285-17-A5.

2.3 RPMI 2650 cell culturing

The RPMI 2650 cells that were used for permeation and histological studies were cultured under ALI conditions according to previously published methods [3,11] with slight modifications. Initially, the cells were cultured in Corning™ T75 culture flasks until confluency of 90 - 95% was reached. Thereafter the cells were washed twice with PBS and trypsinised

for 5 min in an incubator with a 0.25% (w/v) trypsin-EDTA mixture. The resulting suspension of cells was then centrifuged at 200 rpm for 5 min in order to remove the excess trypsin-EDTA, after which cells were counted using a haemocytometer. The cell suspension was diluted and seeded onto ThinCert™ PTP-coated insert membranes at a density of 6×10^5 cells/cm² for cultivation.

The RPMI 2650 cells in ThinCert™ 12-well plates were initially cultured under liquid-covered culture (LCC) conditions for 2 days, where after the inserts were lifted to the ALI for an additional 19 days. During LCC conditions, growth medium was incubated on both the apical and basolateral sides of the insert membranes. In contrast to this, growth medium was only added to the basolateral side of the cells during ALI conditions while the apical side was exposed to air.

MEM fortified with 10% FBS, 1% L-glutamine, 1% NEAA and 1% PenStrep was used as growth medium with medium changes three times a week. All cells were between passages of 25 and 47 when used for seeding and all incubation continued in a humidified 5% CO₂ atmosphere at 37°C.

2.4 Preparation of sheep nasal mucosal tissues

A technique was adapted from a range of previously published methods [9,12-14] to excise sheep nasal mucosa. The nasal mucosal tissue for a single experiment was excised from one sheep head that were collected directly after slaughter from a local abattoir in Potchefstroom, South Africa. The steps in the nasal tissue excision technique are depicted by a series of photographs shown in Figure 1. Thereafter, the excised nasal mucosal tissues were mounted in Sweetana-Grass diffusion chambers as permeation model.

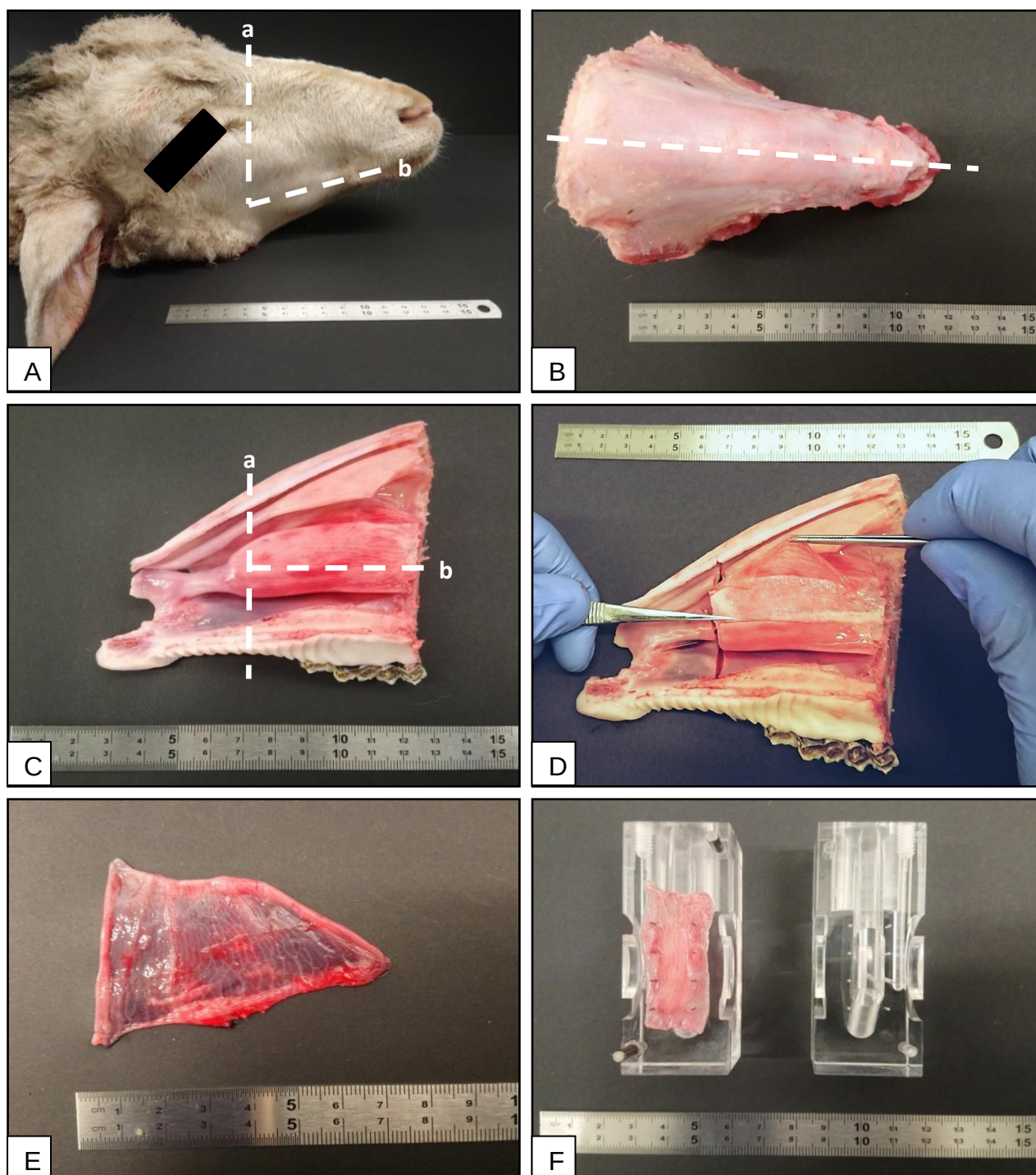


Figure 1: Photographs illustrating the chronological steps in the sheep nasal mucosa excision technique and mounting in a half cell of a Sweetana-Grass diffusion chamber for use as a drug permeation model

Firstly, the front part of the snout of the sheep was removed by a longitudinal incision anterior to the eyes (Figure 1A) followed by removal of the skin via dissection. The snout was rinsed with and submerged in ice-cold KRB ant then transported to the laboratory. In the laboratory, the snout was bisected along the septal midline and the septum was removed

(Figure 1B). After separation into two halves, a vertical incision was made through the lateral wall of the snout to isolate the conchae, with another incision across the conchae (Figure 1C) in order to facilitate removal of the nasal mucosal tissue (Figure 1D). The resulting specimen of sheep nasal epithelial tissue (Figure 1E) was cut into smaller strips and then mounted onto one half-cell of the Sweetana-Grass diffusion apparatus (Figure 1F) ready for assembly and use in the permeation studies.

2.5 Histological analysis

Histological analysis was conducted on RPMI 2650 cells grown for 21 days for both the LCC and ALI techniques as well as on the excised sheep nasal mucosal tissues. Analyses were completed on intact control samples (samples not exposed to permeation). Firstly, tissue specimens were washed with PBS and fixed in 10% buffered formalin at 4°C for 24 h. Following fixation, the specimens were dehydrated in a graded series of ethanol, embedded in paraffin and sliced into 4 – 5 µm sections, stained with hematoxylin-eosin and 1% Alcian Blue. Samples were examined with a Nikon E800 compound microscope equipped with a 60x objective lens. Thickness of the epithelial layer and nucleus areas of the RPMI 2650 cells at the ALI and sheep nasal mucosa were measured and compared using NIS-Elements software, v4.05.

2.6 Fluorescent immunocytochemistry

All samples were fixated in 10% buffered formalin for 1 h at room temperature. The sheep nasal epithelial tissue was cut into slices of approximately 400 µm thickness using an automated tissue chopper. Permeabilisation and non-specific reactivity blocking were performed by incubating samples in a 1% FBS and 0.3% triton-X100 in PBS solution at room temperature for 1 h. Here after the samples were incubated at 4°C overnight with the following antibodies at set dilutions: ZO-1 at 1:50 dilution, Claudin-1 at 1:100 dilution and Cyto Painter Phalloidin-iFluor 488 at 1X solution in PBS. Following incubation with the antibodies, tissue specimens were washed thrice with PBS for 10 min each. Then, samples were incubated with secondary goat-anti rabbit Alexa Fluor 488 antibodies at a dilution of 1:300 for 2 h at room temperature and protected from light. Following nuclear staining with DAPI, slides were mounted using mounting media. Confocal laser scanning microscopic analysis was performed using a Zeiss LSM 800 Meta microscope and images were captured with ZEN blue edition 2.6 software.

2.7 Permeation studies

Lucifer Yellow (LY, 50 µg/ml) was used as hydrophilic marker to determine if membrane integrity was maintained for the duration of the 3 h permeation experiments. FITC-dextran 4400 (FD4, 500 µg/ml) was used as large molecular weight model compound to evaluate paracellular transport. Rhodamine 123 (R123, 5 µM) was used as model compound to determine the extent of P-gp related efflux transport during bi-directional permeation studies.

For both the RPMI 2650 cell line and excised sheep nasal mucosal tissue models, 180 µl samples were withdrawn at time intervals of 5, 10, 15, 30, 60, 90, 120 and 180 min and replaced with equal volumes of pre-warmed KRB. Permeation studies with LY and FD4 were conducted uni-directionally (i.e. absorptive direction only) and for R123 it was conducted bi-directionally (i.e. both absorptive and secretory directions).

When using the RPMI 2650 cells, inserts were firstly transferred to ThinCert™ 12-well plates and acclimatized for 15 min by adding 37°C pre-warmed KRB in volumes of 0.8 ml on the apical side and 1.8 ml on the basolateral side of the membrane. Following acclimatization, the KRB was aspirated and pre-warmed solutions of the specific marker molecule was either added to the apical side for permeation studies in the absorptive direction or to the basolateral side for permeation studies in the secretory direction in the same volumes mentioned above (i.e. 0.8 ml apical and 1.8 ml basolateral). When using the excised sheep mucosal tissue, the same procedure was followed, except at 34°C to simulate *in vivo* conditions and adding a volume of 7 ml to both sides of the tissue in the Sweetana-Grass diffusion chamber.

2.8 Fluorescence spectroscopic analysis

All the selected model compounds in the samples were analyzed using a Spectramax® Paradigm (Serial number: 33270-1142) multi-mode detection platform plate reader by means of validated fluorescence spectroscopy analytical methods. LY was analysed at excitation and emission wavelengths of 485 nm and 535 nm, respectively [15,16], FD4 at excitation wavelength of 485 nm and emission wavelength of 525 nm [17,18], while R123 was measured at excitation and emission wavelengths of 480 nm and 520 nm, respectively [19,20].

209 2.9 Data analysis

210 Samples collected from permeation studies for all three marker molecules and in both
211 models were processed, corrected for dilution and expressed as % transport as shown in
212 equation 1.

$$213 \quad \% \text{ Transport} = \frac{\text{Conc. value at specific time}}{\text{Conc. value of donor solution}} \times 100 \quad (\text{Eq. 1})$$

214 The cumulative percentage transport data were then used to calculate the apparent
215 permeability coefficient (P_{app}) values using equation 2:

$$216 \quad P_{\text{app}} = \frac{dQ}{dt} \left(\frac{1}{A \cdot 60 \cdot 100} \right) \quad (\text{Eq. 2})$$

217 Where P_{app} is the apparent permeability coefficient ($\text{cm} \cdot \text{s}^{-1}$), $\frac{dQ}{dt}$ is the permeability rate
218 constant per minute and A is the area available for molecule diffusion (cm^2) [3,21,22].

219 Following the calculation of P_{app} values for R123, the efflux ratio (ER) was calculated to
220 indicate which direction of transport was most prominent during R123 permeation (Equation
221 3). $\text{ER} > 1$ indicates active efflux, $\text{ER} \approx 1$ indicates passive diffusion, while $\text{ER} < 1$ is
222 indicative of active absorptive uptake [19,23].

$$223 \quad \text{ER} = \frac{P_{\text{app}} (\text{secretory})}{P_{\text{app}} (\text{absorptive})} \quad (\text{Eq. 3})$$

3 Results and discussion

3.1 Histological analysis

Figure 2 depicts micrographs obtained from the histological analyses from RPMI 2650 cell layers grown by means of ALI and LCC techniques as well as excised sheep nasal mucosal tissue specimens.

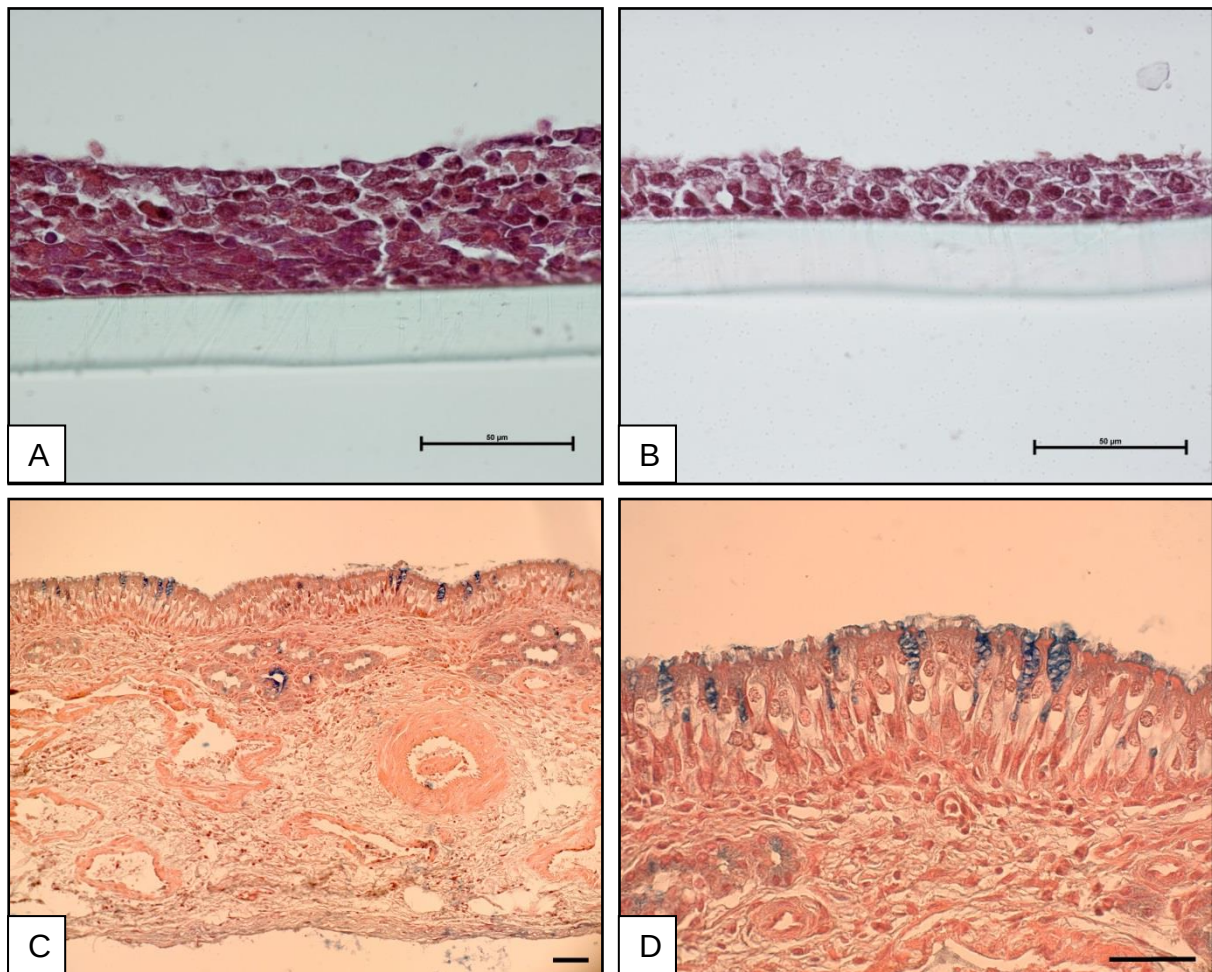


Figure 2. Micrographs of histological sections of: A) RPMI 2650 cell layer grown under air-liquid interface (ALI) conditions, B) RPMI 2650 cell layer grown under liquid-covered culture (LCC) conditions, C) section of whole mucosa of excised sheep nasal tissue and D) epithelial layer of excised sheep nasal mucosa. Scale bars = 50 μm

From Figure 2, distinct differences between RPMI 2650 cells grown using the ALI technique (Figure 2A) and the LCC technique (Figure 2B) can be observed. The RPMI 2650 cells formed a tightly packed, relatively thick multi-layered epithelial barrier when grown under ALI conditions. By comparison, RPMI 2650 cells grown under LCC conditions formed a much thinner epithelial layer composed of similar sized cells, but with consistently larger

intercellular spaces. The morphology of the cell layers in these micrographs may explain why much lower TEER values were obtained in previous studies when RPMI 2650 cells were cultured under LCC conditions compared to those cultured under ALI conditions [3,7,22]. Furthermore, no mucus-producing cells were observed in either of RPMI cell layers irrespective of the technique that was used to grow the cells. Conversely, in the whole mucosal layer of excised sheep nasal tissue (Figure 2C) the presence of thin muscular walled veins, thick muscular walled arteries and serous glands can be seen embedded within collagen enriched connective tissue. Furthermore, intact cilia can be seen on the apical side of epithelial cells (pseudostratified columnar epithelium) along with blue stained mucus producing goblet cells within the epithelial layer of excised sheep nasal mucosal tissue (Figure 2D). In addition, the thickness of the epithelial layer in the RPMI 2650 cell layer grown under ALI conditions and the excised sheep nasal epithelial tissue model is similar (i.e. $53.3 \pm 8.1 \mu\text{m}$ vs $52.9 \pm 4.4 \mu\text{m}$), while it was much thinner in the RPMI 2650 cell layer grown under LCC conditions (i.e. $23.9 \pm 4.1 \mu\text{m}$). However, nuclei in the sheep nasal mucosa were approximately twice the size of those found in the RPMI 2650 cell layer grown under ALI conditions ($14.3 \pm 7.2 \mu\text{m}^2$ vs $36.1 \pm 6.6 \mu\text{m}^2$).

3.2 Fluorescent immunocytochemistry

Confocal laser scanning micrographs were taken of RPMI 2650 cell layers grown under ALI conditions (Figure 3) as well as excised sheep nasal epithelial tissues (Figure 4) after fluorescent staining of claudin-1, F-actin chains and zonula occluden-1 proteins.

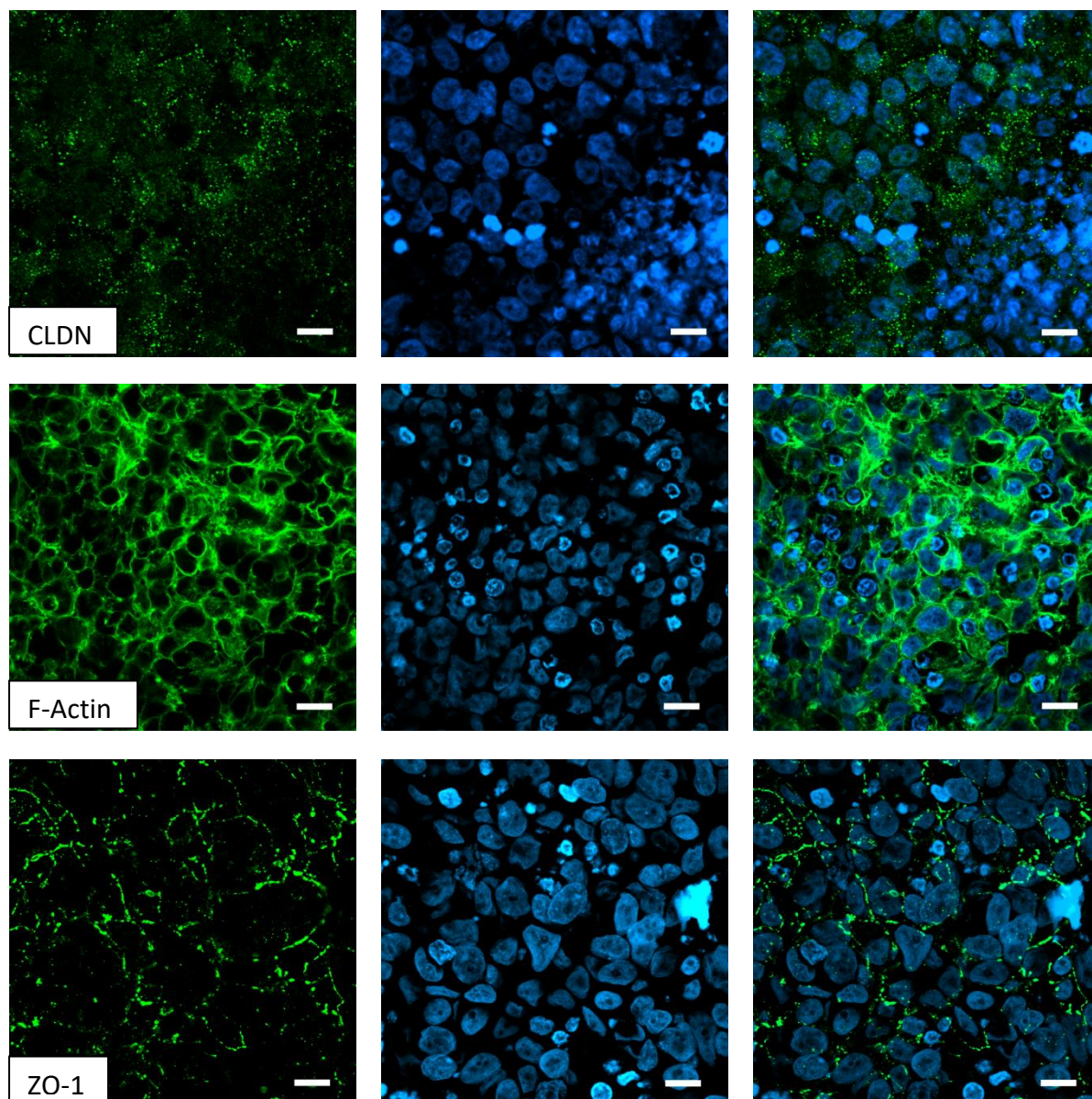


Figure 3. Confocal laser scanning micrograph images indicating the presence and distribution of claudin-1 (CLDN), F-actin chains (F-actin) and zonula occludin-1 (ZO-1) tight junction proteins in the green channel together with DAPI-stained nuclei in the blue channel and a composite image with both channels in the RPMI 2650 cells cultivated under the air-liquid interface (ALI) conditions (Scale bar = 10 μ m)

Figure 3 shows that RPMI 2650 cultured at the ALI presented with diffusely spread claudin-1 and zonula occludin-1. Contrary to this, F-actin chains are more densely distributed throughout the cell layer. Similar tight junction expression in RPMI 2650 cell layers have been shown when the ALI growth technique used in previously reported studies [3,7,11].

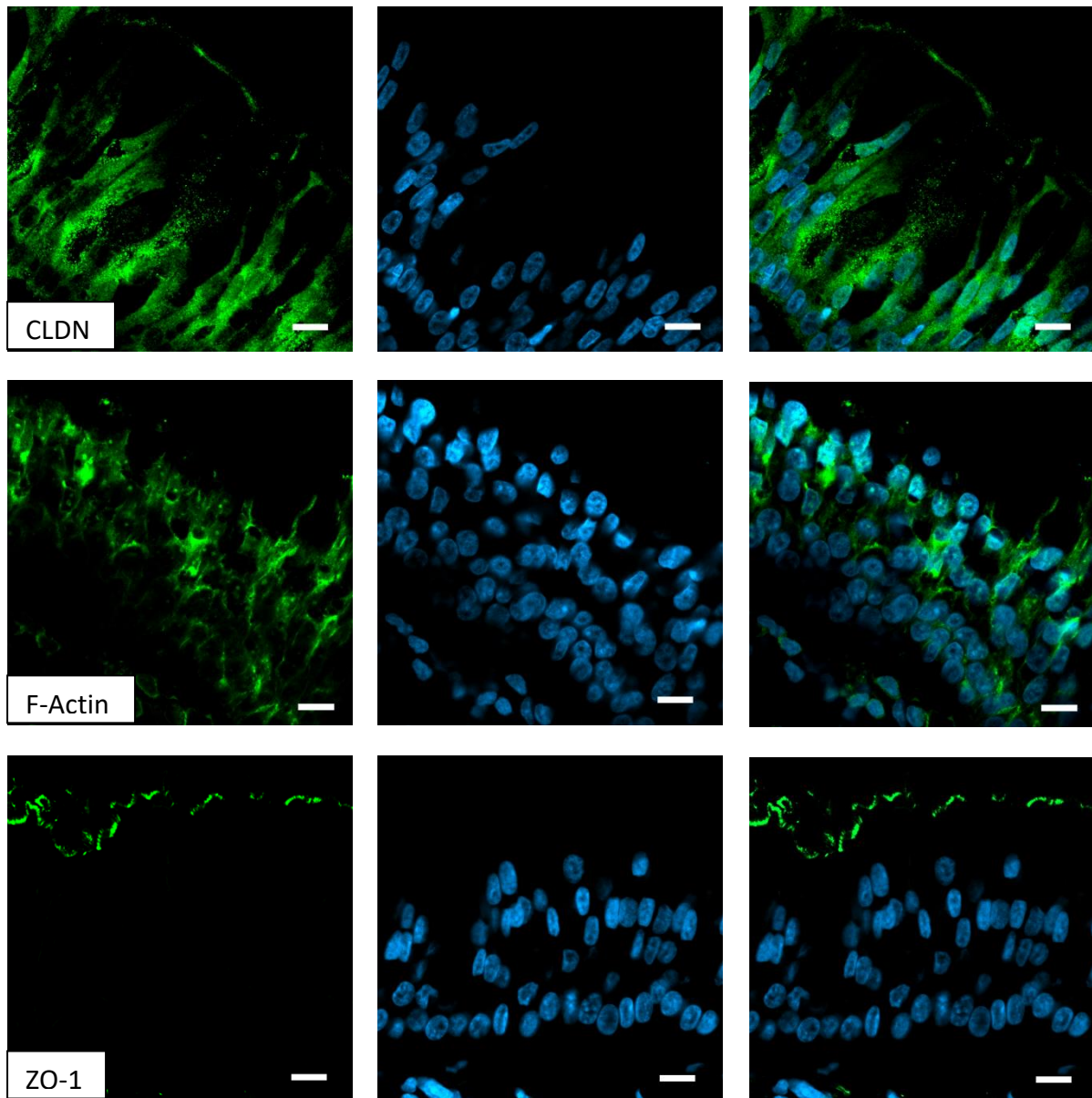


Figure 4. Confocal micrograph images indicating the presence and distribution of claudin (CLDN), F-actin chains (F-actin) and zonula occludin-1 (ZO-1) tight junction proteins in the green channel together with DAPI-stained nuclei in the blue channel and a composite image with both channels in the excised sheep nasal mucosa (Scale bar = 10 μm)

Interestingly, Figure 4 shows that the claudin-1 tended to be present in high densities closer to the basolateral side of the epithelial layer, while zonula occludin-1 proteins were more prominently distributed on the apical region of the epithelial layer. F-actin seemed to be present in high densities in the middle of the epithelial layer. The same pattern of tight junction protein distribution was found in human nasal mucosal tissues [24-26].

In general, the fluorescent immunocytochemistry evaluation showed that tight junction expression was similar in the RPMI 2650 cell model and in the excised sheep nasal

epithelial tissue model. Claudin-1 appeared to be present in slightly higher concentrations (as observed by more dense staining) in the excised sheep nasal mucosa compared to the RPMI cell layer, whereas F-actin chains seemed slightly more diffuse in the excised sheep nasal epithelial tissue.

3.3 Permeation studies

From the P_{app} values obtained for LY across the RPMI 2650 cell layers and excised sheep nasal epithelial tissues ($1.48 \pm 0.51 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ and $1.72 \pm 0.31 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$, respectively), it is clear that both models maintained membrane integrity for the entire period of the permeation studies (i.e. a period of 3 h). The LY permeation across these nasal epithelial models was slightly higher than the P_{app} value of $0.66 - 0.75 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ previously published for intact membranes in intestinal epithelial models [15,16], but this can be attributed to the fact that nasal epithelium is known to be more permeable than the intestinal epithelium, as reported earlier.

P_{app} values for the selected marker molecules (i.e. FD4 and R123) calculated from the permeation data across RPMI 2650 cell layers and excised sheep nasal epithelial tissues are indicated in Figure 5.

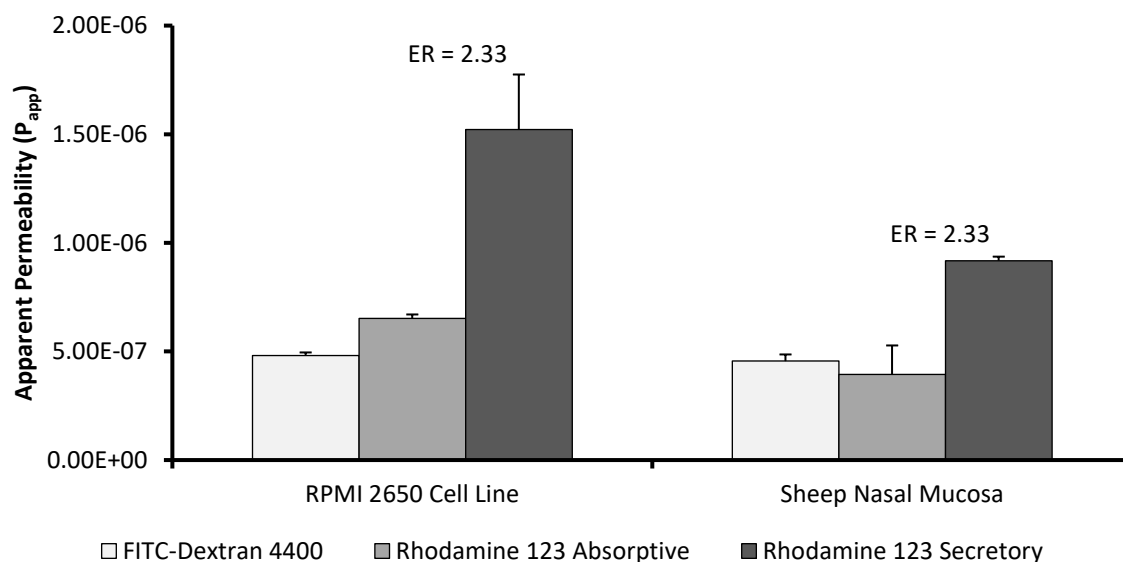


Figure 5. Apparent permeability coefficient (P_{app}) values of the selected marker compounds across RPMI 2650 cell layers cultivated under air-liquid interface conditions and excised sheep nasal epithelial tissue (ER = Efflux ratio)

FD4 is a large, hydrophilic compound (MW $\approx 4400 \text{ g/mole}$), which is often used to simulate the permeation of large molecules such as peptide therapeutics across epithelial layers

303 [4,21]. The P_{app} values for FD4 were very similar in both models with $0.48 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ and
304 $0.46 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ across the RPMI 2650 cell layers and excised sheep nasal mucosal tissue,
305 respectively. This may be explained by the similar expression of tight junction proteins in
306 both the RPMI 2650 cell and excised sheep nasal mucosal tissue models, which resulted in
307 similar regulation of FD4 permeation via the paracellular pathway. Efflux ratio values (ER =
308 2.33) for R123 were exactly the same in both the RPMI 2650 cell and excised nasal mucosal
309 tissue models. However, the P_{app} values of R123 in each direction were slightly higher
310 across the RPMI 2650 cell model than across the excised sheep nasal mucosal tissue
311 model.

312 4 Conclusion

313 Histological analyses indicated that RPMI 2650 cells can be grown to similar epithelial
314 thickness as excised sheep nasal mucosa under ALI conditions, albeit without differentiated
315 mucous-producing cells or cilia. Tight junction proteins (specifically claudin-1, F-actin chains
316 and zonula occluden-1) were similar in terms of expression but differed slightly in terms of
317 morphological location and distribution in the RPMI 2650 cells when compared with the
318 excised sheep nasal tissue model.

319 Both the RPMI 2650 cell line (grown at the ALI) and excised sheep nasal mucosal tissue
320 models showed similar properties with respect to model compound permeation (for both
321 large molecular weight compounds and efflux transporter ligands) and other properties, such
322 as tight junction protein expression as well as epithelial thickness. It was confirmed that the
323 excised sheep nasal epithelial tissue model compares well with a human originated nasal
324 epithelial cell line, namely the RPMI 2650 cell line. The excised sheep nasal epithelial tissue
325 model is therefore presented as the preferable alternative pharmacokinetic model for
326 prediction of nasal drug delivery due to lower cost (RPMI 2650 cells are grown on ThinCert®
327 transwell insert membranes that are relatively expensive), higher throughput rates (RPMI
328 cells need to be grown for 21 days) and easier access (animals readily slaughtered for meat
329 production purposes without the need for specialized laboratories).

330 **Conflict of interests**

331 The authors of the manuscript have no conflict of interests to declare.

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335 or recommendations expressed in this material are those of the authors and therefore the
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CHAPTER 4

RESEARCH ARTICLE: “CAPSAICIN AND PIPERINE AS FUNCTIONAL EXCIPIENTS FOR IMPROVED DRUG DELIVERY ACROSS NASAL EPITHELIAL MODELS”

Chapter 4 is presented in the form of a research article entitled “*Capsaicin and piperine as functional excipients for improved drug delivery across nasal epithelial models*”, which was published in the peer-reviewed journal “*Planta Medica*” in 2019 (volume 85, p 114-1123; DOI: 10.1055/a-0978-5172). The author guidelines can be found in Appendix D, ethical approval for the use of excised animal tissues in Appendix G, purity of the pepper extracts in Appendix H, validation of analytical methods in Appendix I, along with the permeation data, toxicity studies and statistical analyses of results in Appendix J, L and M, respectively.

Pepper extracts have been proven to modulate pharmacokinetic profiles by inhibiting efflux and metabolism. This is the first study to indicate that capsaicin and piperine are able to modulate and improve permeation through nasal epithelium, thus indicating beneficial pharmacokinetic interactions to improve nasal drug delivery in *in vitro* and *ex vivo* models.

Capsaicin and Piperine as Functional Excipients for Improved Drug Delivery across Nasal Epithelial Models

Authors

Werner Gerber¹, Dewald Steyn¹, Awie Kotzé¹, Hanna Svitina¹, Ché Weldon², Josias Hamman¹

Affiliations

- 1 Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom, South Africa
- 2 Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

Key words

Bioenhancer, capsaicin, piperine, FITC-dextran 4400, rhodamine 123, RPMI 2650, nasal delivery

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Bibliography

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Correspondence

Prof. Josias Hamman, PhD
Centre of Excellence for Pharmaceutical Sciences,
North-West University
Private Bag X6001, 2520 Potchefstroom, South Africa
Phone: + 27 1 82 99 40 35, Fax: + 27 1 82 99 22 48
sias.hamman@nwu.ac.za

ABSTRACT

The fruit from various pepper plants has been employed for the seasoning of food, as perfuming agents, and also as traditional medicines. Phytochemicals isolated from different pepper species have been found to modulate the pharmacokinetics of orally administered drugs. This study investigated the possibility to apply capsaicin and piperine (extracted alkaloids) as modulators for drug delivery across the nasal epithelium. Both a nasal epithelial cell line (RPMI 2650) and excised sheep nasal tissue were used as models to investigate the effects of the selected pepper compounds on drug permeation. FITC-dextran 4400 (MW 4400 Da) was used as a large molecular weight marker compound for paracellular transport, while rhodamine 123 was used as a marker compound that is a substrate for P-glycoprotein-mediated efflux. From the permeation results, it was clear that capsaicin inhibited P-glycoprotein efflux to a larger extent, while piperine showed drug permeation enhancement via other mechanisms. The cell cytotoxicity studies indicated that capsaicin was noncytotoxic up to a concentration of 200 μ M and piperine up to a concentration of 500 μ M as indicated by cell viability above 80%. The histological analysis of the excised nasal tissue and cultured RPMI 2650 cell layers indicated that some damage occurred after treatment with 200 μ M capsaicin, but no changes were observed for piperine up to a concentration of 50 μ M.

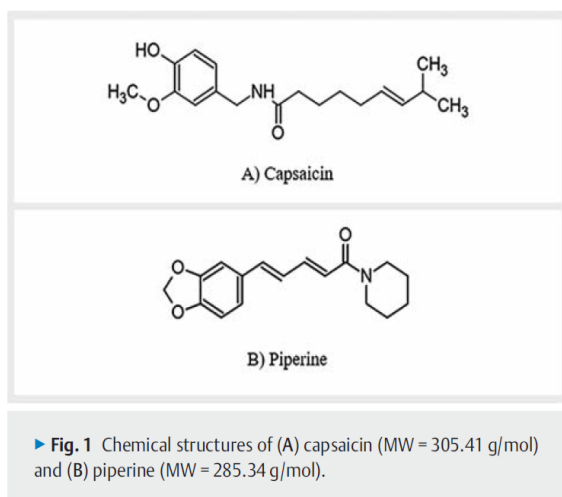
ABBREVIATIONS

ALI	air-liquid interface
FD4	FITC-dextran 4400
KRB	Krebs-Ringer bicarbonate buffer
LCC	liquid-covered culture
MEM	modified Eagle's medium
P _{app}	apparent permeability coefficient
P-gp	P-glycoprotein
PTP	polyethylene terephthalate
R123	rhodamine 123
TEER	transepithelial electrical resistance
TRPV1	transient receptor potential vanilloid 1

Introduction

Chili pepper (family *Solanaceae*) and black pepper (family *Piperaceae*) are annual herbs grown in warm climates. The production thereof is globally regarded as one of the most important economical and agricultural activities in developed and developing countries. The fruits of these plants are widely used in the flavoring of cuisine, especially in Asia and South America, and have also found applications in the cosmetic industry and for medicinal uses [1, 2].

Capsaicin (► **Fig. 1 A**) is one of the main capsaicinoids uniquely present in the fruit of plants from the genus *Capsicum*. These plants have been known for their medicinal value, with Mayan pharmacopoeia reporting the application of chili peppers in respiratory and bowel ailments, relief of earaches, and in the treatment of wounds [3]. It is now also known that capsaicin is able to inhibit melanoma proliferation, induce apoptosis in human mammary glands, and is capable of inhibiting P-gp efflux transporters in the



Caco-2 (human colon adenocarcinoma) and KB-C2 (human papillomavirus-related endocervical adenocarcinoma) cell lines [4]. In addition, it was proven that capsaicin presents with analgesic, antimicrobial, and antioxidant effects [1,3].

Black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.) belong to the genus *Piper* for which piperine (► **Fig. 1B**) is the major alkaloid and is responsible for the pungent taste of the fruits of both. Piperine has long been used as a traditional medicine, preservative, and insecticide in addition to the more commonly known seasoning and perfuming applications. Piperine has also been proven to be effective in improving the pharmacokinetics of poorly bioavailable drugs and other medicinal herbs through inhibiting P-gp and CYP450 metabolism in intestinal epithelial cells [2, 5–7].

The effects of capsaicin and piperine on the permeation of compounds have been tested in different cell models (i.e., Caco-2 and MDCK II cells) for potentially improved delivery of drug compounds, but they have not yet been explored for improved nasal delivery of drug compounds. Nasal delivery of drugs holds certain benefits for the systemic delivery of drugs, including a rich blood supply and an epithelium that is relatively more permeable in comparison with other areas in the body, and it circumvents first-pass metabolism. Furthermore, with a relatively large surface area (150 cm²) and volume (15–20 mL), the nasal cavity provides an alternative route for self-administration of poor orally bioavailable drug products that would have otherwise been administered via the parenteral route [8–10].

In the current study, the effects of capsaicin and piperine were investigated on the permeation of two model compounds in two nasal epithelial models. The RPMI 2650 cell line is currently the only commercially available human nasal epithelial cell line. When grown under the correct ALI conditions, RPMI 2650 cells have been found to express P-gp, multidrug resistance proteins, and tight junctions. Furthermore, even though the model presents with multilayered cells, the TEER and permeability properties are comparable to that of the human nasal mucosa [8, 11, 12]. The excised sheep nasal mucosa model has been proven to be well suited for permeation and metabolism studies related to the nasal delivery of compounds. This model is more cost-effective than

comparable *in vitro* and *in vivo* models, and sheep nasal tissue is easily accessible from abattoirs. The sheep nasal model has been found to closely resemble nasal absorption in humans [13]. In addition, comparable TEER values have been reported between sheep and human nasal epithelium, and the sheep nasal tissue also expresses P-gp efflux transporters [14, 15].

Results and Discussion

Validation results of the analysis by fluorescent spectroscopy of marker compounds used in the permeability studies are shown in ► **Table 1**. R123 was used as a marker for efflux-mediated transport, while FD4 was used as a macromolecular marker for paracellular transport.

Linearity and purity of the two pepper extracts, capsaicin and piperine, that were evaluated/screened for possible pharmacokinetic interactions were determined using HPLC analysis (► **Table 2**). Linearity was determined using reference standards of each compound, whereas purity is expressed as a percentage of raw material in relation to the reference standards. The concentrations of the reference standard were 250 µg/mL for capsaicin and 150 µg/mL for piperine.

The P_{app} values of FD4 across RPMI 2650 epithelial cell layers in the apical-to-basolateral direction are shown in ► **Fig. 2** in the absence (FD4 alone, control group) and presence of different concentrations of capsaicin (50, 100, and 200 µM) and piperine (10, 25, and 50 µM).

From ► **Fig. 2**, it is clear that capsaicin increased the FD4 permeation (P_{app}) across RPMI 2650 nasal epithelial cell layers at all the concentrations applied, which was to some extent inversely proportional to the capsaicin concentration. However, the permeation of FD4 was statistically significantly higher than the control (FD4 alone) only at the lowest concentration of capsaicin (50 µM) applied. In general, these findings in the nasal epithelial cell line are in coherence with previous findings obtained for capsaicin across different epithelial models such as the Caco-2 cell model [16, 17] and the MDCK II model [18, 19]. In both these previous sets of studies, it was found that capsaicin reduced TEER and increased paracellular permeation of different compounds by means of tight junction modulation. It was suggested that capsaicin can open tight junctions via a unique and reversible dual-action mechanism. Firstly, capsaicin caused an influx of Ca²⁺ into the cells, which led to cofilin dephosphorylation that directly induced depolymerization of F-actin leading to the opening of tight junctions [1, 16]. Secondly, the total concentration of occludin expression was reduced, while a redistribution of actin at the bicellular tight junctions was seen [17, 19]. These two mechanisms seemed to occur independently from one another, but both contributed to the increased permeability through the paracellular transport pathway.

In this study, capsaicin had a negligible effect on the TEER (data not shown) of the RPMI 2650 nasal epithelial cell layers. This phenomenon may be explained by the fact that RPMI 2650 cells grow in multilayers (please refer to histology below) and not in monolayers like other epithelial cell models (e.g., Caco-2 and MDCK II cells). The study by Shiobara et al. [19] concluded that capsaicin acted differently on actin at bicellular tight junctions than at tri-

► **Table 1** Fluorescent spectroscopy validation parameters of marker compounds used for permeation studies.

	R123			FD4		
Linearity	$r^2 = 0.999$			$r^2 = 0.999$		
Limit of detection	0.002 μM			0.005 $\mu\text{g/mL}$		
Limit of quantification	0.005 μM			0.016 $\mu\text{g/mL}$		
	5 μM	2.5 μM	0.125 μM	125 $\mu\text{g/mL}$	62.5 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$
Accuracy	98.21 %	98.14 %	116.64 %	95.77 %	98.27 %	104.67 %
Intraday precision (%RSD)	4.97 %	2.37 %	6.45 %	2.77 %	3.69 %	2.63 %
Inter-day precision (%RSD)	4.52 %	2.16 %	7.22 %	2.89 %	3.52 %	2.63 %

► **Table 2** Linearity and purity results of the selected pepper extracts (i.e., capsaicin and piperine) as determined by HPLC.

	Capsaicin	Piperine
Linearity	$r^2 = 0.999$	$r^2 = 0.999$
Purity	91.82 %	97.62 %

cellular tight junctions. In a multilayer model, such as the RPMI 2650 cells grown on insert membranes, there will be a larger amount of tricellular tight junctions than bicellular tight junctions as compared to that found in monolayer cell models. A probable explanation for the inverse proportion in FD4 permeation as a function of capsaicin concentration across the multilayered RPMI 2650 cells could be attributed to an overall increase in tricellular tight junctions found in the model. Due to this, a saturation of the interactions between the capsaicin and bicellular tight junctions may have already been reached at the lowest concentration (50 μM) applied and higher concentrations therefore did not cause any higher permeation enhancement effect.

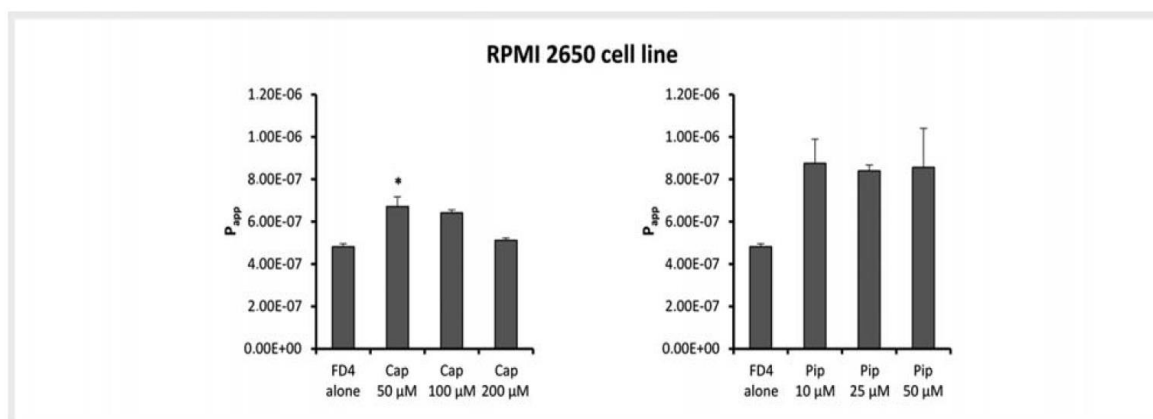
► **Fig. 2** indicates that piperine increased FD4 permeation pronouncedly across the RPMI cell layers, albeit not statistically significantly ($p > 0.05$). The effect by piperine on FD4 permeation across the RPMI 2650 cell layers did not increase with an increase in piperine concentration over the concentration range investigated (10–50 μM). It was previously proposed that piperine led to polarized paracellular opening of the blood-brain barrier [20] and increased drug absorption by fluidizing the brush border membrane while increasing microvilli length [21]. Furthermore, TEER measurements during the current permeation studies indicated a 8.99, 8.57, and 18.69% decrease in the presence of 10, 25, and 50 μM piperine, respectively. Since piperine only had a slight decreasing effect on TEER of the RPMI nasal epithelial cell layers, it may therefore have increased FD4 permeation across the RPMI 2650 nasal epithelial cell layers, most probably by a combination of the mechanisms previously proposed. Similar to that observed with capsaicin, piperine also exhibited the maximum effect at the lowest concentration applied and this effect did not increase further with an increase in concentration. Once again, this may be explained by a saturation of the interaction between piperine and the tight junctions at the lowest concentration applied.

The P_{app} values of the efflux substrate, R123, across RPMI 2650 nasal epithelial cell layers in the apical-to-basolateral (absorptive) and basolateral-to-apical (secretory) directions are shown in ► **Fig. 3**. The P_{app} values for R123 are shown in the absence (R123 alone, control group) and presence of different concentrations of capsaicin (50, 100, and 200 μM) as well as piperine (10, 25, and 50 μM).

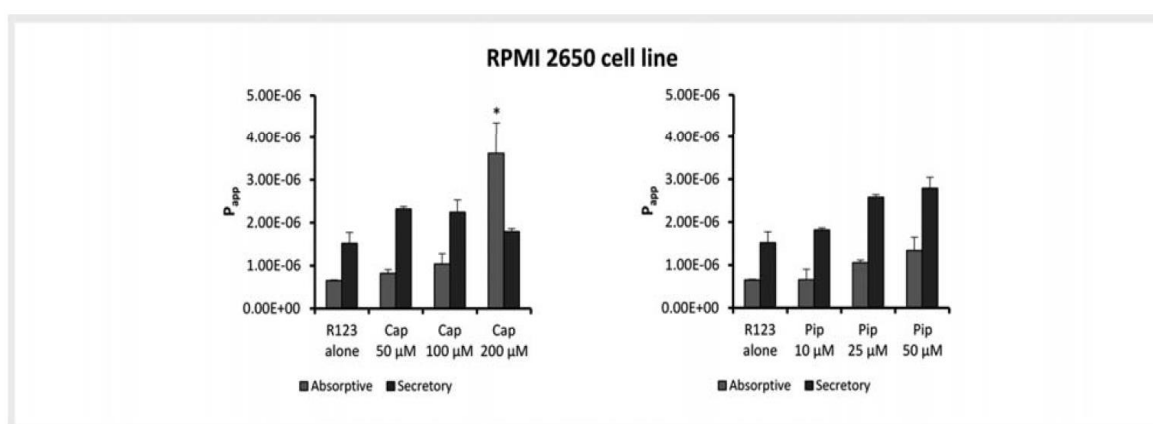
Capsaicin increased the permeation of R123 across nasal epithelial RPMI 2650 cell layers in the absorptive direction compared to the control in a concentration-dependent manner, which was statistically significant ($p \leq 0.05$) at the highest concentration (200 μM) applied (► **Fig. 3**). Although the secretory permeation was slightly higher than that of the control group, it decreased with an increase in capsaicin concentration and the effect is clearly visible in the efflux ratio values (► **Table 3**). The slightly higher permeation in the secretory direction may be explained by other mechanisms, but this was overshadowed by efflux inhibition at higher concentrations of capsaicin. From the permeation results obtained for both FD4 and R123 in the presence of capsaicin, it seems that capsaicin exhibited a larger effect on efflux-related permeation than paracellular permeation. This efflux inhibition effect of capsaicin on nasal epithelial cells correlates well with the efflux inhibition exhibited by capsaicin in other *in vitro* epithelial cell models such as Caco-2 cells [3] and KB-C2 cells [22]. Additionally, it has also been proven with *in vivo* models [23].

In the presence of piperine, both absorptive and secretory permeation of R123 increased in a concentration-dependent manner together with a decrease in the efflux ratio value at the highest concentration applied (► **Table 3**). It was previously shown in the intestinal epithelium that piperine caused efflux inhibition when applied together with a P-gp transporter substrate [6, 24, 25]. However, the results of this study indicated that piperine had a larger modulatory effect on paracellular permeation than on efflux inhibition across the nasal epithelial cells (although efflux inhibition was not explicitly ruled out as seen by the reduced efflux ratio value at the highest concentration). Since R123 is also able to diffuse paracellularly [26], the potential opening of tight junctions by the coapplied piperine correlated well with the increase in P_{app} values of R123.

The effect of capsaicin and piperine on the permeation of model compounds was also evaluated across excised sheep nasal tissues. The P_{app} values of FD4 across excised sheep nasal epithelial tissues in the apical-to-basolateral direction are shown in ► **Fig. 4** in the absence (FD4 alone, control group) and presence of differ-



► **Fig. 2** P_{app} values of FD4 in the absence (control) and presence of different concentrations of capsaicin (Cap) and piperine (Pip) across RPMI 2650 nasal epithelial cell layers in the absorptive direction (* $p \leq 0.05$, Kruskal-Wallis test followed by Dunn's post hoc test).



► **Fig. 3** P_{app} values of R123 in the absence (control) and presence of different concentrations of capsaicin (Cap) and piperine (Pip) across RPMI 2650 nasal epithelial cell layers in the absorptive and secretory directions (* $p \leq 0.05$, Kruskal-Wallis test followed by Dunn's post hoc test).

► **Table 3** Efflux ratio values of R123 in the absence (R123 alone, control) and presence of different concentrations of capsaicin and piperine across RPMI 2650 nasal epithelial cell layers.

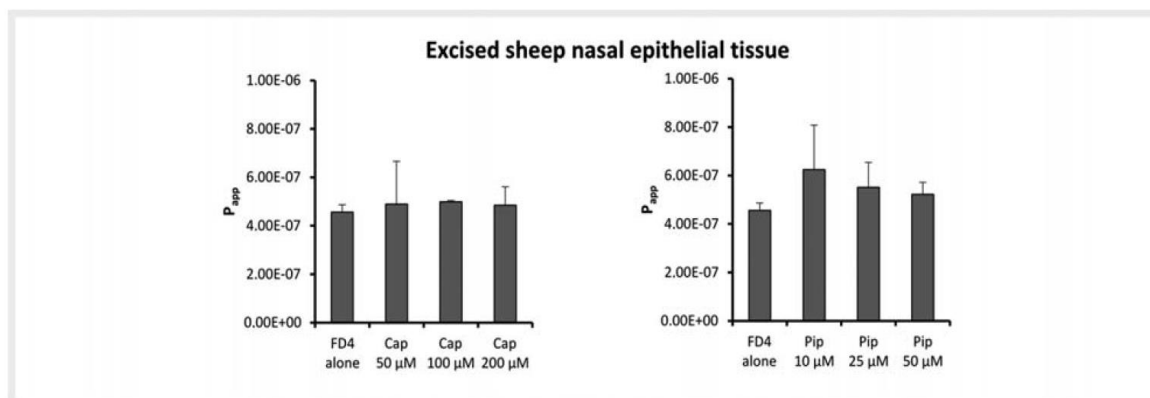
	R123 alone	Capsaicin			Piperine		
		50 μM	100 μM	200 μM	10 μM	25 μM	50 μM
Efflux ratio value	2.33	2.83	2.17	0.50	2.75	2.44	2.08

ent concentrations of capsaicin (50, 100, and 200 μM) and piperine (10, 25, and 50 μM).

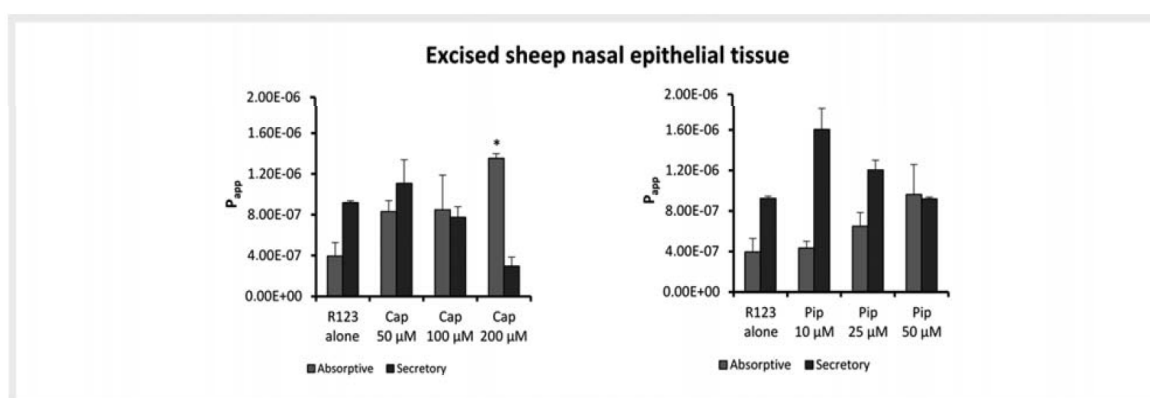
Capsaicin mediated a slight increase in the permeation of FD4 across the excised nasal epithelial tissues at the concentrations applied in this study, as shown in ► **Fig. 4**, and no statistically significant differences were evident. This indicated that the tight junction modulation effect of capsaicin to increase the paracellular permeation of FD4, a macromolecular model compound, was much lower in the excised sheep nasal tissue model compared to the nasal epithelial RPMI cell model (► **Fig. 2**). Piperine increased FD4 permeation in the absorptive direction to a larger extent than capsaicin, but it was still not statistically significant. The less pro-

nounced effect of the selected pepper extracts on FD4 permeation across the excised sheep nasal tissue as compared to the nasal epithelial RPMI 2650 cell layers may be explained by the differences in the inherent properties of the two models. In contrast to nasal epithelial cell models (e.g., RPMI 2650), animal excised tissues have ciliated cells and goblet cells, and mucus can be present [15]. Mucus plays an important role in the permeation of compounds, especially large molecules such as FD4, across biological membranes.

Both capsaicin and piperine caused pronounced concentration-dependent increases in the absorptive permeation of R123 and mediated a reduction in R123 permeation in the secretory



► **Fig. 4** P_{app} values for FD4 in the absence (FD4 alone, control) and presence of different concentrations of capsaicin (Cap) and piperine (Pip) across excised sheep nasal epithelial tissue in the absorptive direction.



► **Fig. 5** P_{app} values of R123 in the presence of capsaicin (Cap) and piperine (Pip) across sheep nasal mucosa in absorptive and secretory directions (* $p \leq 0.05$, Kruskal-Wallis test followed by Dunn's post hoc test).

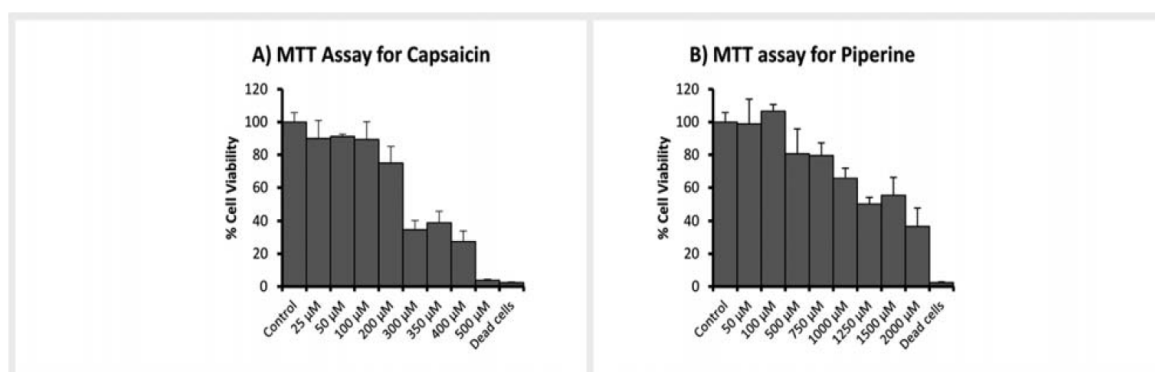
► **Table 4** Efflux ratios of R123 in the absence (control) and presence of different concentrations of capsaicin and piperine across excised sheep nasal epithelial tissue.

	R123 alone	Capsaicin			Piperine		
		50 μ M	100 μ M	200 μ M	10 μ M	25 μ M	50 μ M
Efflux ratios	2.34	1.33	0.91	0.22	3.70	1.86	0.95

direction across excised sheep nasal epithelial tissues (► **Fig. 5**). When the experimental transport values are compared to that of the controls it is evident that the efflux ratio values had decreased (► **Table 4**). These results clearly show that both capsaicin and piperine exhibit P-gp-related efflux inhibitory properties in excised nasal epithelial mucosa, which means that the absorption of efflux transporter substrates can be improved by coadministration of these pepper extract components. The improved absorption of efflux transporter substrates in the presence of capsaicin and piperine suggests that these substrates can be administered via the nasal route at lower concentrations and still attain similar blood plasma levels.

It has been shown previously that piperine has the ability to inhibit CYP3A4 metabolism, which has been proven in a variety of *in vitro* and *in vivo* models [2, 6, 25]. This has also been shown for capsaicin in an *in vivo* model [23]. The metabolism inhibition effects of these pepper extracts should also be investigated after nasal administration in future studies to elucidate if the metabolism inhibitory effects contribute to their drug absorption enhancing effects.

MTT assays were employed on the RPMI 2650 cell line to evaluate the cytotoxicity effects of capsaicin and piperine on nasal epithelial cells. This is an important consideration in order to establish if the selected pepper extract components have cytotoxic-



► **Fig. 6** Percentage cell viability as determined with an MTT assay on RPMI 2650 cells after 24 h exposure to a range of (A) capsaicin and (B) piperine concentrations.

ity effects that can cause damage at the concentrations where they are effective as drug absorption enhancers across nasal epithelial surfaces.

According to the ISO 10993-5:2009 standards determined for “Tests for *in vitro* cytotoxicity” [27], from ► **Fig. 6A**, one can see that capsaicin is not cytotoxic to RPMI 2650 cells up to a concentration of 100 µM (cell viability above 80%), weakly cytotoxic at a concentration of 200 µM (cell viability between 60–80%), and highly cytotoxic from 300 µM and above (cell viability below 40%). Piperine, on the other hand (► **Fig. 6B**), is considered non-cytotoxic to RPMI 2650 cells up to a concentration of 750 µM. The difference in toxicity between the two pepper extract components can potentially be related to the pungency of the extracts measured in Scoville heat units. The Scoville heat unit scale is used to depict the relative pungency of a pepper. Pure capsaicin possesses a pungency of 16×10^6 Scoville heat units [1], which is much higher than that of piperine at 2×10^5 Scoville heat units [5]. Furthermore, capsaicin and piperine are both proven agonists of the TRPV1 ion channel, which is responsible for the transmittance of multiple noxious stimuli. It is known that the pungency (i.e., the total Scoville heat units) of TRPV1 agonists is directly proportionate to the activation of the receptor [28,29]. Caterina et al. [28] found that capsaicin kills both neuronal and non-neuronal cells that express vanilloid receptors through cytoplasmic swelling, an amalgamation of the contents with eventual lysis due to increased receptor activation.

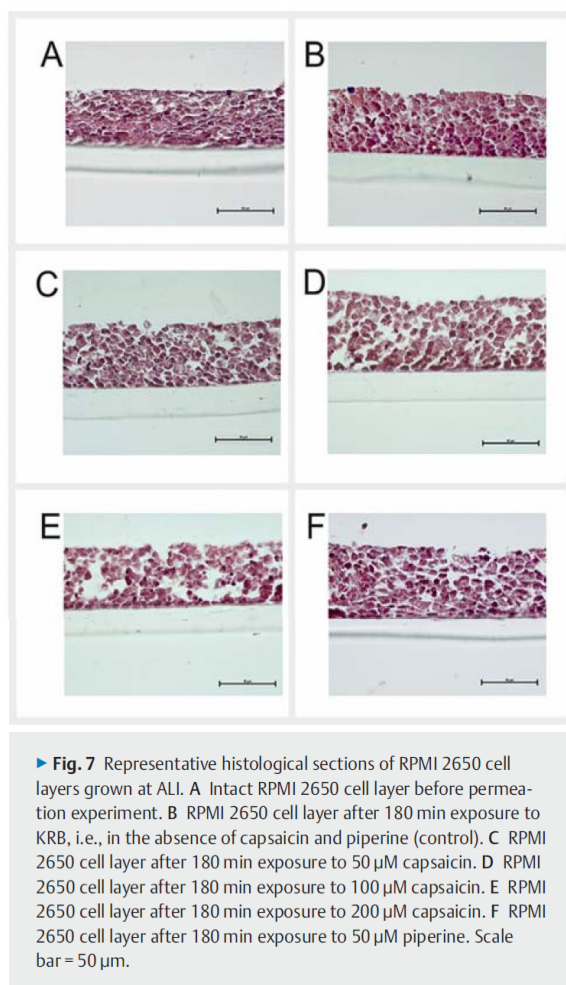
Histological analysis of both RPMI 2650 cell layers grown with the ALI technique and excised sheep nasal epithelial tissues were conducted in the absence (control) and presence of capsaicin and piperine to establish whether any physical changes occurred in the cells or tissues during the permeation experiments.

The histological investigation revealed that RPMI 2650 cells cultured under ALI conditions on porous membranes formed multilayered epithelial surfaces without differentiation into different types of cells that are found in the normal nasal epithelial mucosa. There is no evidence of columnar cells with any specialized modifications, such as goblet and ciliated cells. All cells have a round or slightly elliptical shape. Nuclei are dense without heterochromatin and euchromatin segregation, which is usually associated with the absence of secretory activity. Indeed, no mucus secretion was ob-

served, which was confirmed by the absence of Alcian Blue staining. On some areas of the RPMI epithelial cell layers, spaces are visible between the cells, resulting in a less dense aggregation of cells. Exposure to KRB for 180 min as well as 50 µM capsaicin and 50 µM piperine did not show any signs of adverse effects on the cell layers in any way (► **Fig. 7B, C, F**). Signs of cell loosening or detachment started to appear after exposure to 100 µM capsaicin for 180 min (► **Fig. 7D**), while exposure to 200 µM capsaicin for 180 min resulted in damage to the cell layers, evidenced by the formation of sinusoids (► **Fig. 7E**), which is in coherence with MTT results.

The excised sheep nasal epithelial tissues presented as pseudostratified ciliated columnar epithelium with mucus-producing goblet cells present. Epitheliocytes have an elongated shape with cilia on the apical side (free surface) of the tissue. Nuclei are loose with visible nucleoli that indicate an active synthesis process in the tissue cells. Underneath the ciliated epithelial layer, connective tissue with serous glands and blood vessels are visible (► **Fig. 8A**). After exposure to KRB, all the epithelial tissue structures remained intact with no obvious signs of tissue damage (► **Fig. 8B**). Furthermore, the underlying tissue layers including the connective tissue, blood vessels, and serous glands did not show signs of necrosis, edema, or hemorrhage after 180 min exposure to 200 µM capsaicin and 50 µM piperine. However, the epithelial layer was altered after exposure to 200 µM capsaicin with excessive cell loss (► **Fig. 8C**). The columnar cell layer appears to be separated from the rest of the tissue distal to the germ layer. Incubation of sheep nasal tissue with piperine 50 µM did not have any adverse effects on the excised sheep nasal epithelium and all structures appeared to be the same as in the intact control tissue (► **Fig. 8D**).

A histometrical analysis was conducted on both the RPMI 2650 and excised sheep nasal epithelial models, and the values of the parameters that were measured are shown in ► **Table 5**. The thickness of the whole excised sheep mucosal tissues ranged between 553.4 ± 65.8 and 683.6 ± 336.1 µm, while the thickness of the sheep nasal epithelial layer alone was 52.9 ± 4.4 µm. This compares well with the RPMI 2650 cell layers, where the epithelial thickness ranged between 47.7 ± 2.5 and 58.5 ± 4.34 µm. The epithelial thickness and average epitheliocyte nuclei areas were comparable between the intact control and after piperine expo-



sure for both the excised sheep tissue and RPMI 2650 cell models. However, the thickness of the epithelial layer and the nucleus area of epitheliocytes was dramatically decreased after exposure to capsaicin.

Materials and Methods

Cell lines and chemicals

The RPMI 2650 (nasal septum carcinoma) cell line was purchased from the ATCC. FITC-dextran (MW 4400, FD4), R123, capsaicin (purity >91% by HPLC against a reference standard), piperine (purity >97% by HPLC against a reference standard), hematoxylin-eosin, and 1% Alcian Blue were purchased from Sigma-Aldrich. Krebs-Ringer bicarbonate buffer was prepared according to Sigma-Aldrich's product information sheet [30]. PBS, MEM, and FBS were purchased from Thermo Fischer, while L-glutamine, Pen-Strep, nonessential amino acids, and trypsin-EDTA (0.25% w/v) were purchased from Whitehead Scientific. MTT, hematoxylin solution (GHS132), Eosin Y solution, and aqueous (HT110216) and Alcian Blue solution (B8438) were purchased from Sigma-Aldrich.

ThinCert 12-well plates (catalogue number 665110) and PTP-coated inserts (catalogue number 665641) were purchased from Separation Scientific. Sheep (Merino, Dorper or Île-de-France subject to availability) nasal tissue was collected from a local abattoir in Potchefstroom, South Africa.

Chemical characterization of capsaicin and piperine raw materials

The purity of the purchased piperine and capsaicin raw materials was determined by means of liquid chromatography analysis using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, UV detector, and OpenLab CDS Chemstation Rev. C.01.07 SR3 data acquisition and analysis software (Agilent Technologies). HPLC conditions were adapted from the literature and were as follows: USP L1, Venusil XBP C18 column, 150 $\times$ 4.5 mm, 5 μ m (Agela Technologies) with the mobile phase as ACN/H₂O with 0.1% H₃PO₄ 60:40 at a flow rate of 1.0 mL/min for both capsaicin and piperine. Both injection volumes were set at 10 μ L per sample. For capsaicin, the detection wavelength was set at 225 nm and it eluted at a retention time of $\pm$ 5.28 min [31], whereas a wavelength of 325 nm was used for piperine with a retention time of $\pm$ 5.18 min [5].

The linearity of capsaicin and piperine reference standards were determined first followed by a purity analysis. The purity of the raw materials was determined against the reference standard for each extract using Equation 1 where the reference standard concentrations were 250 μ g/mL for capsaicin and 150 μ g/mL for piperine.

$$\% \text{ Purity of pepper extract} = \frac{\text{experimental concentration}}{\text{reference standard concentration}} \times 100 \quad (\text{Eq. 1})$$

Fluorescence spectroscopic analysis of FITC-dextran and rhodamine 123

Fluorescence spectroscopic analysis was used to determine FD4 and R123 in the permeation samples and the analyses were done using a Spectramax Paradigm (serial nr. 33270–1142) multimode detection platform plate reader. FD4 was analyzed with excitation and emission wavelengths set at 485 nm and 525 nm [10, 32], respectively, while R123 was analyzed using 480 nm excitation and 520 nm emission wavelengths [33, 34]. The fluorescence analytical methods were validated for linearity, accuracy, and precision.

Cell culturing

RPMI 2650 cell layers were cultured on ThinCert insert membranes by means of the ALI technique using previously published methods [8, 11, 35] with some slight modifications.

Briefly, RPMI 2650 cells were first grown in culture flasks using MEM fortified with 10% FBS and 1% each of Pen-Strep, nonessential amino acids, and L-glutamine. Cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C with media changes every 2 days. After confluency of approximately 90% was reached in the culture flasks, cells were washed twice with 10 mL PBS and then trypsinized with 0.25% (w/v) trypsin-EDTA for 5 min in an incubator. Cells were then counted using a hemocytometer and seeded into PTP-coated ThinCert inserts (113.1 mm² culture surface, 0.4 μ m pore size, 2 $\times$ 10⁶ $\cdot$ cm⁻² pore density) at a density of

6×10^5 cells/cm². Seeding was done with cells between the passages of 25 and 47.

Culturing in the 12-well ThinCert culture plates took place for a total of 21 days with media changes every 2 days. Cells were cultured under LCC conditions for the first 2 days, allowing cells to adhere to the membrane. LCC conditions entail media being present at both the apical (0.8 mL) and basolateral (approximately 4 mL) side of the membrane. After 2 days, during the first media change, cells were lifted to ALI conditions. ALI conditions entail media (approximately 4 mL) to only be added to the basolateral side of the membrane for a period of 19 days. After a total of 21 days, the inserts were carefully transferred to 12-well ThinCert transport plates, the TEER across each cell layer on insert membrane was measured, and permeation studies were then initiated.

Collection and excision of sheep nasal epithelial tissue

Approval from the North-West University's animal ethics committee (AnimCare) was obtained for the use of excised sheep nasal tissue in the permeation experiments (ethics approval certificate nr. NWU-00285-17-A5). The removal of sheep nasal mucosal tissue was accomplished via a method that was adapted from previously published methods for excision of nasal epithelial tissues from different animals [15, 36–38].

After slaughter, the anterior portion of the skull (i.e., the snout) was removed via a longitudinal incision along the frontal plane, anterior to the eyes. The skin was then removed from the snout via dissection, and the snout specimen was rinsed and submerged in ice-cold KRB and transported to the laboratory. In the laboratory, a vertical incision was made along the septal midline in order to remove the septum. After separation into two halves, a vertical incision was made through the lateral wall of the snout, just posterior to the *incisura nasoincisiva* in order to isolate the nasal conchae. Hereafter, the nasal epithelial tissue layer was separated from the conchae cartilage via blunt dissection.

The pieces of epithelium were laid out on moistened filter paper in order to facilitate easier handling and to prevent damage to the epithelial cells. Strips of tissue were then cut in widths of approximately 1 cm each and mounted in Sweetana-Grass diffusion chambers after filter paper removal.

Ex vivo permeation studies

All permeation studies of R123 and FD4 in the absence (control groups) and presence of capsaicin or piperine were done in both of the nasal epithelial models (i.e., RPMI 2650 cell layers grown on insert membranes and excised sheep nasal tissues mounted in Sweetana Grass diffusion chambers) in triplicate.

For the RPMI 2650 cell model, succeeding the 21 days in culture, permeation studies were slightly adapted from previously published methods [8, 10, 12]. Firstly, the PTP-coated ThinCert inserts were transferred from 12-well culture plates to 12-well transport plates while maintaining sterile conditions. A volume of 0.8 mL preheated (37°C) KRB was added to the apical compartment and 1.8 mL KRB to the basolateral compartment. The 12-well transport plates were then left to equilibrate for 15 min in an incubator. For permeation studies in the absorptive (apical-to-basolateral) direction, 0.8 mL of the selected marker compound (i.e., either FD4 or R123) in the presence of the selected pepper

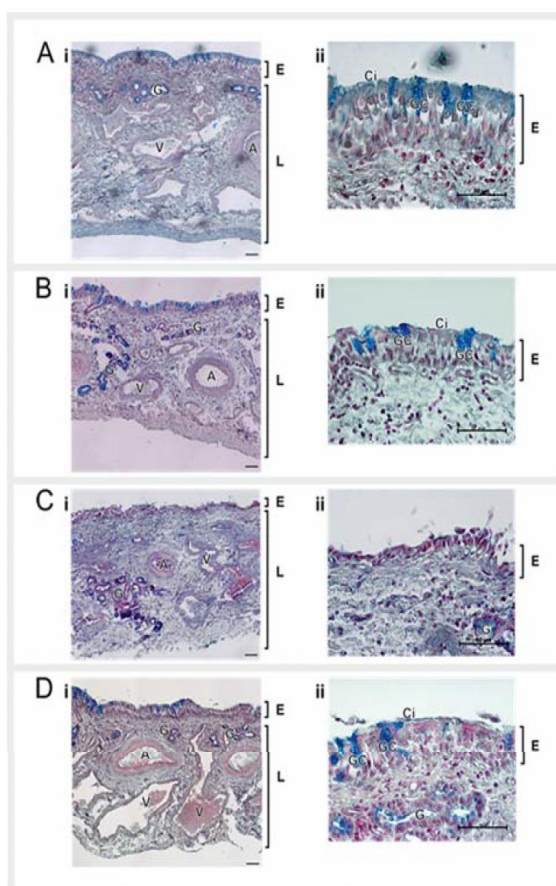


Fig. 8 Representative histological sections of excised sheep nasal epithelial tissues with column i indicating the whole excised tissue and column ii only the epithelial layer at a higher magnification. A Intact excised sheep nasal epithelial tissue before permeation experiment. B Excised sheep nasal epithelial tissue after 180 min exposure to KRB. C Excised sheep nasal epithelial tissue after 180 min exposure to 200 μM capsaicin. D Excised sheep nasal epithelial tissue after 180 min exposure to 50 μM piperine. Legend: A – arteries, Ci – cilia, E – epithelial layer, G – serous glands, GC – goblet cells, L – lamina propria, V – veins. Scale bar = 50 μm.

extract component (i.e., either capsaicin or piperine) suspended in KRB was applied and incubated on the apical side of the membrane. Samples of 180 μL were withdrawn from the basolateral side at the time intervals of 5, 10, 15, 30, 60, 90, 120, and 180 min and immediately replaced with an equal volume of fresh KRB after each withdrawal. Similarly, for permeation studies in the secretory (basolateral-to-apical) direction, 1.8 mL test solution was applied and incubated on the basolateral side. Samples of 180 μL were withdrawn from the apical side at the time intervals of 5, 10, 15, 30, 60, 90, 120, and 180 min and immediately replaced with an equal volume of fresh KRB after each withdrawal.

Permeation studies across excised sheep nasal epithelial tissue in the Sweetana-Grass diffusion chambers were done in a similar fashion. After assembly of the diffusion chambers, 7 mL preheated (34°C) KRB were incubated at both sides of the membrane, connected to a heating block and carbogen (95% O₂:5%

► **Table 5** Parameters of epithelia of two different nasal tissue models (*statistically significant differences compared to the control group with $p \leq 0.01$).

	Intact control	Capsaicin, 200 μ M	Piperine, 50 μ M
Excised sheep tissue			
Excised mucosa thickness (μ m)	683.6 $\pm$ 336.1	656.3 $\pm$ 93.1	553.4 $\pm$ 65.8
Epithelial thickness (μ m)	52.9 $\pm$ 4.4	18.8 $\pm$ 7.4*	47.5 $\pm$ 9.1
Average epitheliocyte nucleus area (μ m ²)	36.1 $\pm$ 6.6	22.2 $\pm$ 8.2*	35.1 $\pm$ 7.3
RPMI 2650 cell layers grown with ALI technique			
Epithelial thickness (μ m)	53.3 $\pm$ 8.1	47.7 $\pm$ 2.5*	58.5 $\pm$ 4.34
Average epitheliocyte nuclei area (μ m ²)	14.3 $\pm$ 7.2	10.8 $\pm$ 3.7*	13.1 $\pm$ 5.4

CO₂) supply to equilibrate for 15 min. Permeation studies in the absorptive direction contained 7 mL test solution in the apical chamber and 7 mL KRB in the basolateral chamber, while for secretory permeation studies, the 7 mL test solution was applied to the basolateral chamber and 7 mL KRB in the apical chamber. Samples were withdrawn at the same volume and time intervals as described for the permeation studies on the RPMI 2650 cell model.

Following analysis of samples using a Spectramax Paradigm plate reader for either FD4 or R123 concentrations, the percentage transport (Eq. 2) and the P_{app} values were calculated (Eq. 3).

$$\% \text{ Transport} = \frac{\text{Conc. value at specific time}}{\text{Conc. value of donor solution}} \times 100 \quad (\text{Eq. 2})$$

$$P_{app} = \frac{dQ}{dt} \left(\frac{1}{A \cdot 60 \cdot C_0} \right) \quad (\text{Eq. 3})$$

Where P_{app} is the apparent permeability coefficient ($\text{cm} \times \text{s}^{-1}$), $\frac{dQ}{dt}$ is the permeability rate per minute, A is the diffusion area of the membrane (cm^2), and C_0 is the initial concentration of each marker compound used.

The efflux ratio of the P-gp substrate, R123, was also calculated in order to determine the extent to which peppers are able to modulate efflux proteins (Eq. 4).

$$\text{ER} = \frac{P_{app} \text{ (secretory)}}{P_{app} \text{ (absorptive)}} \quad (\text{Eq. 4})$$

Cytotoxicity and histology

An MTT assay was performed to determine cytotoxicity on RPMI 2650 cells based on previously published [9,39] methods. Cells were seeded in a clear bottom 96-well plate at a density of 3×10^5 cells/cm² and left to attach for 24 h. Both capsaicin (25–500 μ M) and piperine (50–2000 μ M) were prepared in MEM, added to the cells after attachment, and left to incubate at 37 °C in a 5% CO₂ humidified atmosphere for 24 h. Following drug exposure, the cells were washed twice with 100 μ L PBS. Thereafter, 180 μ L non-additive MEM were added to the cells, followed by 20 μ L of 5 mg/mL MTT in PBS. Plates were then returned to the incubator for 4 h. Following the assay, the media was aspirated from all wells and crystals were dissolved in 200 μ L DMSO. The

plates were then placed on an orbital shaker for 1 h and absorbance was measured at two wavelengths, 560 and 630 nm, for cell signal and background noise, respectively, using a Spectramax Paradigm® plate reader. All experiments were done in triplicate.

Histological analysis was conducted to determine the effects of the pepper extract components on both the RPMI 2650 cell layers and the excised sheep nasal epithelial tissues following exposure during the permeation studies in triplicate. Excised sheep nasal tissue samples before permeation (intact control) and after the 3 h permeation studies were fixed in 10% buffered formalin at +4 °C for 24 h. RPMI 2650 ALI cell layers on ThinCert insert membranes before permeation (intact control) and after the 3 h permeation studies were washed with PBS and fixed in 10% buffered formalin at room temperature for 1 h. The samples were then dehydrated in a graded series of ethanol and embedded in paraffin, sliced into 4–5 μ m sections, stained with hematoxylin-eosin and 1% Alcian Blue, and then examined under a Nikon E800 compound microscope using 60 $\times$ objective. The sheep mucosa thickness, epithelial thickness, and nucleic area of the epitheliocytes were measured from five different sections per sample using NIS-Elements, Version 4.05 software.

Statistical analysis

Statistical analysis was performed using Statistica v. 13.3 (StatSoft, Inc.). Data are presented as the mean $\pm$ SD. Data analysis of the P_{app} values for all test groups and in both models as well as histological parameters was performed using Kruskal-Wallis tests for comparison of multiple groups followed by Dunn's test for comparison of two groups. $P \leq 0.05$ was considered to represent a statistically significant difference.

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Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER 5

RESEARCH ARTICLE: “PERMEATION ENHANCEMENT EFFECTS OF LEAF MATERIALS FROM DIFFERENT ALOE SPECIES ON IN VITRO AND EX VIVO NASAL EPITHELIAL MODELS”

Chapter 5 is presented in the form of a research article that was submitted to the “*Journal of Herbmmed Pharmacology*” for peer-review and publishing in 2019. Information with regards to this article can be found in the following appendices: Author guidelines for the journal (Appendix E), certificate of submission to the journal (Appendix F), ethical approval for the use of excised animal tissue (Appendix G), certificates of analysis of *Aloe muth-muth* materials (Appendix H), validation of analytical methods (Appendix I), permeation data (Appendix J), viscosity values of aloe materials (Appendix K), toxicity studies (Appendix L) and statistical analysis (Appendix M).

Along with the known medicinal properties of aloe species, numerous materials and species of aloe species have proven to enhance permeation and inhibit efflux in a variety of models. However, the effects of aloe leaf materials have never been tested on nasal delivery of drugs. This is the first study to indicate the enhancement of nasal drug permeation in the presence of leaf materials of *Aloe vera*, *A. ferox* and *A. muth-muth* by beneficial pharmacokinetic interactions.

Permeation enhancement effects of leaf materials from different aloe species on *in vitro* and *ex vivo* nasal epithelial models

Werner Gerber¹, Johan Dewald Steyn¹, Awie Frederik Kotzé¹, Hanna Svitina¹, Ché Weldon², Josias Hendrik Hamman^{1*}

¹ *Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa*

² *Unit for Environmental Sciences and Management, Faculty of Natural Sciences, North-West University, Potchefstroom, South Africa*

Nasal epithelial permeation enhancement by aloe materials

*Address correspondence to this author at the Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Private Bag: X6001, Potchefstroom, 2520, South Africa; Tel/Fax: +27-18-299-4035, +27-18-299-2248; E-mails: sias.hamman@nwu.ac.za

Abstract: Introduction: The nasal route of drug administration offers an alternative for oral drug delivery and has the benefit of avoiding first-pass metabolism through drug delivery directly into the systemic circulation. The drug absorption enhancing effects of selected aloe leaf materials have been shown across the intestinal epithelium, but their efficacy in this regard across nasal epithelia has not yet been investigated. The aim of this study was to determine the effects of gel and whole leaf extract materials from three selected aloe species (Aloe vera, Aloe ferox and Aloe muth-muth) on FITC-dextran 4400 permeation across two nasal epithelial models. **Method:** Permeation of FITC-dextran 4400 and histological studies were conducted on both RPMI 2650 cell layers and excised sheep nasal mucosa, while toxicity studies were conducted using a neutral red assay on the RPMI 2650 cells and histological studies on both models. **Results:** All the selected aloe materials enhanced the permeation of FITC-dextran 4400 to different extents. Similar permeation enhancement trends were found in the RPMI 2650 cell model and the excised sheep nasal tissue model. Histological and neutral red analysis showed that certain of the selected aloe leaf materials exhibited detrimental effects to some extent on the nasal epithelium, but not by A. muth-muth. **Conclusion:** This in vitro study showed for the first time the potential of A. muth-muth leaf materials to enhance nasal drug delivery without causing damaging effects on the epithelium.

Key words: Absorption enhancement; Aloe; Nasal delivery; Pharmacokinetics

Introduction

Pharmacokinetic modulation has been the focus of numerous research studies in recent years, especially research on drug-herb interactions. Substances responsible for the enhancement of pharmacokinetic profiles (i.e. bioenhancers) may be substrates, inhibitors and/or inducers of absorption, metabolism, distribution and excretion¹. Beneficial pharmacokinetic interactions, which may occur at any of these processes, can lead to increased co-administered drug bioavailability. This is especially applicable to drugs with unfavourable physico-chemical properties such as large molecular weight drugs that experience difficulty to diffuse across biological membranes, as well as drugs that exhibit a high affinity for efflux transporters or metabolic enzymes. Two medicinal plants with proven drug membrane permeation enhancing effects are *Aloe vera* and *A. ferox*. The gel and whole leaf extracts of *A. vera* have the ability to open tight junctions between adjacent epithelial cells and thereby increases drug permeation across the intestinal epithelium^{2,3}, buccal epithelium⁴ and the skin^{5,6}. Furthermore, there is also an indication that P-gp inhibition takes place in gastro-intestinal epithelium in the presence of precipitated polysaccharides from *A. vera* gel^{7,8}. Permeation enhancement in the presence of *A. ferox* leaf materials also took place across the intestinal epithelium⁹ and the skin⁶, while reducing trans-epithelial electrical resistance in excised rat intestinal tissue¹⁰. In the current study, leaf materials from a new plant hybrid, namely *A. muth-muth*, created through forced pollination between *A. vera* and *A. ferox*, was also investigated for the possibility of drug delivery enhancement effects.

So far, aloe leaf materials have not yet been evaluated for pharmacokinetic modulation of drug delivery via the nasal route. The nasal route of drug administration provides many advantages over other routes of administration, which may be further optimised by bioenhancers that can facilitate delivery of poorly bioavailable drugs. After nasal administration, drugs are delivered directly into the systemic circulation, thereby circumventing first-pass metabolism, it has a large surface area of approximately 150 cm² and has a high blood supply along with a leaky epithelial layer¹¹⁻¹³.

The RPMI 2650 cell line is the only indefinitely proliferating human nasal epithelial model currently commercially available. Although initially regarded to be unsuitable as a transport model when grown under liquid-covered conditions (LCC), further refinement of the growth procedures proved otherwise when the model was grown at the air-liquid interface (ALI). When grown under ALI conditions, the RPMI 2650 cell line presents as a uniform multi-layered cell formation that expresses tight-junctions, multi-drug resistance proteins and trans-epithelial resistance and permeability values similar to that of excised human nasal

mucosa^{11,14-16}. In contrast to *in vitro* cell models, excised animal models are much more affordable, relatively uncomplicated to deploy as a transport model and readily accessible. *Ex vivo* animal models, such as excised sheep nasal mucosa, have been used in nasal delivery studies due to the comparable absorption parameters, relative surface area and mucosal thickness as compared to humans^{17,18}.

The purpose of this study was to determine if the gel and whole leaf extract of selected aloe species namely *A. vera*, *A. ferox* and *A. muth-muth* have any drug permeation enhancement effects across RPMI 2650 nasal epithelial cell layers as well as across excised sheep nasal tissues.

Materials and methods

Plant material and extract obtaining

A. vera (*Aloe barbadensis* Mill.) gel and whole leaf extract were donated by Improve USA (De Soto, Texas, USA, authenticated by the International Aloe Science Council). *A. ferox* Mill. (= *Aloe candelabrum* A. Berger) gel and de-bittered fibre material were both donated by Organic Aloe (Pty) Ltd (Albertina, South Africa, endorsed by the South African Aloe Council). *A. ferox* whole leaf extract was constituted by mixing freeze-dried gel:de-bittered fibre (rind) in a 3:1 ratio¹⁹. *Aloe muth-muth* (*Aloe ferox* x *vera*) leaves were identified and generously provided by botanists Jaap and Hannes Viljoen from Rooiklip Nursery (-34.029293, 20.431906, Swellendam, South Africa) over a period of time and a voucher was deposited at the Mildred van der Merwe herbarium, North-West University, South Africa with accession number PUC0014886 (Collector: JNJ Viljoen 1). The fresh *A. muth-muth* leaves were processed according to a previously published method^{6,20}. In brief, the thorns were firstly stripped from the leaves. Thereafter the green rind was separated from the inner pulp and the yellow latex was swiftly rinsed from the gel fillets with distilled water. Both compounds were then finely processed and freeze-dried. The *A. muth-muth* whole leaf extract was also constituted in a 3:1 gel:rind ratio.

Chemicals and materials

FITC-dextran (MW = 4400, FD4, Cat. number: 60842-46-8), non-essential amino acids (NEAA) (Cat. number: M7145), neutral red (Cat. number: N2889), hematoxylin (Cat. number: GHS132), Eosin Y (Cat. number: HT110216) and Alcian Blue (Cat. number: B8438) were purchased from Sigma-Aldrich (Johannesburg, South Africa). KRB was prepared according to Sigma-Aldrich's product information sheet²¹. RPMI 2650 (nasal septum carcinoma) cells (Lot number: 60525161) were purchased from American Type Culture Collection (Manassas,

Virginia, USA). Phosphate buffered saline (PBS) (Cat. number: SH30256.01), Earle's minimum essential media (MEM) (Cat. number: 21090-022), fetal bovine serum (Cat. number: 10270-106), 0.25% w/v trypsin-EDTA (Cat. number: SV30031.01) were purchased from ThermoFischer (Johannesburg, South Africa) while L-glutamine (Cat. number: G7513) and Pen-Strep (Cat. number: DE17-602E) were purchased from Lonza (Johannesburg, South Africa). ThinCert™ 12-well plates (Cat. number: 665110) and polyethylene terephthalate coated inserts (Cat. number: 665641) with 0.4 µm pore size, $2 \times 10^6 \text{ cm}^{-2}$ pore density and 1.131 cm^2 culture surface were purchased from Greiner Bio-One (Frickenhausen, Germany). Sheep nasal tissue was sourced from animals slaughtered for meat purposes at a local abattoir (Potchefstroom, South Africa).

¹H-NMR analysis

Quantitative ¹H-NMR spectroscopic analysis was performed on all the aloe materials investigated in this study to quantify marker molecules including aloverose, glucose, malic acid and lactic acid. A Bruker Avance III HD 500 MHz with automated sample changer and BBFOPLUS SmartProbe was used with nicotinic acid as internal standard according to a previously published method²².

Fluorescence spectroscopic analysis

Fluorescence spectroscopic analysis was used to determine the FD4 concentration in the samples obtained from the permeation studies and the analyses were done using a Spectramax Paradigm® (San Jose, California, USA, Serial nr: 33270-1142) multi-mode detection platform plate reader. FD4 was analyzed with excitation and emission wavelengths set at 485 nm and 525 nm, respectively^{13,23}. The fluorescence spectroscopic analysis method for FD4 was validated in terms of linearity, accuracy, precision, limit of detection and limit of quantification.

RPMI 2650 cell culturing

RPMI 2650 cells were initially cultured in 75 cm² culture flasks with MEM fortified with 10% FBS, 1% NEAA, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine. As soon as confluency of approximately 90% was reached, flasks were washed twice with 10 ml PBS before being trypsinized in an incubator for 5 min with 0.25% (w/v) trypsin-EDTA. Following trypsinization, the cells were counted using a haemocytometer and seeded on the apical side of the 12-well plate membranes (ThinCert™) at a density of $6 \times 10^5 \text{ cells/cm}^2$. All cells used for seeding were between passages 25 and 47.

Following seeding of the cells into the 12-well plates, the cells were cultured for 2 days under LCC conditions and then cultured for a further 19 days at the air-liquid interface (ALI). During the first 2 days, LCC conditions included adding 0.8 mL growth medium to the apical side and 4 mL growth medium to the basolateral side. After 2 days, the growth medium on the apical side was removed by aspiration, while the medium changes on the basolateral side continued every second day. Following the ALI growth phase, the inserts with the cell layers were transferred to 12-well transport plates in which the permeation studies were conducted.

All the cell flasks and plates were kept in an incubator at 37°C humidified atmosphere containing 5% CO₂ throughout the cell culturing period, while media was changed every second day^{11,15,24}.

Sheep nasal mucosa excision

Immediately following slaughter of a sheep, the snout of the animal was removed by a cross sectional incision just anterior to the eyes. The skin was removed and the tissue sample was rinsed, submerged and transported to the laboratory using ice-cold KRB. The snout was bisected via a vertical, longitudinal incision along the septal midline. The septum was completely removed and discarded. From each half, the conchae were isolated by a vertical incision through the lateral wall of the nasal cavity, just posterior to the *incisura nasoincisiva*. The epithelial tissue sheets were then removed from the surface of the cartilage via blunt dissection. Strips of approximately 1 cm in width were cut from the nasal epithelial tissue sheets and mounted in Sweetana-Grass diffusion chambers for permeation studies^{17,25-27}.

Permeation studies

In both models (i.e. RPMI 2650 cells at ALI and excised sheep nasal tissue), FD4 (500 µg/mL) dissolved in KRB was used as a marker molecule for paracellular transport. Permeation studies were done in the absorptive direction only (i.e. unidirectional). During the FD4 absorptive permeation studies in the RPMI 2650 cell model, 0.8 mL of marker compound solution (in the presence and absence of different aloe materials) was added to the apical side of the membrane and 1.8 mL KRB was added to the basolateral side. In the excised tissue model, the same method was followed, but volumes of 7 mL were used in both the apical and basolateral chambers.

The RPMI 2650 cell layers (37°C) and the excised nasal epithelial tissues (34°C) were acclimatised in pre-heated KRB for 15 min before permeation studies commenced. All aloe gel and whole leaf extracts were tested in experimental solutions at concentrations of 0.5, 1.5 and 3% w/v, respectively in triplicate. Following the application of the experimental

solutions to the donor chambers, samples (180 μL) were withdrawn from the acceptor chambers at time intervals of 5, 10, 15, 30, 60, 90, 120 and 180 min, replaced with equal volumes of pre-heated KRB and analysed for the presence of marker compound using fluorescent spectroscopy as described above.

Data analysis

Following fluorescence spectroscopic analysis of the samples obtained from the permeation studies, the data were corrected for dilution and the percentage transport for each experimental solution was calculated²⁸ with equation 1.

$$\text{Transport} = \frac{\text{Concentration at specific time}}{\text{Concentration of donor solution}} \times 100 \quad (1)$$

The apparent permeability (P_{app}) coefficient values of each marker compound were calculated using equation 2^{11,28,29}.

$$P_{\text{app}} = \frac{dQ}{dt} \left(\frac{1}{A \cdot 60 \cdot C_0} \right) \quad (2)$$

Where P_{app} is the apparent permeability coefficient ($\text{cm} \cdot \text{s}^{-1}$), $\frac{dQ}{dt}$ is the permeability rate per minute, A is the diffusion area of the membrane (cm^2) and C_0 is the initial concentration of each marker compound used.

In vitro cytotoxicity and histology

Neutral red viability assay

Cell viability of the RPMI 2650 cells when exposed to the aloe materials investigated in this study was determined using a neutral red assay. RPMI 2650 cells were seeded at 250 000 cells/ cm^2 in 96 well-plates. After a 24 h recovery period allowing cells to attach to the plates and reach confluency of 80%, the cells were treated with different aloe materials at a range of concentrations (0.25 to 6% w/v) in KRB with 10% FBS supplementation for 3 h. Since many plant extracts contain compounds with intrinsic reductive potential such as antioxidants, the neutral red assay was performed instead of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay to avoid false positive results³⁰.

For the neutral red assay, the aloe material containing experimental solutions were removed after the 3 h exposure time and the cells were washed three times with 200 μL KRB. Following the washing step, a volume of 100 μL of a 0.033% neutral red solution in KRB was added to each well and the plate was placed back in the incubator. After a further 2 h of

incubation, the medium was removed and the cells were washed with 100 μL of a fixation solution (1% CaCl_2 in 0.5% formaldehyde). Then, a volume of 150 μL of a 1% v/v acetic acid in 50% v/v ethanol solution was added to each well for the solubilisation of the neutral red dye. The plates were protected from light and gently shaken for 10 min for complete dissolution of the dye. The neutral red absorbance of each well treated with aloe was measured at 540 nm with a SpectraMax[®] plate reader, after which the background noise was subtracted following measurement at a wavelength of 690 nm. The percentage cell viability was determined using Equation 3.

$$\text{Cell viability \%} = \frac{(\Delta\text{Sample} - \Delta\text{Solubilisation blank})}{(\Delta\text{Untreated control} - \Delta\text{Solubilisation blank})} \times 100 \quad (3)$$

Histology

Histological analysis of the RPMI 2650 cell layers and excised nasal epithelial tissues after exposure to the highest concentration (i.e. 3% w/v) aloe materials investigated in this study, were conducted. Excised sheep nasal tissue samples before the permeation study (intact control) and samples at the end of each 3 h permeation study with 3% (w/v) of each aloe material were fixated in 10% buffered formalin at 4°C for 24 h. RPMI 2650 cell layers before the permeation study (intact control) and at the end of each 3 h permeation study with 3% (w/v) aloe materials were washed with PBS and fixated in 10% buffered formalin at room temperature for 1 h. The tissue and cell samples were subsequently dehydrated in a graded series of ethanol solutions and thereafter embedded in paraffin wax. Embedded samples were sliced into 4 – 5 μm sections that were stained with hematoxylin-eosin and 1% Alcian Blue. After staining, the sections were examined under a Nikon E800 compound microscope using the 60 $\times$ objective. The sheep nasal epithelium and RPMI 2650 cells' height and nuclei area in the cells were measured from five different slices per sample using NIS-Elements, Version 4.05 software.

Viscosity

The viscosity of all the aloe experimental solutions (including the gel and whole-leaf extract materials of all the selected species) was determined at three concentrations (i.e. 0.5, 1.5 and 3% w/v) using a TA Instruments ARES-G2 (New Castle, Delaware, USA, Serial number: 4010-0677) viscometer. A stainless steel 40 mm parallel plate geometry was used with gap width set at 1 mm. These parameters resulted in the use of a minimum volume of 1.26 ml sample. An oscillatory mode was used at 37°C with the logarithmic shear rate ranging from 10 to 100 rad/s^{12} . With pseudoplastic flow, the rheogram follows a straight line at higher

shear rates, thus a minimum viscosity value can be attained from the slope of the straight line between 0 and 5 rad/s ($r^2 \geq 0.990$) on the graph³¹.

Statistical analysis

Statistical data analysis of the P_{app} values and histological parameters for all test groups in both models was performed with software from RStudio: Integrated development environment for R (Version 1.1.453) software (RStudio, Inc., Boston, MA). The analysis was done by means of non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test for comparison of multiple groups. Differences were considered statistically significant when $p < 0.05$.

Ethics approval

The use of nasal epithelial tissue obtained from sheep slaughtered at an abattoir was approved by the animal ethics committee (AnimCare) of the North-West University as a category 0 project (i.e. use of biological samples of animals that are already dead for *in vitro* experimentation) (ethics approval certificate number: NWU-00285-17-A5).

Results

¹H-NMR analysis results

The chemical composition of the *A. muth-muth* gel and whole leaf extracts in terms of quantities (% dry weight) of selected marker compounds obtained from quantitative ¹H-NMR analysis are summarized in Table 1. The chemical composition of the *A. ferox* and *A. vera* gel and whole leaf extracts as determined by the same quantitative ¹H-NMR analysis method have already been published previously^{6,10}.

Table 1. Percentage content of selected marker molecules in *Aloe muth-muth* gel and whole leaf extracts as determined by ^1H -NMR spectroscopy.

	Percentage content	
	<i>A. muth-muth</i> gel	<i>A. muth-muth</i> whole-leaf extract
Aloverose	10.4	9.3
Glucose	9.1	8.4
Malic acid	18.3	16
Citric acid	Not detected	2.2
Whole leaf marker	Not detected	3.0

The *A. muth-muth* gel material contained all the marker compounds (i.e. aloverose, glucose and malic acid) commonly used for the identification of *A. vera* gel material. Aloverose (also known as acetylated polymannose or acemannan) is one of the major bioactive compounds in *A. vera* responsible for certain medicinal properties of this plant^{10,32}. The aloverose and malic acid contents were found to be similar in *A. muth-muth* gel compared to that in *A. vera* gel (10.4% vs 12.7% for aloverose and 18.3% vs 20.0% for malic acid, respectively). Interestingly, the whole-leaf material extract of *A. muth-muth* contained higher quantities of aloverose and malic acid than *A. vera* whole leaf extract (9.3% vs 5.5% for aloverose and 16% vs 1.2% for malic acid) [2,10].

Fluorescence spectroscopic verification

All the validation results are summarised in Table 2 and this analytical method complied with the limits set for the different validation parameters.

Table 2. Fluorescent spectroscopic validation results for FITC-dextran 4400.

	FITC-dextran 4400		
Linearity (r^2)	0.999		
Limit of detection ($\mu\text{g/ml}$)	0.005		
Limit of quantification ($\mu\text{g/ml}$)	0.016		
	125 $\mu\text{g/ml}$	62.5 $\mu\text{g/ml}$	12.5 $\mu\text{g/ml}$
Accuracy (%)	95.77	98.49	104.67
Intra-day precision (% RSD)	2.77	2.75	2.53
Inter-day precision (% RSD)	2.89	3.08	2.51

Permeation studies**FITC-dextran permeation across RPMI 2650 nasal epithelial cell layers**

The apparent permeability coefficient (P_{app}) values of FD4 in the absence (control) and in the presence of three concentrations (0.5%, 1.5% and 3% w/v) of the selected aloe gel materials across RPMI 2650 nasal epithelial cell layers are shown in Figure 1.

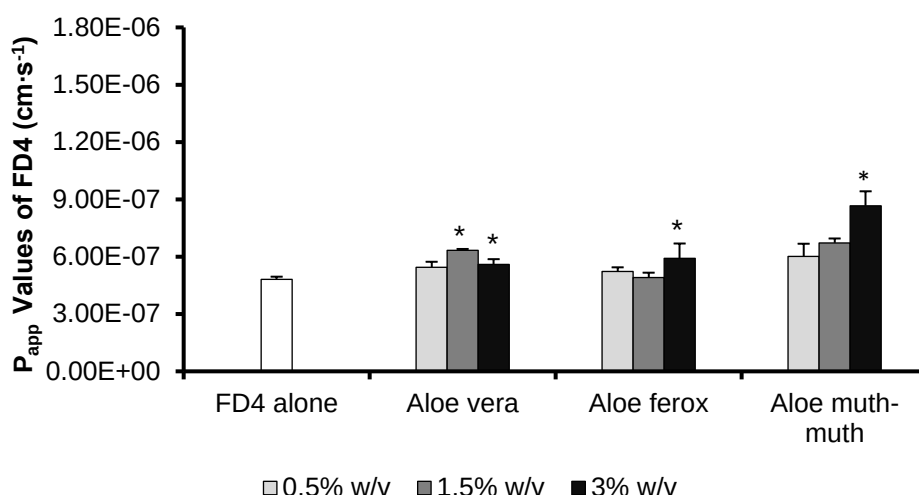


Figure 1. Apparent permeability coefficient (P_{app}) values of FITC-dextran 4400 (FD4) alone (control) and in the presence of different concentrations of *Aloe vera*, *A. ferox* and *A. muth-muth* gel across RPMI 2650 nasal epithelial cell layers in the absorptive direction (* indicates $p \leq 0.05$, Kruskal-Wallis tests followed by Dunn's post-hoc test)

A. vera gel increased FD4 permeation across the RPMI 2650 cell layers at all three concentrations tested, with the two highest concentrations' permeation being statistically significantly ($p < 0.05$) higher than that of the control. However, it is simultaneously found that the permeation of FD4 in the presence of 3% w/v *A. vera* gel is slightly lower than that of the 1.5% w/v concentration. *A. ferox* gel mediated a slight increase in FD4 permeation across the RPMI 2650 cell layers, presenting with an inverse-bell curve across the concentration range. However, only the 3% w/v suspension indicated a statistically significant increase in permeation. *A. muth-muth* gel caused a pronounced increase in FD4 permeation across the RPMI 2650 nasal epithelial cell layers in a concentration dependent manner and the highest concentration (3% w/v) applied caused a statistically significant increase in the P_{app} of FD4, which was also much higher than those of *A. ferox* and *A. vera* gel.

The P_{app} values of FD4 in the presence of three concentrations (0.5%, 1.5% and 3% w/v) of the selected aloe whole leaf extracts are shown in Figure 2.

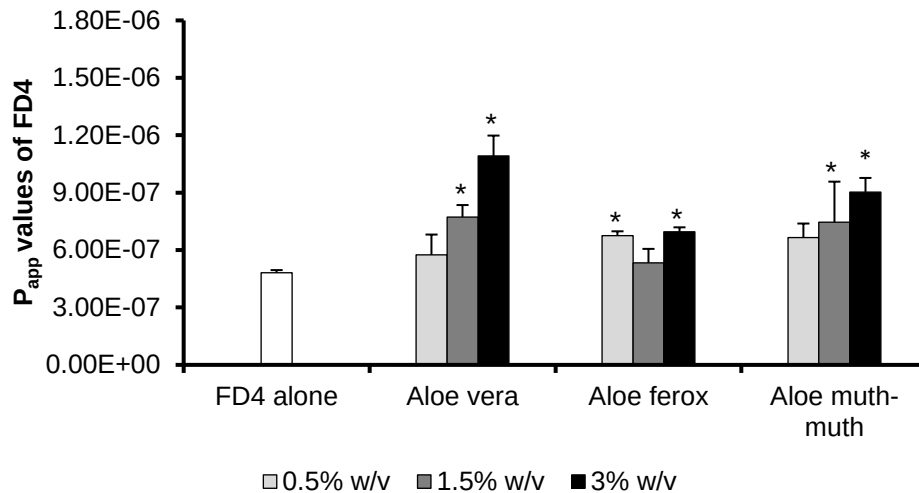


Figure 2. Apparent permeability coefficient (P_{app}) values of FITC-dextran (FD4) alone (control) and in the presence of different concentrations of *Aloe vera*, *A. ferox* and *A. muth-muth* whole leaf extract across RPMI 2650 nasal epithelial cell layers in the absorptive direction (* indicates $p \leq 0.05$, Kruskal-Wallis tests followed by Dunn's post-hoc test)

Both *A. vera* and *A. muth-muth* whole leaf extract mediated a concentration dependent increase in FD4 permeation across the RPMI 2650 nasal epithelial cell layers, with both species causing a statistically significant difference in FD4 P_{app} values from that of the control ($p < 0.05$) at the two highest concentrations applied (1.5% and 3% w/v). Interestingly, *A. ferox* whole leaf extract indicated that statistically significant increases in permeation were found at the lowest and highest concentrations, presenting an inverse bell-shaped permeation pattern similar to that found with the *A. ferox* gel material.

FITC-dextran permeation across excised sheep nasal epithelial tissues

The P_{app} values of FD4 in the absence (control) and in the presence of three concentrations (0.5%, 1.5% and 3% w/v) of the selected aloe gel materials across excised sheep nasal epithelial tissues are shown in Figure 3, while the P_{app} values of FD4 in the presence of three concentrations (0.5%, 1.5% and 3% w/v) of the selected aloe whole leaf extract are shown in Figure 4.

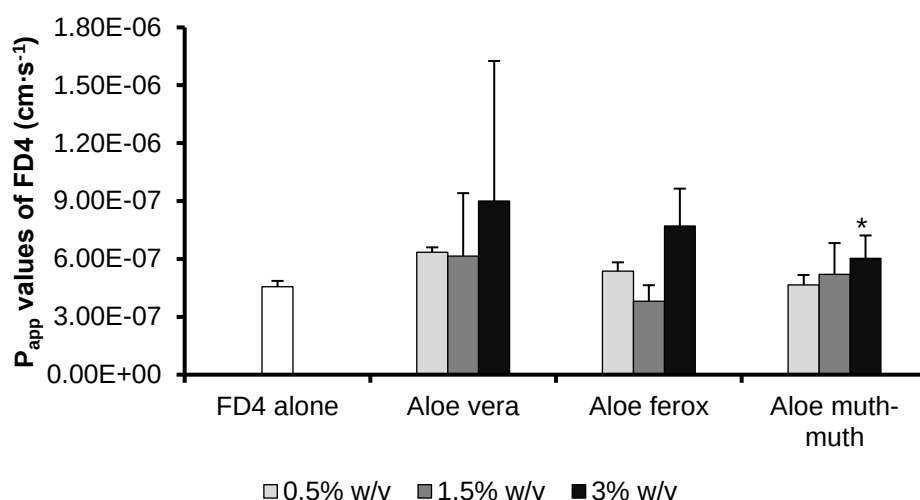


Figure 3. Apparent permeability coefficient (P_{app}) values of FITC-dextran (FD4) alone (control) and in the presence of different concentrations of *Aloe vera*, *A. ferox* and *A. muth-muth* gel across excised sheep nasal epithelial tissues in the absorptive direction (* indicates $p \leq 0.05$, Kruskal-Wallis tests followed by Dunn's post-hoc test)

In Figure 3 one can see that *A. vera* gel caused a more pronounced increase in FD4 permeation across excised sheep nasal mucosa as compared to the RPMI 2650 cell model. In this excised tissue model, a concentration dependent increase is apparent. The *A. ferox* gel showed a permeation enhancement effect on FD4 in the same inverse bell-shaped curve as found with the RPMI 2650 cell model, although in the excised tissue model no statistical significant differences were found. The *A. muth-muth* gel exhibited a concentration dependent increase in FD4 permeation across the excised sheep nasal epithelial tissue similar to the results obtained in the RPMI 2650 cell layer model.

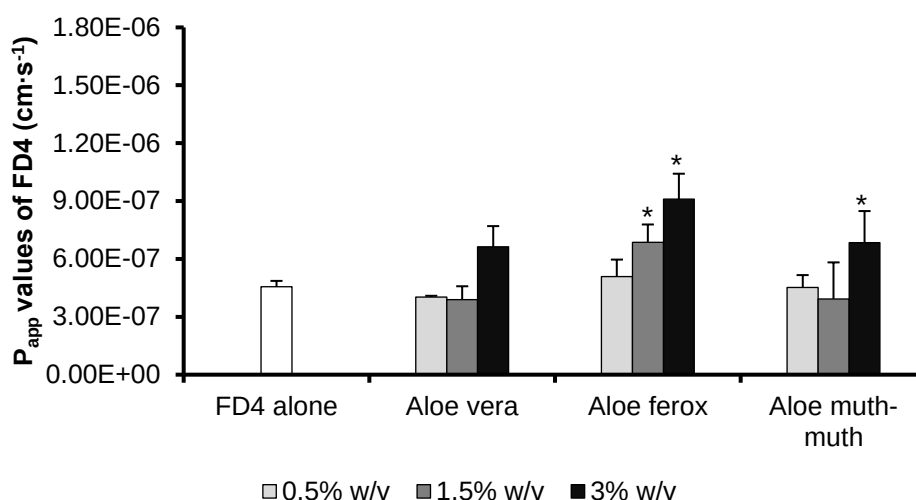


Figure 4. Apparent permeability coefficient (P_{app}) values of FITC-dextran (FD4) alone (control) and in the presence of different concentrations of *Aloe vera*, *A. ferox* and *A. muth-muth* whole leaf extract across excised sheep nasal epithelial tissue in the absorptive direction (* indicates $p \leq 0.05$, Kruskal-Wallis tests followed by Dunn's post-hoc test)

In general, the whole leaf extracts from all three the aloe species investigated in this study caused an increase in FD4 permeation across the excised sheep nasal epithelial tissues in a concentration dependent manner. In addition, similar trends in absorption enhancement are seen in Figure 4 for *A. vera* and *A. muth-muth* than was seen in the RPMI 2650 cell model (Figure 2). However, with the excised sheep nasal mucosa model only *A. ferox* at the two highest concentrations and *A. muth-muth* at the highest concentration presented with statistically significant increases in FD4 permeation when compared to that of the control.

Although this study showed the drug absorption enhancement potential of the gel and whole leaf extracts from selected aloe species across the nasal epithelium in two different models, it was important to determine if they caused any cytotoxic or damaging effects on the nasal epithelium.

***In vitro* cytotoxicity**

***Aloe vera* gel and whole leaf extract**

The percentage cell viability of RPMI 2650 cells after exposure to *A. vera* gel and whole leaf extract as a function of concentration is shown in Figure 5.

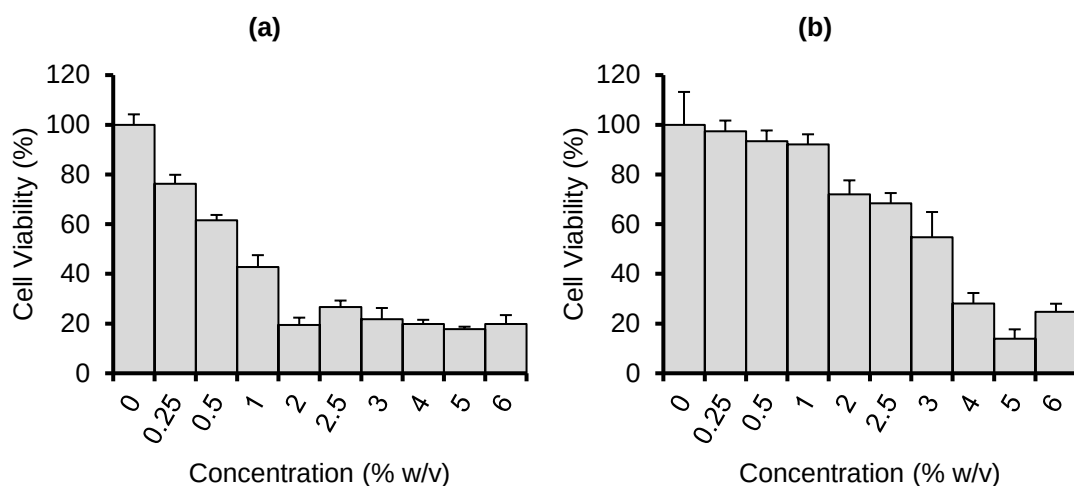


Figure 5. Percentage cell viability as determined with a neutral red assay on RPMI 2650 cells after 3 hour exposure to a range of *Aloe vera* gel (a) and whole leaf extract (b) concentrations

According to a previously published classification system, a substance can be considered as non-cytotoxic if cell viability remains above 80%, weakly-cytotoxic if cell viability ranges from 60% to 80%, moderately cytotoxic between 40% and 60% and strong or severe cytotoxicity is indicated by cell viability below 40%³⁴. Figure 5 indicates that *A. vera* gel was weak to moderate cytotoxic at concentrations up to 1% w/v and severely cytotoxic at concentrations higher than 2% w/v, while *A. vera* whole leaf extract was non-cytotoxic up to concentrations of 1% w/v and only moderately cytotoxic up to a concentration of 3% w/v.

***Aloe ferox* gel and whole leaf extract**

The percentage cell viability of RPMI 2650 cells after exposure to *A. ferox* gel and whole leaf extract as a function of concentration is shown in Figure 6.

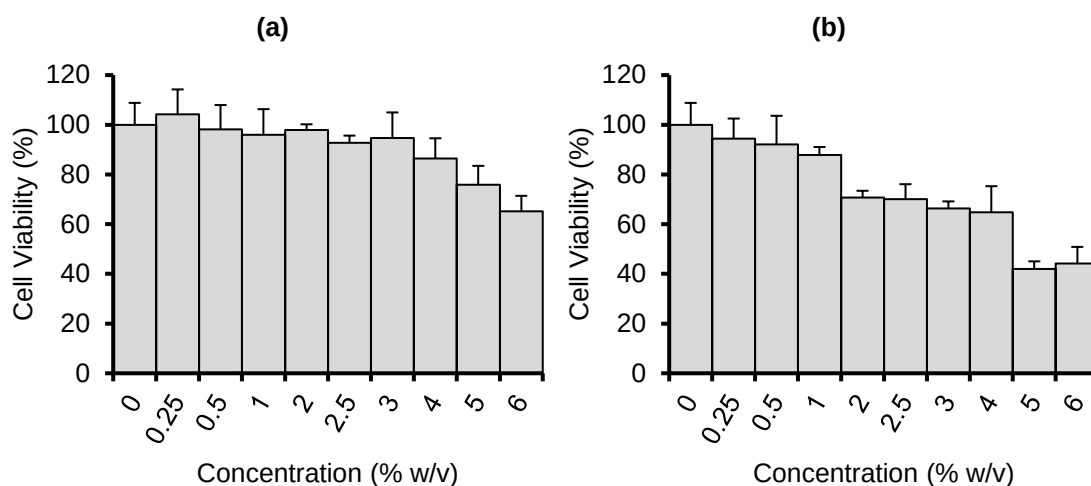


Figure 6. Percentage cell viability as determined with a neutral red assay on RPMI 2650 cells after 3 hour exposure to a range of *Aloe ferox* gel (a) and whole leaf extract (b) concentrations

A. ferox gel showed no to weak cytotoxic effects for all the concentrations tested (i.e. up to 6% w/v, Figure 6), whereas *A. ferox* whole leaf extract exhibited no to weak cytotoxicity up to a concentration of 4% w/v and moderate cytotoxic effects at concentrations between 5 and 6% w/v.

***Aloe muth-muth* gel and whole leaf extract**

The percentage cell viability of RPMI 2650 cells after exposure to *A. muth-muth* gel and whole leaf extract as a function of concentration is shown in Figure 7.

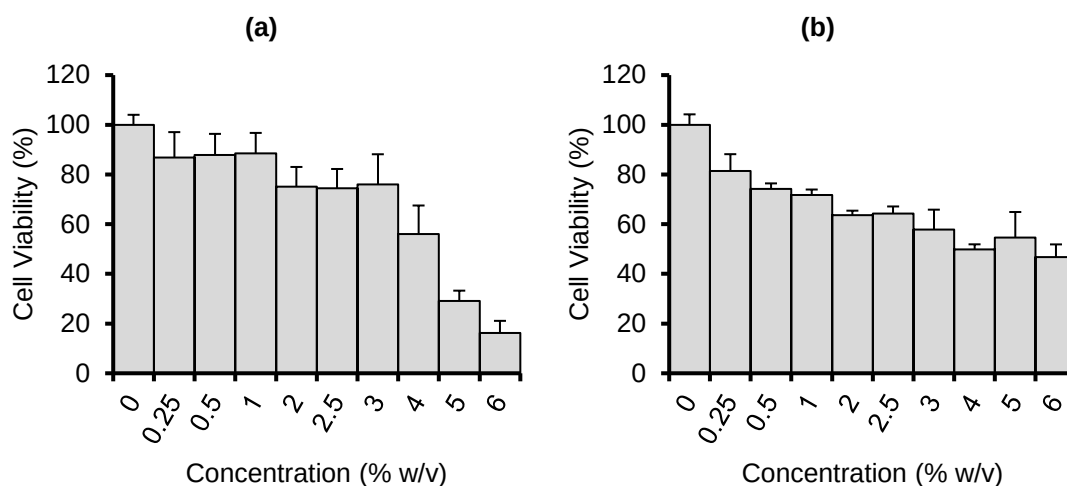


Figure 7. Percentage cell viability as determined with a neutral red assay on RPMI 2650 cells after 3 hour exposure to a range of *Aloe muth-muth* gel (a) and whole leaf extract (b) concentrations

A. muth-muth gel showed no cytotoxic effects up to 1% w/v, moderately cytotoxic effects up to 4% w/v and was strongly cytotoxic at 5 and 6% w/v (Figure 7). *A. muth-muth* whole leaf extract was only moderately cytotoxic up to the highest concentration tested, namely 6% w/v.

Histology

Histological analyses of the RPMI 2650 cell layers as well as the excised sheep nasal epithelial tissues were conducted in the absence and presence of the selected aloe materials and the micrographs are shown in Figures 8 and 9, respectively. Histological parameters of the RPMI 2650 cell layers as well as excised nasal epithelial tissues are shown in Table 3.

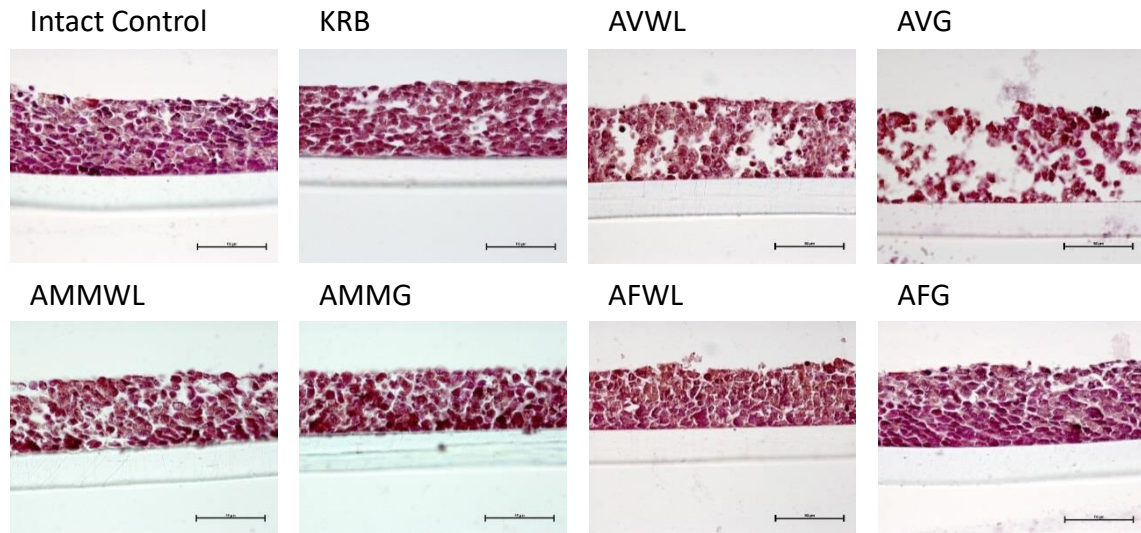


Figure 8. Representative micrographs of RPMI 2650 cell layers grown with the air-liquid interface technique before and after 180 min exposures in the permeability study. Intact control: RPMI 2650 cell layer before the permeation experiment started. KRB: after exposure to Krebs-Ringer Bicarbonate buffer only. AVWL: after exposure to 3% w/v *A. vera* whole leaf extract. AVG: after exposure to 3% w/v *A. vera* gel. AMMWL: after exposure to 3% w/v *A. muth-muth* whole leaf extract. AMMG: after exposure to 3% w/v *Aloe muth-muth* gel. AFWL: after exposure to 3% w/v *Aloe ferox* whole leaf extract. AFG: after exposure to 3% w/v *Aloe ferox* gel. Scale bar = 50 μ m

From Figure 8, it is clear that the RPMI 2650 cells cultured under ALI conditions developed multi-layered epithelia on the insert support filter membranes without differentiation into different types of cells as visible in the excised sheep nasal epithelial tissue (Figure 9). Morphological changes, which may indicate cell damage, can be observed on the micrographs of the RPMI 2650 cell layers exposed to *A. vera* gel and whole leaf extract. Sinusoidal gaps appeared within the RPMI 2650 cell layer after treatment with *A. vera* gel and whole leaf extract. The RPMI 2650 cell layer thickness did not change after treatment with *A. vera* whole leaf extract, but it increased ($p < 0.01$) after treatment with *A. vera* gel. On the other hand, the excised sheep nasal epithelial tissue that was exposed to *A. vera* gel exhibited a reduction in thickness (Table 2). This may be explained by the differences in the morphology of the two models, whereby excised sheep nasal epithelial tissue consists of a pseudostratified monolayer, while the multi layered *in vitro* RPMI 2650 cell model provides a network of cells that collectively allow some adhesion for detached cells and thereby expands the overall thickness of the cell layers. The widening of intercellular spaces was characteristic of cell layers exposed to both *A. muth-muth* whole leaf extract and gel, but

without concurrent increase of epithelial thickness. A slight reduction in epithelial thickness was observed after treatment with *A. ferox* whole leaf extract. The RPMI 2650 cell layers exposed to Krebs-Ringer Bicarbonate buffer (KRB) and *A. ferox* gel were morphologically comparable with that of the intact control without any signs of cell disruption.

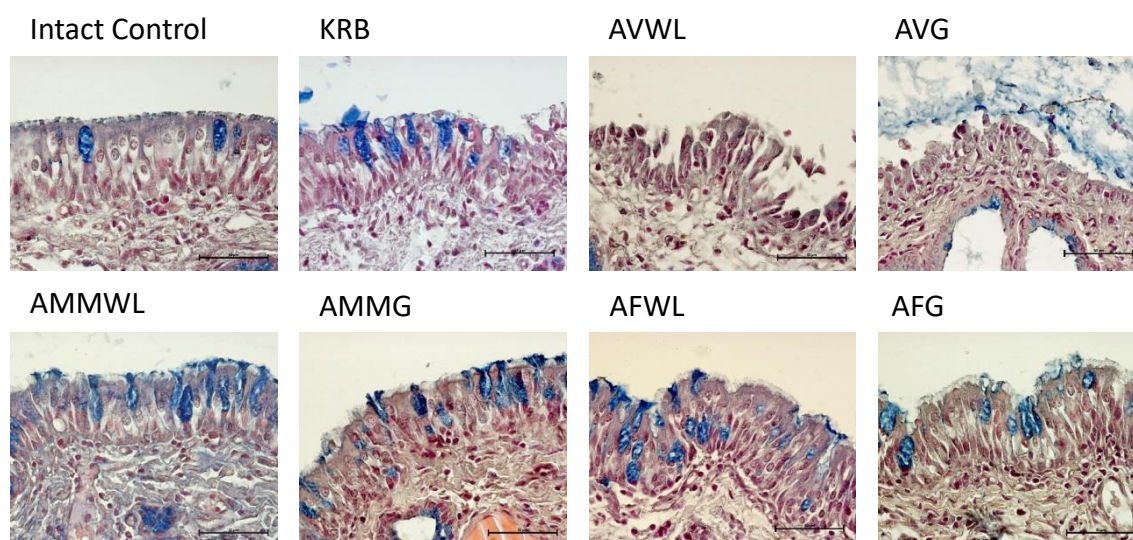


Figure 9. Representative micrographs of excised sheep nasal mucosa before and after 180 min exposures in the permeability study. Intact control: Excised sheep nasal mucosa before the permeation experiment started. KRB: after exposure to Krebs-Ringer Bicarbonate buffer only. AVWL: after exposure to 3% w/v *A. vera* whole leaf extract. AVG: after exposure to 3% w/v *A. vera* gel. AMMWL: after exposure to 3% w/v *A. muth-muth* whole leaf extract. AMMG: after exposure to 3% w/v *Aloe muth-muth* gel. AFWL: after exposure to 3% w/v *Aloe ferox* whole leaf extract. AFG: after exposure to 3% w/v *Aloe ferox* gel. Scale bar = 50 μ m

The excised sheep nasal epithelial tissues consist of pseudostratified ciliated columnar epithelial cells with interspaced mucus producing goblet cells (Figure 9). There is no visible sign of tissue alteration of the excised nasal epithelial tissues after exposure to KRB, *A. ferox* gel and whole leaf extract, *A. muth-muth* gel and whole leaf extract when compared to the intact control group. However, the excised epithelial tissue exposed to *A. vera* whole leaf extract has a heterogeneous appearance consisting of some loosely connected as well as detached epithelial cells. This effect is even more pronounced after exposure to *A. vera* gel with only the germ layer of the epithelial cells remaining intact. The epithelial thickness and average epitheliocyte nucleus area were significantly reduced ($p < 0.001$) compared to the control group (Table 3) after exposure to the *A. vera* gel and whole leaf extract materials.

Table 3. Parameters of epithelia of two different nasal tissue models. (Data represented as mean $\pm$ SD, Kruskal-Wallis test followed by Dunn's post-hoc test. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$).

	Excised sheep tissue		RPMI 2650 cell line	
	Epithelia thickness (μm)	Average epitheliocyte's nucleus area (μm^2)	Epithelia thickness (μm)	Average epitheliocyte nuclei area, (μm^2)
Intact control	52.9 $\pm$ 4.4	36.1 $\pm$ 6.6	53.3 $\pm$ 8.1	14.3 $\pm$ 7.2
<i>A. vera</i> gel 3%	31.1 $\pm$ 20.2 ***	24.0 $\pm$ 8.5 ***	61.6 $\pm$ 10.6 **	13.3 $\pm$ 5.7
<i>A. vera</i> whole leaf extract 3%	32.8 $\pm$ 15.8 ***	18.2 $\pm$ 6.8 ***	50.1 $\pm$ 3.1	15.9 $\pm$ 5.6
<i>A. ferox</i> gel 3%	51.3 $\pm$ 15.3	36.2 $\pm$ 7.2	54.2 $\pm$ 9.9	15.2 $\pm$ 4.6
<i>A. ferox</i> whole leaf extract 3%	55.4 $\pm$ 8.3	31.7 $\pm$ 7.6	47.6 $\pm$ 4.6 *	15.9 $\pm$ 5.2
<i>A. muth-muth</i> gel 3%	48.0 $\pm$ 11.6	34.2 $\pm$ 8.8	53.4 $\pm$ 5.1	15.7 $\pm$ 5.1
<i>A. muth-muth</i> whole leaf extract 3%	47.1 $\pm$ 9.7	36.0 $\pm$ 10.9	51.1 $\pm$ 6.2	15.4 $\pm$ 5.4

Viscosity

All of the aloe materials that were tested exhibited pseudoplastic flow behaviour in both the gel and whole leaf extract form, which is expected of aqueous suspensions of natural hydrocolloids³¹. The viscosity values of the selected aloe gel and whole leaf extracts in the state that it was used are shown in Table 4.

Table 4. Viscosities ($\text{Pa}\cdot\text{s}^{-1}$) of the selected aloe gel and whole leaf extracts at different concentrations in Krebs-Ringer Bicarbonate buffer.

		Concentration		
		0.5% w/v	1.5% w/v	3% w/v
Viscosity ($\times 10^{-3} \text{ Pa}\cdot\text{s}^{-1}$)	<i>A. vera</i> gel	1.83	5.57	18.09
	<i>A. vera</i> whole leaf extract	0.56	1.12	1.69
	<i>A. ferox</i> gel	1.16	0.96	1.17
	<i>A. ferox</i> whole leaf extract	0.83	1.73	6.03
	<i>A. muth-muth</i> gel	3.43	4.29	14.17
	<i>A. muth-muth</i> whole leaf extract	5.32	3.92	13.11
	KRB	0.00		

The viscosity values in Table 4 indicate that there is dramatic increase in viscosity with an increase in concentration of the *A. vera* gel, *A. ferox* whole leaf extract and *A. muth-muth* gel and whole leaf extracts. Viscosity of *A. vera* whole leaf extract and *A. ferox* gel remained relatively low over all three concentrations, while the viscosity of the solvent (i.e. KRB) remained virtually undetected under the experimental parameters.

Discussion

Although a slight decreased FD4 P_{app} value was found at the highest concentration *A. vera* gel (i.e. at 3.0% w/v) compared to that of the 1.5% w/v concentration during permeation across the RPMI 2650 cell layers, the permeation trends are generally in coherence with previous results where *A. vera* gel increased the transport of different molecules in a concentration dependent manner across other epithelial membranes including intestinal and buccal mucosal surfaces^{2,4}. The slight decrease in FD4 permeation across the RPMI 2650 nasal epithelial cell layers observed at the highest concentration of *A. vera* gel can possibly be explained by the relatively high viscosity of the *A. vera* gel at this concentration. The relatively high viscosity at the highest *A. vera* gel concentration has probably hampered FD4 diffusion. Previous findings also supported this explanation, since decreased percentage ketoprofen permeation across a synthetic membrane was evident with an increase in *A. vera*

gel concentration⁶. Another potential reason for decreased FD4 permeation may include blocking of the intercellular spaces by aloe components (e.g. large polysaccharides) at higher concentrations or chemical interaction (e.g. complex formation) between aloe components and the FD4. Furthermore, the lack of aloverose in *A. ferox* gel^{6,10} may be the reason for its relatively small effect on FD4 permeation across the RPMI 2650 cell line. As mentioned before, aloverose is one of the bioactive constituents in *A. vera* gel³² that may be predominantly responsible for modulation of tight junctions. These results for *A. ferox* gel across the nasal epithelial cell layers are similar to the results previously found across excised rat intestinal tissues¹⁰ and excised human skin tissues⁶. Since FD4 is a macromolecular compound that is not normally transported by transcellular diffusion, the increases in permeation of FD4 that was observed in the presence of aloe materials indicated enhancement of paracellular movement possibly due to tight junction modulation. Tight junction modulation in intestinal epithelial cell monolayers was previously shown for *A. vera* gel³³. The higher effect of the *A. muth-muth* on FD4 permeation compared to the other aloe gel materials can possibly be related to the higher aloverose content as compared to that of *A. vera* gel and *A. ferox* gel, but this requires more research to be conclusive.

With regards to the aloe whole leaf extracts, the results found in the RPMI 2650 cell model are in line with previously published data on *A. vera* that increased paracellular permeation of insulin in a concentration dependent manner across human intestinal epithelial cell monolayers *in vitro*². *A. vera* whole leaf material showed in general the highest effect on FD4 permeation across the RPMI 2650 epithelial cell layers.

The considerable higher increase in FD4 permeation obtained in the excised sheep nasal mucosa as compared to the RPMI 2650 cell line for *A. vera* gel may be attributed to the differences in these two models. The RPMI 2650 cell line grows in multi-layers, whereas the sheep nasal mucosa has a single layer of epithelial cells (also indicated in the histology results above). A higher effect was observed in the excised tissue model with a single layer of epithelial cells.

The cytotoxic effects of *A. vera* gel that were found *in vitro* are similar to those previously found in Caco-2 cells⁸, and in HeLa, HepG2 and SY-5Y cells³⁵. One explanation for the higher cytotoxic nature of the *A. vera* gel as compared to the *A. vera* whole leaf extract is the higher viscosity of the gel suspensions. The higher viscosity suspensions may have influenced the oxygen exchange and diffusion through the growth medium and prevented the cells from receiving sufficient quantities of oxygen. The *in vitro* results indicated that *A. muth-muth* leaf materials are safe to be used at concentrations where they are effective as

functional excipients and/or bioenhancers, and that their increases on FD4 permeation (Figures 1 & 2) can be credited to permeation enhancing effects and not tissue damage.

Conclusions

This study is one of the first to show the drug absorption enhancement potential of the gel and whole leaf extracts from different selected aloe species across the nasal epithelium.

In general, *A. vera* gel increased paracellular permeation of a macromolecular compound across nasal epithelial models in a concentration dependent manner, which is in coherence to most previous findings across other mucosal surfaces (e.g. intestinal epithelium). However, the high viscosity of *A. vera* gel may have contributed to delayed permeation time, physical blockade of intercellular spaces and/or chemical interactions may also have occurred between phytochemicals in the *A. vera* gel and the permeate, which would explain the slight reduction in FD4 permeation in the RPMI 2650 cell model at the highest concentration as well as relatively high degree of deviation in the sheep nasal mucosa. *A. vera* whole leaf extract, on the other hand, had mediated a significant increase in paracellular transport, while both *A. ferox* gel and whole leaf extract showed enhancement of paracellular permeation in an inverse bell-shaped curve. Further studies would be needed with an extended concentration range to determine the trend of the permeation as a function of concentration.

Aloe muth-muth, a hybrid aloe species cultivated through forced pollination between *A. vera* and *A. ferox*, was also tested for its ability to enhance drug permeation across nasal epithelial membranes. Both *A. muth-muth* gel and whole leaf extract increased paracellular permeation of FD4 in a concentration dependent manner. The cytotoxicity of the *A. muth-muth* gel and whole leaf extract materials was mild and the neutral red experimental results were supported by the histological results, which indicated an absence of tissue damage.

The experimental results showed a relatively high potential for use of *A. muth-muth* gel and/or whole leaf extract in nasal dosage forms as functional excipients to enhance the bioavailability of certain drugs via tight junction modulation, but this should first be investigated with *in vivo* models to determine the clinical significance of their absorption enhancing effects. The experimental results also indicated that *A. muth-muth* gel or whole leaf extract have no damaging effects on the excised epithelial tissues.

Conflict of interests

The authors declare no conflict of interest.

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CHAPTER 6

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

1 Final conclusions

The nasal route of drug delivery has often been overlooked when developing new drug formulations, even though it is an alternative route of drug administration with numerous exploitable benefits. The relatively large mucosal surface area together with a relatively leaky epithelial layer and high vascularity provides the opportunity to deliver, amongst others, large molecular compounds that are otherwise destined for parenteral delivery (Johnson & Quay, 2005; Kim, 2008). An important, but often neglected aspect of drug delivery is the potential of pharmacokinetic interactions of various compounds with co-administered drugs. Although such interactions with drug compounds are sometimes unwanted or detrimental to the patient, the possibility also exists to use pharmacokinetic interactions to the advantage of the patient (Edwards, 2012).

In the current contribution, the work intended to show that herb-drug interactions can also be used to the pharmaceutical advantage of patients by increasing the absorption of active pharmaceutical ingredients. Such pharmacokinetic interactions include opening of tight junctions, inhibition of efflux proteins, ion-transporters and metabolism in different regions of the body, but also induction of ion-transporters and metabolism to be used in drugs with pharmaceutically active metabolites. This was accomplished by publication of a peer-reviewed literature review article in the journal "*Pharmaceutics*" (Gerber *et al.*, 2018).

Based on the literature review that pharmacokinetic interactions can be advantageously employed to increase drug bioavailability, a collection of natural extracts/compounds was selected to elucidate the pharmacokinetic effects they may exert on large molecular compounds (mimicked by FITC-dextran 4400 (FD4)) and on efflux substrates (mimicked by rhodamine 123 (R123)). These selected extracts/compounds of natural origin have never been tested for nasal drug delivery enhancement, which included two pepper extracts (capsaicin and piperine) and gel as well as whole leaf extracts of three aloe species (i.e. *Aloe vera*, *A. ferox* and a novel cultivated hybrid: *A. muth-muth*).

The current study also investigated and compared the *in vitro* RPMI 2650 cell model with an *ex vivo* sheep nasal mucosa model in terms of histology, epithelial thickness, tight junction protein expression and transport of above-mentioned model compounds namely FD4 and R123. Taken together, it was concluded that the models were comparable in all aspects and

could be used interchangeably to assess drug permeation through nasal mucosa. This work was submitted for publication in “*Journal of Drug Delivery Science and Technology*”, November.

Two selected pepper extracts, capsaicin and piperine, have been investigated in terms of their effects on the transport of selected model compounds across both models mentioned above with regards to paracellular and efflux-mediated transport. Capsaicin, in both models, caused a slight increase in paracellular permeation of FD4, but showed extensive concentration dependent P-glycoprotein (P-gp) inhibitory effects. Efflux ratio values were decreased from 2.33 to 0.50 in the RPMI 2650 cell line and to 0.22 in the excised sheep nasal mucosa, respectively. Piperine, on the other hand, mediated an increase in FD4 paracellular permeation across both models, but showed only slight P-gp inhibitory effects on the RPMI 2650 cell line. Contrastingly, piperine lowered the efflux ratio from 2.33 to 0.95 in the sheep nasal mucosa, but simultaneously caused increased R123 P_{app} values in both directions in a concentration dependent manner in the RPMI 2650 cell model. For both pepper extracts, this was the first experimentation on nasal drug delivery, while the potential for piperine to mediate paracellular transport mechanisms was indicated for the first time. Furthermore, the cytotoxicity of capsaicin and piperine on the RPMI 2650 cell line was conducted using a MTT toxicity assay. Capsaicin was not cytotoxic at concentrations up to 100 μ M, but extensive cell death with cell viability dropping below 40% was seen at 300 μ M and higher. Piperine can be categorized as a non-toxic substance with weakly cytotoxic only becoming evident from concentrations of 750 μ M and higher. The cytotoxicity was confirmed with whole histology of both models indicating the same trends. This work on the nasal epithelial transport enhancement effects of the pepper extracts was published as an original research article in the journal “*Planta Medica*” (Gerber *et al.*, 2019).

Gel and whole leaf materials from the three selected aloe species that were investigated for nasal epithelial drug permeation enhancement in this study included *Aloe vera*, *A. ferox* and a newly created hybrid of these two species, namely *A. muth-muth*. With regards to the permeation experiments, all the plant materials were tested for effects pertaining to paracellular transport of FD4 across both the RPMI 2650 cell model and the excised sheep nasal mucosal model. Both *A. vera* gel and whole leaf extract presented with an concentration dependent increase in paracellular FD4 permeation, except for the highest concentration of the gel in the RPMI 2650 cell line. Although the total permeation of FD4 was higher than that of the control when co-applied with 3% w/v *A. vera* gel, it tended to be lower than the permeation obtained when co-applied with 1.5% w/v *A. vera* gel. This phenomenon was accredited to the high viscosity of *A. vera* gel at the highest concentration, which may

have caused difficulty for FD4 to permeate efficiently. *A. ferox* gel and whole leaf extracts interestingly presented mainly with a invers-bell shaped curve of FD4 permeation when plotted as a function of concentration, with exception of the whole leaf extract that caused a concentration dependent increase across excised sheep nasal mucosa. Both the gel and whole leaf extract of *A. muth-muth* caused a concentration dependent increase in FD4 permeation in both the cell and excised tissue models, albeit to a higher extent across the RPMI 2650 cell layers than across the excised sheep nasal mucosal tissue. This was the first time the permeation enhancement effects of any aloe species were tested on nasal tissue, as well as the first time any pharmacokinetic properties of *A. muth-muth* were presented. Toxicity determination of selected aloe species through neutral red assays indicated that *A. vera* gel was already weakly cytotoxic at 0.25% w/v with 2% w/v being severely toxic. In contrast to this, *A. vera* whole leaf extract was non-toxic to a concentration of approximately 1.5% w/v. For *A. ferox* gel, no toxicity was observed in a concentration range up to 5% w/v, while weak toxicity was observed from 2% w/v for the whole leaf extract. *A. muth-muth* gel indicated no toxicity up to 1% w/v and only weak cytotoxicity to up to 3% w/v. In contrast to this, the whole leaf extract showed a linear increase in toxicity, but it was only moderately cytotoxic at 6% w/v. Whole histology images reflected the same weak tissue damage for 3% w/v *A. muth-muth* gel as observed with the neutral red assay. This work was submitted for publication in the journal “*Journal of Herbmmed Pharmacology*”, November 2019.

In conclusion, the current contribution indicates that the selected pepper and aloe extracts/materials have the potential to be included in nasal dosage forms to increase bioavailability of active compounds, which are either transported paracellularly or are substrates for efflux proteins, by means of beneficial pharmacokinetic interactions.

2 Future recommendations

Some recommendations for further studies with regards to the current study include the following:

- Refining and/or extending the concentration ranges tested for the functional excipients from natural origin as investigated in this study in order to determine the most efficient concentration for pharmacokinetic interactions with the highest drug absorption enhancement potential via the nasal route of administration.
- Determination of the specific mechanisms of action of the pharmacokinetic interactions that lead to tight junction modulation (e.g. interaction with which tight

junction protein) and P-glycoprotein inhibition (e.g. competitive inhibition or mechanism based inhibition) in an effort to be able to manipulate the interactions more accurately.

- Extracting and refining pure single or fractions of polysaccharide molecules from aloe gel and whole leaf extracts, each to be studied along an extended concentration range in order to single out bioactive compounds with a higher efficacy and repeatability.
- Formulation, development and evaluation of nasal dosage forms containing the functional excipients from natural origin as investigated in this study, each in the optimized concentration as mentioned in the first recommendation.
- Evaluation of the functional excipients from natural origin as investigated in this study by means of *in vivo* animal testing and also in clinical trials in humans to determine if clinical significant drug absorption enhancement can be achieved.

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APPENDIX A

AUTHOR GUIDELINES “*PHARMACEUTICS*”

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1. read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
2. use the Microsoft Word template or LaTeX template to prepare your manuscript;
3. make sure that issues about publication ethics, research ethics, copyright, authorship, figure formats, data and references format have been appropriately considered;
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Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, ...) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME)

guidelines published by the Microarray Gene Expression Data Society must be followed.

- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (<https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/>), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

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The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Pharmaceutics endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: <http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf>.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1⁺ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

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threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

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An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopsis mutant lines (SALKxxxx, SAILxxxx,...) were kindly provided by Dr. XXX , institute, city, country).

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The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Pharmaceutics* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

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- Plagiarism, data fabrication and image manipulation are not tolerated.

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- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE).

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

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Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

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- *Reconsider after Major Revisions:*
The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reject and Encourage Resubmission:*
If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject:*
The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

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Clinical Trials Registration

Registration

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Pharmaceutics requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (<http://www.consort-statement.org>) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

APPENDIX B

AUTHOR GUIDELINES “*JOURNAL OF DRUG DELIVERY SCIENCE AND TECHNOLOGY*”



Before You Begin

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Article structure

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

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Results should be clear and concise.

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Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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Examples:

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, *J. Sci. Commun.* 163 (2010) 51–59. <https://doi.org/10.1016/j.Sc.2010.00372>.

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Reference to a book:

[3] W. Strunk Jr., E.B. White, *The Elements of Style*, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), *Introduction to the Electronic Age*, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13 March 2003).

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APPENDIX D

AUTHOR GUIDELINES “*PLANTA MEDICA*”

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Submission of a manuscript signifies acceptance of the journal's Guidelines for Authors. Submissions which are not in line with these principles will be returned directly to the authors by the Editorial Office.

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4.2. The Abstract should contain brief information on purpose, methods, results and conclusion (without subheadings). **Abstracts should not exceed 250 words**. Please note that during the upload of the manuscript files you will be asked to insert the abstract. This abstract needs to be identical to the abstract in the manuscript file itself.

4.3 The Key words should include the scientific name and family of the organism(s) investigated (as separate key words). 4–6 key words should be listed.

4.4. Abbreviations should generally be used sparingly. Abbreviations should be introduced only when repeatedly used. **Standard abbreviations** such as m. p., b. p., K, s, min, h, μL , mL, μg , mg, g, kg, nm, mm, cm, ppm, mmol, HPLC, TLC, GC, UV, CD, IR, MS, NMR, ELISA, PCR can be used throughout the manuscript (for a more extensive list follow this link: <https://www.thieme.de/de/planta-medica/authors-5605.htm>). **Non-standard abbreviations** must be defined in the text following their first use. Provide a list of **all nonstandard abbreviations** after the key words. Define all symbols used in equations and formulas. If symbols are used extensively, provide a list of all symbols together with the list of abbreviations.

4.5. The Introduction should state the purpose of the investigation and relate to current knowledge in the specific topic addressed.

4.6. Results should be presented in a concise manner. The **Discussion** should provide an interpretation of the data and relate them to existing knowledge. The discussion should not be a repetition of the results. **Results and Discussion** may be combined. **No subheadings are allowed within these sections**. There should be no separate conclusions paragraph, the conclusion should be incorporated into the discussion.

4.7. In Materials and Methods specific details about test materials and test compounds, instrumentation and experimental protocols should be given. This section should contain sufficient details so that others are able to reproduce the experiment(s). Purity (%) of all reference and standard compounds should be mentioned, as well as the method of how it was determined. Previously reported methods should be referenced only. Suppliers for major equipment, cell lines, chemicals, biochemical reagents and major disposables should be indicated. It should read in the manuscript for example “Quercetin (purity > 98%) was purchased from Sigma” and **not** “was purchased from Sigma (St. Louis, USA)”.

4.7.1. Documentation of plants and other organisms or starting materials. Use the correct scientific nomenclature. For plants, the Index Kewensis (electronic Plant Information Centre ePIC, Royal Botanic Gardens, Kew, UK: <http://www.kew.org/epic/>), and/or the International Code of Botanical Nomenclature (www.bgbm.fu-berlin.de/)

berlin.de/iapt/nomenclature/code/tokyo-e/default.htm) or The Plant List (www.theplantlist.org) should be followed. Give the scientific name (in italics), the author of this name and the family. **Indicate the person who identified the material as well as date and place of collection.** The manuscript must include references to **voucher specimens** of the plants (deposited in a major regional herbarium) or the material examined including their registration number(s). It should be mentioned which plant parts have been used.

4.7.2. Description of the preparation of extracts and isolation of compounds. The kind and amount of starting material, solvents (including volumes) and extraction methods (including temperature and extraction time) must be indicated. The description of chromatographic systems should contain the quantitative information that allows the reader to repeat the work. Column dimensions, stationary phase, particle size, mobile phase composition, flow rate, sample amount, and elution volumes (or retention times, k' values) of fractions should be given. E.g.: "MPLC on silica gel (40–63 μ m; 2 $\times$ 50 cm), MeOH/EtOAc 8: 2, 3 mL/min; tR of 1: 60–70 mL, 2: 120–140 mL, 3: 145–175 mL; detection of eluates by TLC (SiO₂, MeOH/H₂O 9: 1; Dragendorff reagent), Rf 1: 0.35, 2: 0.55, 3: 0.73)." When using gradients the volumes of solvents should be presented; fractions should be defined by their elution volume. Similar information is necessary for HPLC, GLC, DCCC, MLCC and all other methods of purification. Figures of chromatograms will only be accepted if they are essential for understanding the methods or the results described. GC identifications of constituents of essential oils must be supported by retention indices on a polar and an apolar column. Identification by GC-MS is preferred.

4.7.3. Chemical nomenclature used should be based on the systematic rules adopted by Chemical Abstracts and IUPAC. Trivial names should be avoided unless they are definitely advantageous over the corresponding systematic names. Trivial names are not accepted for close analogues and derivatives of known compounds. For reference drug substances the INN names should be used.

4.7.4. Physico-chemical characterisation of compounds. Data provided for new compounds should enable an unambiguous identification of the substance and have to appear in the following order, if available: visual appearance, chromatographic mobility in TLC, GC, or HPLC, mp, UV-vis, specific optical rotation, CD, IR, ¹H-NMR, ¹³C-NMR, low resolution MS, high resolution MS, elemental analysis. Note that for specific optical rotation $[\alpha]_D^{temp}$ the symbol c is defined as mass of substance (in g) in 100 mL of solution. For specific optical rotation no unit should be given; the "degree" symbol "°" should not be used. In case of spectroscopic work on known substances refer, if possible, to published data; the manuscript should then contain the following indication: Copies of the original spectra are obtainable from the corresponding author. Original spectra for new compounds should be provided as Supporting Information (see 4.14), IR, NMR, mass, and UV spectra should normally not be given in the manuscript as figures, unless the listing of characteristic signals is not sufficient.

4.7.5. X-Ray crystallographic data must include a line drawing of the structure, a perspective drawing, and a discussion of bond

lengths and angles. A supplement describing full details of the structure and methods and means of its determination in a form suitable for deposition must be submitted to the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0) 1223 33 60 33 or e-mail: deposit@ccdc.cam.ac.uk). Deposition of the data has to be prior to submission of the manuscript, and appropriate reference has to be made in the Materials and Methods section, including the deposition number.

4.7.6. Analytical studies. Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. For more information regarding validation issues, prospective authors should also refer to ICH guidelines. Analytical studies of a routine nature will not be considered for publication.

4.7.7. Pharmacological investigations. *Planta Medica* will only consider manuscripts in which conclusions are based on adequate statistics that incorporate the appropriate tests of significance, account for the type of data distribution, and are based on the number of experimental observations required for the application of the respective statistical method. In each case, positive controls (reference compounds) have to be used and the dose-activity dependence should be shown. If IC₅₀ values are given, the dose-response relation should be displayed graphically at least as supplementary data, and the method of calculation should be given. Authors should be conscious of the differences between EC₅₀, IC₅₀, TC₅₀, LC₅₀, ED₅₀, LD₅₀ values. Compounds should follow accepted guidelines when represented as "active". For example, the cytotoxic effect of a pure substance when tested against a cancer cell line would exhibit an IC₅₀ value of <10 μ M. Authors should pay attention to the following definitions: Compounds that suppress the growth of, or kill, isolated tumor cell lines grown in culture should be referred to as either "cytostatic" or "cytotoxic", as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called "antitumor". The term "anticancer" should be reserved for compounds that show specific activity in human-based clinical studies.

When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations and to approval by the local ethical committee. **The protocol approval number and the exact date of approval (e.g. January 1st 2016) must be provided.**

Pharmacological investigations of extracts require detailed extract characterization. This includes botanical characterization of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). For starting material coming from a company/commercially obtained samples the batch/Lot. Number has to be provided. The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typ-

ical constituents should be provided. Altogether the phytochemical standardization of an extract and/or fraction(s) requires state-of-the-art methods.

4.7.8. Biological screening. Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in *Planta Medica*. Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections 4.7.1 and 4.7.7). Biological activities should be reported by listing IC₅₀ or EC₅₀ values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

4.8. Acknowledgements should list persons who made minor contributions to the investigation and organisations providing support.

4.9. Conflict of Interest Disclosure. A statement describing any financial conflicts of interest or lack thereof is published with each manuscript. The statement should describe all potential sources of bias, including affiliations, funding sources, and financial or management relationships, that may constitute conflicts of interest (please see the ACS Ethical Guidelines to Publication of Chemical Research). The statement will be published in the final article. If no conflict of interest is declared, the following statement will be published in the article: **"The authors declare no conflict of interest."**

4.10. References, including those in tables and figure legends, should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1–3] or [1,2,5]. The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. **Original Research articles should not have more than 45 references.**

The following examples illustrate the format for references:

a) Journals

Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in *Hedera helix*. *Phytochem Anal* 1996; 7: 204–208

Article in press **without** doi:

Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. *Biotech Bioeng*, in press

Note: If reference is made to papers that are in press, authors are requested to add the galley proof or acceptance letter to the online submission. Avoid references to unpublished personal communications. These have to be included in the body of the text as "unpublished data".

Article in press **with** doi:

Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. *EMBO J*, advance online publication 8 July 2004; doi:10.1038/sj.emboj.7600295

b) Books

Citation to complete book:

Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520–521

Citation to article within a book:

Lechtenberg M, Nahrstedt A. Cyanogenic glycosides. In: Ikan R, editor. Naturally occurring glycosides. Chichester: Wiley & Sons; 1999: 147–191

Lorberg A, Hall MN. TOR: the first ten years. In: Thomas G, Sabatini DM, Hall MN, editors. TOR – target of rapamycin. Heidelberg: Springer Verlag; 2004: 1–18

Multi-volume books and encyclopedias:

Warren SA. Mental retardation and environment. In: International encyclopedia of psychiatry, psychology, psychoanalysis and neurology, Vol. 7. New York: Aesculapius Publishers; 1977: 202–207
Pharmacopoeia of China, Part 1. Beijing: People's Health Press; 1977: 531–534

c) PhD and Diploma Theses

Dettmers JM. Assessing the trophic cascade in reservoirs: the role of an introduced predator [dissertation]. Columbus: Ohio State University; 1995

d) Patents

Cookson AH. Particle trap for compressed gas insulated transmission system. US Patent 4 554 399; 1985

e) Conference Paper

Okada K, Kamiya Y, Saito T, Nakagawa T, Kaawamukai M. Localization and expression of geranylgeranyldiphosphate synthases in *Arabidopsis thaliana*. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD; 1999

f) Electronic Sources

Agatep R, Kirkpatrick RD, Parchaliuk DL, Woods RA, Gietz RD. Transformation of *S. cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at <http://research.bmn.com/tto>. Accessed September 22, 2005

If no author is given, the title is used as the first element of the citation.

4.11. Chemical structures should be prepared with ChemDraw or a similar program using the following settings: bond lengths 0.508 cm, bond width 0.021 cm, bold bond width 0.071 cm, bond spacing 18% of length, hash spacing 0.088 cm, atom labels Arial 10, compound numbers Arial 10 bold. These settings correspond to American Chemical Society document settings preset in ChemDraw. The configuration of all stereocenters present should

be indicated; use of bold and dashed lines rather than solid and dashed wedges is recommended. **They will be reproduced without reduction and the charts should be prepared with maximum widths of up to 8.5 cm for single column print and up to 17.5 cm for double column print.**

Authors using other drawing packages should modify their program's parameters so that they reflect the above guidelines.

4.12. Graphics: Figures are numbered with Arabic numerals. The quality of the illustrations depends on the quality of the originals provided. Graphics cannot be modified or enhanced by the journal production staff. **The graphics must be submitted as separate files. The legend should not appear under the figures but should be included as a separate figure legend after the references in the manuscript file.** The figure legend needs to be self explanatory.

Acceptable file formats are TIFF, EPS (vector artwork), or CDX (ChemDraw file). Labeling of all figure parts should be present, and the parts should be assembled into a single graphic.

TIFF files should have the following resolution requirements:

Line Art (Black and White, Color):	800–1200 dpi
Greyscale Art:	600 dpi
Color Art (RGB mode):	300 dpi

For efficient use of journal space, single-column illustrations are preferred.

	Single (preferred)	Double
Width		
minimum	5.5 cm (2.16 in)	11.5 cm (4.5 in)
maximum	8.5 cm (3.33 in)	17.5 cm (7 in.)
Maximum depth	25.4 cm (10 in.)	25.4 cm (10 in.)

For best results, illustrations should be submitted in the actual size at which they should appear in the Journal. Consistently sized letters and labels in graphics throughout the manuscript will help ensure consistent graphic presentation for publication. Lettering should be no smaller than 5 points. (Arial type works well for lettering.) Lines should be exactly 0.5 point. Lettering and lines should be of uniform density. If artwork that should be reduced must be submitted, larger lettering and thicker lines should be used so that, when reduced, the artwork meets the above-mentioned parameters.

Complex textures and shading to achieve a three-dimensional effect should be avoided. Different grey scale tones to show group differences are preferred.

4.13. Tables: These should be numbered consecutively with Arabic numerals. Tables should be placed in the manuscript or uploaded as separate file (file format DOC, DOCX) after the figure legends. Footnotes in tables should be given lowercase letter designations and be cited in the table by italic superscript letters. The sequence of letters should proceed by line rather than by column. If a footnote is cited both in the text and in a table, insert a lettered footnote in the table to refer to the numbered footnote in the text.

Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. Arrangements that leave many columns partially filled or that contain much blank space should be avoided. **The table legend should appear directly under the tables.**

4.14. Supporting Information: To keep articles as concise and at the same time as informative as possible, authors are strongly encouraged to submit part of their tables and figures as Supporting Information. The following type of data will be preferentially published as Supporting Information rather than in the print article: Spectra, chromatograms, structural drawings outlining NMR correlations, experimental procedures of secondary importance, tables summarizing data that are non-essential but useful to the understanding of an article. Tables, figures and text provided as Supporting Information must be referred to in the manuscript as follows: (Table 1S, Supporting Information, etc.). The cover page for Supporting Information should be identical to the cover page of the manuscript. Legends for Figures and Tables must appear directly on the respective figure pages. Pages have to be numbered consecutively. All figures and tables should be referenced in the main manuscript. Supporting Information has to be submitted as a separate file.

Supporting Information is published on the journals homepage at <http://www.thieme-connect.de/ejournals/toc/plantamedica>.

5. Proofs and Reprints

Galley proofs will be sent to the corresponding author as a PDF file. An electronic author reprint will be supplied free of charge after online publication.

APPENDIX E

AUTHOR GUIDELINES “*JOURNAL OF HERBMED PHARMACOLOGY*”

Author Guidelines

The benefits of publishing in the Journal of Herbmед Pharmacology:

- Free access to all articles
- Fast and constructive peer review process (one month)
- Easy and quick online submission
- Rapid publication
- Indexing in high quality databases

Terms of submission

Unsolicited manuscripts will be reviewed for publication with the following understanding:

1. The paper represents an original work.
2. The paper neither has been published already nor is being under review elsewhere.
3. Upon acceptance, the paper may not be published elsewhere without the permission of Journal of Herbmед Pharmacology.
4. The published paper is the sole property of Journal of Herbmед Pharmacology and may be edited before publication.

Manuscripts should be submitted by one of the authors of the manuscript through the online system or sending to Editor-in-Chief's Email (**rafieian@yahoo.com**). The submitting author is responsible for ensuring that the article's publication has been approved by all the other coauthors. It is also the authors' responsibility to ensure that the articles emanating from a particular institution are submitted with the approval of the necessary institution. The submitting author takes responsibility for the paper during submission and peer review.

Correspondence and proofs will be sent to the author(s) before publication unless otherwise indicated. It is a condition of submission of a paper that the authors permit editing of the

paper for readability. All enquiries concerning the publication of accepted papers should be addressed to editor@herbmedpharmacol.com. If for some technical reasons submission through site is not possible, the author can contact editor@herbmedpharmacol.com for support.

All manuscripts are subject to peer review and are expected to meet standards of academic excellence. Submissions will be considered by the technical editor and section editor and “if not rejected right away” by peer-reviewers,

Article Processing Charge

Journal of Herbmed Pharmacology is an open-access journal. Open access publication is not without costs. To cover a section of the costs, papers that are accepted for publication following peer review incur a publishing fee of 100 USD for Original and Review papers, and 50 USD for Case reports and letters (10 Million and 5 Million Rials for Iranian authors, respectively).

The publication fee for manuscripts received from 1 Nov 2019 is 200 USD for Original and Review articles, 120 USD for short communications, and 70 USD for Case reports and letters.

Withdrawal Policy for authors

Authors are free to withdraw an article at no charge – as long as it is withdrawn within 15 days of its initial submission.

If you have concerns or questions about it, please contact us for further discussion. We welcome your input.

Structures

Units of measurement should be presented simply and concisely using System International (SI) units.

Types of Articles

Manuscripts should be presented as one of the following formats.

Full Original Researches

A full-length original research article (up to ~5000 words, including tables, figures, and references) presents novel findings relevant to the aims and scope of the journal.

Reviews

A full-length critical review (up to ~8000 words, including tables, figures and references) provides an abstract and discussion of the relevant literature about any topic covered within the aims and scope of the journal. Review articles should be sharply focused, well-focused, well-documented examinations of timely related issues.

Mini-Reviews

Mini-Reviews are sharply focused, well-focused, well-documented examinations of timely related issues (up to ~4000 words, including tables, figures and references). The issues may be of a controversial nature, or may address a more narrowly focused area than those typically covered in a review.

Short Communications

Short Communications are preliminary reports (up to ~2000 words, including tables, figures and references).

Commentaries

Commentaries present the author's considered opinion (up to ~1000 words limited to one figure/table and limited references) on an original article to be published in the journal and usually submitted by the reviewers.

Case reports

These reports should include an introduction, case description, discussion, and conclusions. Patient confidentiality must be maintained. Any identifying information must not be published, and any specific details or description that may compromise patient anonymity, should be omitted. The patient's consent should be obtained when possible.

Letters to Editor

Letter to Editor presents the author's opinion (up to ~1000 words limited to one figure/table with limited references). If such a letter criticizes an article already published in the journal, then the authors of the original article will be given a chance to respond in the same issue in which the letter is published.

Preparation of Manuscripts

Text

Submitted manuscripts to JHP must be in DOC format as follows. **Authors are strongly recommended to consult a recently published article in this journal** and consider the following instructions.

Title page

Title: Lengthy systematic names and complicated/numerous chemical formulae should be avoided where possible.

Authors' names: Full names (First, Middle and Last) for all the authors of an article should be given and specified with superscript number(s) for the affiliation(s) [e.g., Mark Junior Smiths¹]. The name of the corresponding author(s) should be specified with an asterisk after name (e.g., Mark Junior Smiths*). Where the family name may be ambiguous (e.g., a double name), please indicate this clearly.

Affiliation: Affiliation of all the authors should be given and specified with superscripted number before address (e.g., ¹Faculty of).

Running title: A very short running title should be given.

Corresponding author: Full address, telephone, and fax numbers (with country and area code) and email of the corresponding author(s) should be given.

Abstract page

Abstract: A factual concise abstract (up to 250 words) is required for every manuscript. The abstract should briefly state the **Introduction**, *Methods*, **Results** and **Conclusion**. An abstract is often presented separately from the article; hence it must be able to stand alone. Referencing should be avoided. Also, non-standard or uncommon abbreviations should be avoided, however, if essential they must be defined at their first mention in the abstract itself.

Key words: Immediately after the abstract, three to six relevant keywords should be included. (Notice: Readers are increasingly used search engines to find literature using keywords; thus, recognizable and searchable keywords should be given to maximize the visibility of the article.)

Notice: Original research papers can also be published in a brief format. Submitted papers that are of interest but are not acceptable as a full-length original contribution are offered by the editor to be published in this section. Also, the authors can primarily submit their papers for consideration of publication in this section. An unstructured abstract not longer than 200 words is required for this section. The body of the manuscript should not exceed 2000 words, and no heading or subheading should be used. Tables and/or Figures should be limited to 2 ones and references to 15 in maximum.

Introduction

This section should clearly and briefly (up to 600 words) provide an adequate background with relevant references, avoiding a detailed literature survey or a summary of the results. The last paragraph should address the main objectives of the work.

Materials and Methods (Patients and Methods for clinical investigations)

This section should provide sufficient details to allow the work to be reproduced, with details of the supplier (i.e., company's name, city, country) and catalogue number when appropriate. Methods already published, should be indicated by a reference: only relevant modifications should be described. The company's name, city and country of manufacturer of the major equipment should be given. Unexpected hazards encountered during the experimental work should be noted. Any unusual hazards inherent in the use of chemicals, procedures or equipments in the investigation should be clearly identified. In cases where a study involves the use of live animals or human subjects, the author should include a statement that all experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that has approved the experiments. They should also include a statement that informed consent was obtained for any experimentation with human subjects.

Results

Results should be clear, descriptive and concise. Attention should be paid to the matter of significant figures and tables. The same data should not be presented in more than one figure or in both a figure and a table. Basically, as a rule, interpretation of the results should be reserved for the discussion section of a full original research article.

Discussion

The discussion should explore the significance of the results of the work (without repeating them) in comparison with others similar reports. Extensive citations and discussion of published literature should be avoided.

Conclusion

The main question of the work should be very concisely stated and the final conclusions of the study may be presented in a short “Conclusions” section, which may stand alone or form a subsection of a Discussion or Results and Discussion section(s).

References

The authors are responsible for the accuracy of all references. These should be numbered sequentially as superscripts in order of their appearance in the text and listed in a separate section following the text, double-spaced. All authors and inclusive page numbers should be limited to published works; unpublished data or personal communications should be indicated parenthetically in the text.

Numbered references should appear at the end of the article and should consist of surnames and initials of all authors when six or less (when seven or more list the first six and add et al). Also title of article, name of the journal, year, volume, first and last page numbers should be given.

Authors. Title. Journal. Year; Volume(Issue): Pages.

For books, names and initials of all authors, the full title, place of publication, publisher, year of publication, and page numbers should be given.

Authors. Title. Place Published: Publisher; Year. Number of Pages.

Tables

Tables should be created with a word processor and saved in either DOC or RTF format.

Notice: Please embed tables in your text.

Figures

The figures must be minimum 300 dpi resolution at full size (size when printed in journal).

Notice: Please embed figures in your text.

Ethical Issues

Please see the ethical issue provided in the below link

Every experimental or clinical study may raise controversial ethical issues (e.g., Institutional Ethical Approval for working with animal or human subjects). Thus, the Journal of Herbmmed Pharmacology expects all authors, reviewers, and editors to consider COPE, ICMJE and Equator Network's reporting guidelines in medical ethics plus scientific writing. If any, authors should state related declaration(s), otherwise the following sentence should be given "None to be declared". Please take a look at the review process in Journal of Herbmmed Pharmacology.

The cover letter must include a statement declaring that the study complies with current ethical considerations.

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or redundancy) must completely considered by the authors.

Authors reporting experimental studies on human subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that: (1) informed consent was obtained from each patient enrolled in the study and (2) the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. In studies involving animal experimentation, provide assurance that all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Every experimental or clinical study may raise controversial ethical issues (e.g., Institutional Ethical approval for working with animal or human subjects).

http://en.wikipedia.org/wiki/Declaration_of_Helsinki

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://publicationethics.org/>

<http://www.icmje.org/>

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Ethical considerations

Human subjects

Authors reporting experimental studies on human subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that, the project was done with consideration of ethical issues and obtaining license from the ethics committee of local university/research center and obtaining the written consent of participants. Also, it was done according to ethical standards of human experimentation in accordance to the Helsinki Declaration (www.cirp.org/library/ethics/helsinki).

Animal subjects

Authors reporting experimental studies on animal subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that, the project was done with consideration of ethical issues and obtaining license from the ethics committee of local institute. Also, the general care of the experimental animals used for this study was done in compliance with the Animal Welfare Act (http://www.nap.edu/openbook.php?record_id=5140&page=114).

Informed Consent

In the case of research on human subjects, informed consent and other ethical considerations should be mentioned in the "methods" section of the manuscript. The author should include a statement that informed consent was obtained for any experimentation with human subjects. As *JHP* follows ICMJE, please consider their guidelines for more information. In cases where a study involves the use of live animals or human subjects, the author should also include a statement that all experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that has approved the experiments. Moreover, the templates can be seen from WHO.

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http://www.who.int/rpc/research_ethics/informed_consent/en/

Conflict of Interests

The authors must declare any conflict of interest of contributed authors very briefly in a separate paragraph at the end of the paper. All sources of funding should be declared; unless otherwise the following sentence should be given “Authors declare no conflict of interests”.

To prevent the information on potential conflict of interest for authors from being overlooked or misplaced, mention this information in the cover letter. The authors must identify any potential financial conflicts of interest before the review process begins. Declared conflict of interest will not automatically result in rejection of paper but the editors reserve the right to publish any declared conflict of interest alongside accepted. The following would generally be regarded as potential conflicts of interest:

1. Direct financial payment to an author for the research or manuscript production by the sponsor of a product or service evaluated in an article.
2. Ownership of shares by an author in the company sponsoring a product service evaluated in an article (or in a company sponsoring a competing product).
3. Personal consultant for companies or other organizations with a financial interest in the promotion of particular health care products and services.

Source of Funding

Authors are required to specify the source of funding for their research when submitting a paper. Suppliers of materials should be named and their location (town, state/county, country) included. The information will be disclosed in the Acknowledgements section of the published article.

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Authors should acknowledge any scientific, technical, statistical and financial supports. Contributors other than coauthors may be very briefly acknowledged in a separate paragraph at the end of the paper. All sources of funding should be declared.

APPENDIX F

**CERTIFICATE OF SUBMISSION “*JOURNAL OF HERBMED
PHARMACOLOGY*”**



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Dear Mr Werner Gerber,

A manuscript entitled Permeation enhancement effects of leaf materials from different aloe species on in vitro and ex vivo nasal epithelial models was submitted to Journal of Herbmed Pharmacology (JHP) and you were mentioned as a co-author of the manuscript. Please let us know if you are not a co-author of the manuscript.

Yours Sincerely ,

Administrator

Shahrekord University of Medical Sciences

APPENDIX G

ETHICAL APPROVAL CERTIFICATE

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Email: Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **AnimCare Animal Research Ethics Committee (AREC-130913-015)** on **22/09/2017** after an expedited review, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Ex vivo tissue model for nasal drug delivery research														
Study Leader/Supervisor: Prof Josias Hendrik Hamman														
Ethics number:														
N	W	U	-	0	0	2	8	5	-	1	7	-	A	5
Institution			Study Number			Year			Status					
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation														
Application Type: New Application – Large Project														
Commencement date: 2017-09-26														
														Category: 0
Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.														

Special conditions of the approval (if applicable):

- Any permits/ permission must still be obtained from relevant authorities and provided to the AnimCare, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions: <i>While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:</i> - The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare: - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study. - Annually a number of studies may be randomly selected for an external audit. - The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviation from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited. - The date of approval indicates the first date that the study may be started. - In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to: - request access to any information or data at any time during the course or after completion of the study; - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process. - withdraw or postpone approval if: - any unethical principles or practices of the study are revealed or suspected, - it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented, - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately, - new institutional rules, national legislation or international conventions deem it necessary. - AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Prof LA
Du Plessis

Digitally signed by
Prof LA Du Plessis
Date: 2017.09.27
08:45:27 +02'00'

Prof Linda du Plessis

Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

APPENDIX H

CERTIFICATES OF ANALYSIS

H.1 Introduction

This Appendix indicates the composition of both *Aloe muth-muth* gel and whole materials as determined using ^1H -NMR analysis in order to determine similarities between the newly cultivated hybrid and the two parent species (*A. vera* and *A. ferox*). Furthermore, the purity of the pepper extracts deployed in permeation and toxicity studies was determined using HPLC as compared to a reference standard of each.

H.2 Aloe muth-muth gel ¹H-NMR-analysis certificate

Analysis Certificate

NWU73919-1



Spectral Service AG Postfach 560122 D-50986 Köln

North-West University

Mr. Prof. Hamman

11 Hofman streetn Private Bag X6001

ZA 2520 Potchefstroom

Tel.: +49/(0)2236/96947-0

Fax: +49/(0)2236/96947-11

eMail: Info@spectralservice.de

http: //www.spectralservice.de

Analysis method: quantitative ¹H-NMR spectroscopy

Instrument: Bruker Avance III HD 500 MHz with autom. sample changer & BBFOPLUS SmartProbe

Internal Standard: Nicotinic acid amid (NSA; Sigma-Aldrich, Buchs (CH), Art.-Nr. 72340; RG 9)

Sample Ident.: Aloe gel		Batch: —	
Description: beige powder		Lab. No: —	
Spectral Service Code: NWU73919-1		Results from: 13/06/2018	
	Content [%]*	Content [mg/l]*	Origin of component
Aloverose (polysaccharide)	10.4	730.2	fresh Aloe Vera
Glucose	9.1	639.6	fresh Aloe Vera
Malic acid	18.3	1281.0	fresh Aloe Vera
Lactic acid	0.1	8.6	degradation (bacterial)
Citric acid	Not detected		WLM or added acidifier
WLM	Not detected		whole leaf marker (WLM)
Fructose	Not detected		fresh Aloe Vera
Mg	0.8	55.0	fresh Aloe Vera
Ca	2.7	186.5	fresh Aloe Vera
Maltodextrin	Not detected		formulation aid for drying
Acetic acid	Traces		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**	Detected		unknown
Dry matter	powder		pH-Value: not determined
Aloin***		not determined	

* 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.

*** Limit of quantification 0.1 ppm.

The sample is a 200:1 powder of Aloe Vera origin without preservative.

It shows no degradation.

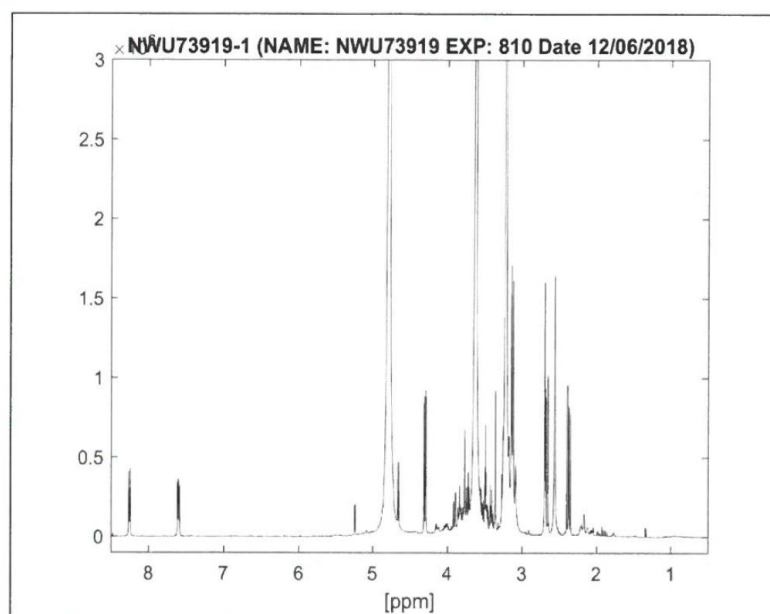


Fig. 1 ^1H -NMR spectrum of test item NWU73919-1

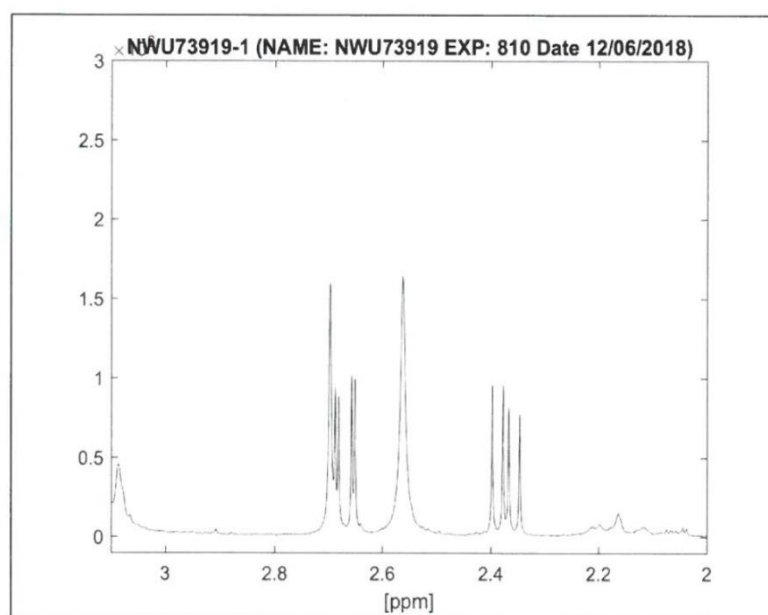


Fig. 2 ^1H -NMR spectrum of test item NWU73919-1: details

Cologne, 13 June 2018

Spectral Service AG
Emil-Hoffmann-Str. 33 · D-50996 Köln
Andreas Formes  0 22 36 / 9 69 47-0 · Fax 0 22 36 / 9 69 47-11
(Master of Science) www.spectralservice.de

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H.3 *Aloe muth-muth* whole leaf extract ¹H-NMR-analysis certificate

Analysis Certificate

NWU73919-2



Spectral Service AG Postfach 560122 D-50986 Köln

North-West University

Mr. Prof. Hamman

11 Hofman streetn Private Bag X6001

ZA 2520 Potchefstroom

Tel.: +49/(0)2236/96947-0

Fax: +49/(0)2236/96947-11

eMail: Info@spectralservice.de

http: [//www.spectralservice.de](http://www.spectralservice.de)

Analysis method: quantitative ¹H-NMR spectroscopy

Instrument: Bruker Avance III HD 500 MHz with autom. sample changer & BBFOPLUS SmartProbe

Internal Standard: Nicotinic acid amid (NSA; Sigma-Aldrich, Buchs (CH), Art.-Nr. 72340; RG 9)

Sample Ident.:	Aloe whole leaf		Batch: —	
Description:	beige powder		Lab. No: —	
Spectral Service Code: NWU73919-2			Results from: 13/06/2018	
	Content [%]*	Content [mg/l]*	Origin of component	
Aloverose (polysaccharide)	9.3	651.5	fresh Aloe Vera	
Glucose	8.4	587.4	fresh Aloe Vera	
Malic acid	16.0	1122.5	fresh Aloe Vera	
Lactic acid	0.1	7.4	degradation (bacterial)	
Citric acid	2.2	156.6	WLM or added acidifier	
WLM	3.0	212.3	whole leaf marker (WLM)	
Fructose	Not detected		fresh Aloe Vera	
Mg	0.7	47.5	fresh Aloe Vera	
Ca	2.7	191.1	fresh Aloe Vera	
Maltodextrin	Not detected		formulation aid for drying	
Acetic acid	Traces		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**	Detected		unknown	
Dry matter	powder		pH-Value:	not determined
Aloin***		not determined		

* 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.

*** Limit of quantification 0.1 ppm.

The sample is a 100:1 powder of Aloe Vera origin without preservative.

The sample contains significant amounts of WLM. It therefore is at least partly produced of whole leaf juice. It shows no degradation.

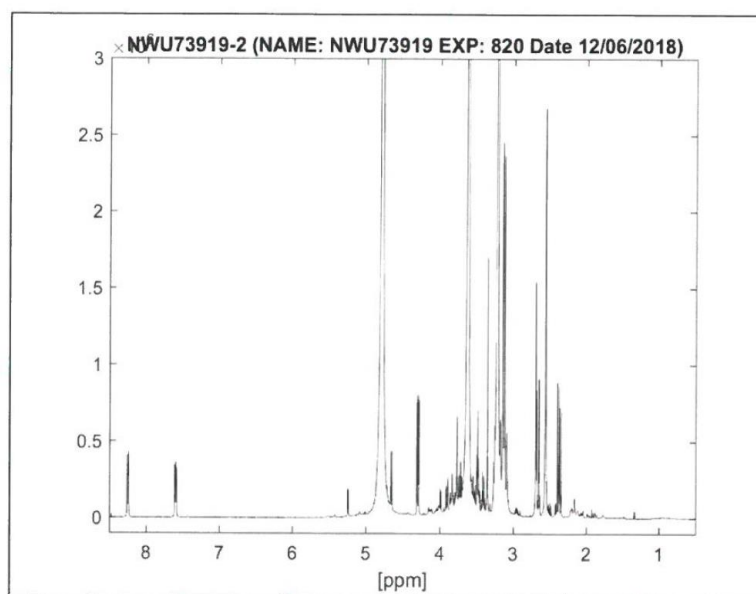


Fig. 1 ^1H -NMR spectrum of test item NWU73919-2

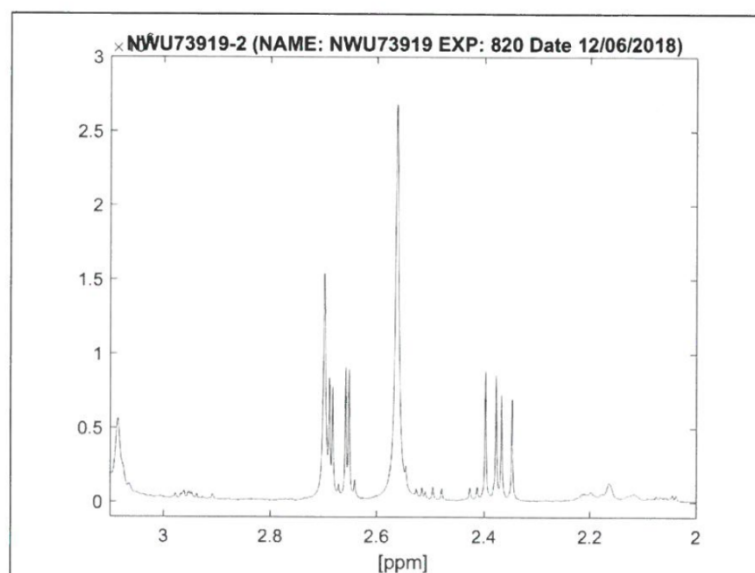


Fig. 2 ^1H -NMR spectrum of test item NWU73919-2: details
Cologne, 13 June 2018

 **Spectral Service AG**
Emil-Hoffmann-Str. 33 · D-50996 Köln
Andreas Formes ☎ 0 22 36 / 9 69 47-0 · Fax 0 22 36 / 9 69 47-11
(Master of Science) www.spectralservice.de

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H.4 Determination of capsaicin purity by HPLC

USP Reference standard: 50 mg/200 ml methanol (250 µg/ml)

Sigma standard: 12.2 mg/50 ml (244 µg/ml)

Table H.1: Validation results for high performance liquid chromatography method for analysis of capsaicin

Linearity				
Standard (µg/ml)	Injection volume	Mean peak area	Concentration (µg/ml)	r ²
250	5	1750.0	62.5	0.999
	10	3454.2	125.0	
	15	5179.1	187.5	
	20	6861.3	250.0	
	25	8583.8	312.5	
Purity				
Theoretical concentration (µg/ml)		Mean peak area	Actual concentration (µg/ml)	Accuracy (%)
244.0		6164.0	224.0	91.82

H.5 Determination of Piperine purity by HPLC

USP Reference standard: 30 mg/200 ml methanol (150 µg/ml)

Sigma standard: 15.08 mg/100 ml (150.8 µg/ml)

Table H.2: Validation results for high performance liquid chromatography method for analysis of piperine

Linearity				
Standard (µg/ml)	Injection volume	Mean peak area	Concentration (µg/ml)	r ²
150	5	4083.0	37.5	0.999
	10	8128.4	75.0	
	15	12142.1	112.5	
	20	16105.7	150.0	
	25	19979.7	187.5	
Purity				
Theoretical concentration (µg/ml)		Mean peak area	Actual concentration (µg/ml)	Accuracy (%)
150.8		15769.0	147.2	97.62

APPENDIX I

VALIDATION OF ANALYTICAL METHODS

I.1 Introduction

Marker compounds' (FITC-dextran 4400 and rhodamine 123) analytical methods for permeation studies were validated on a Spectramax Paradigm® multi-mode detection plate reader (Serial nr: 33270-1142) with regards to linearity, limit of detection, limit of quantification, accuracy and precision. Furthermore, the specificity of FITC-dextran 4400 in the presence of the aloe materials was determined by proving linearity of the suspensions.

I.2 Rhodamine 123 validation

Table I.1: Data obtained for the determination of linearity of rhodamine 123 (R123)

R123 concentration (µM)	Mean fluorescent value	Slope	r ²
0.0005	1115773.875	257521099.067	0.999
0.0025	1694006.875		
0.005	2945221.875		
0.025	7704382.625		
0.05	15422277.125		
0.25	63802136.875		
0.5	130965142.875		
2.5	651411728.875		
5	1283575376.875		

Table I.2: Data obtained for the determination of limit of detection (LOD) and limit of quantification (LOQ) values of rhodamine 123

Background Noise	Average	Standard deviation	LOD (µM)	LOQ (µM)
1123949	1137071.125	124014.211	1.589×10^{-3}	4.816×10^{-3}
936498				
999560				
1178331				
1224816				
1369836				
1125145				
1138434				

Table I.3: Data obtained for the determination of accuracy of rhodamine 123

Theoretical concentration (μM)	5	2.5	0.125
Mean fluorescent value	1264564268.304	631858016.875	37545375.275
Standard deviation	39431773.469	14341321.591	1655718.240
Actual concentration (μM)	4.911	2.454	0.146
Accuracy (%)	98.211	98.145	116.636
% RSD	3.118	2.270	4.410

Table I.4: Data obtained for the determination of intra-day precision of rhodamine 123

Concentration (μM)	Repeat	Mean fluorescent values	Standard deviation	% RSD
5	1	1264564268.304	59698682.709	4.965
	2	1168995545.575		
	3	1148363095.125		
2.5	1	631858016.875	14782848.318	2.371
	2	623923340.775		
	3	609311255.125		
0.125	1	37545375.275	2246841.371	6.453
	2	33691983.575		
	3	33224037.925		

Table I.5: Data obtained for the determination of inter-day precision of rhodamine 123

Concentration (μM)	Repeat	Mean fluorescent values	Standard deviation	% RSD
5	1	1202279004.801	53659183.279	4.518
	2	623390950.806		
	3	34820465.592		
2.5	1	1149502970.350	13396601.812	2.163
	2	613651527.150		
	3	32733258.083		
0.125	1	1176273307.700	2526831.385	7.220
	2	609832475.700		
	3	38257630.100		

I.3 FITC-Dextran 4400 validation

Table I.6: Data obtained for the determination of linearity of FITC-dextran 4400 (FD4)

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
0.9765625	23378509	14513230.396	0.999
1.953125	36664526		
3.90625	66967550		
7.8125	129977439		
15.625	247110833		
31.25	486021209		
62.5	940828297		
125	1785054665		

Table I.7: Data obtained for the determination of limit of detection (LOD) and limit of quantification (LOQ) values of FITC-dextran 4400

Background Noise	Average	Standard deviation	LOD (mg/ml)	LOQ (mg/ml)
967073	935319	23294.355	5.297×10^{-3}	16.050×10^{-3}
929805				
911984				
922003				
907955				
921011				
949728				
972993				

Table I.8: Data obtained for the determination of accuracy of FITC-dextran 4400

Theoretical concentration (μM)	125	62.5	12.5
Mean fluorescent value	1737339778.600	893342505.000	189885052.200
Standard deviation	12638638.644	13592814.959	2669156.626
Actual concentration (μM)	119.707	61.554	13.084
Accuracy (%)	95.766	98.486	104.669
% RSD	0.727	1.522	1.406

Table I.9: Data obtained for the determination of intra-day precision of FITC-dextran 4400

Concentration (μM)	Repeat	Mean fluorescent values	Standard deviation	% RSD
125	1	1737339778.600	47249955.343	2.772
	2	1736404504.525		
	3	1639059191.500		
62.5	1	893342505.000	24035281.096	2.750
	2	881776933.325		
	3	846703927.500		
12.5	1	189885052.200	4902987.669	2.537
	2	199095010.125		
	3	190781831.500		

Table I.10: Data obtained for the determination of inter-day precision of FITC-dextran 4400

Concentration (μM)	Repeat	Mean fluorescent values	Standard deviation	% RSD
125	1	1704267824.875	49459280.427	2.893
	2	1767776781.750		
	3	1667376693.600		
62.5	1	873941121.942	27062273.506	3.082
	2	909688858.550		
	3	858614133.600		
12.5	1	193253964.608	4862932.656	2.506
	2	194676644.150		
	3	195800575.200		

I.4 FITC-dextran 4400 specificity in aloe compounds

Table I.11: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe vera* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	35758529.625	7722210.369	0.986
3.90625	64489129.625		
7.8125	116578454.625		
15.625	231794590.625		
31.25	392595066.625		
125	1244502098.625		
250	2238470386.625		
500	3623184306.625		

Table I.12: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe vera* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	7509502.375	1300452.556	0.998
3.90625	11571941.375		
7.8125	16149325.125		
15.625	27302102.625		
31.25	47379977.625		
125	142169510.625		
250	311234850.625		
500	661458962.625		

Table I.13: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe vera* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	5498122.250	340907.783	0.989
3.90625	6397748.500		
7.8125	8191479.500		
15.625	11248612.500		
31.25	17842313.500		
125	52673379.250		
250	95505404.250		
500	162020484.250		

Table I.14: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe vera* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	33606735.750	8227229.462	0.984
3.90625	59378245.750		
7.8125	130985514.750		
15.625	203968510.750		
31.25	415539254.750		
62.5	722673502.750		
125	1325685534.750		
250	2389320862.750		
500	3833395678.750		

Table I.15: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe vera* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	5185960.250	995103.523	0.998
3.90625	7750087.000		
7.8125	12324720.250		
15.625	21525072.750		
31.25	39415032.750		
62.5	75492706.750		
125	136259146.750		
250	258138750.750		
500	487419590.750		

Table I.16: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe vera* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	5979362.500	648818.007	0.996
3.90625	7496772.500		
7.8125	10644595.500		
15.625	16498797.000		
31.25	27512976.250		
62.5	49656861.750		
125	90087976.750		
250	172305270.750		
500	315178554.750		

Table I.17: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe ferox* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	40363895.250	7860420.248	0.974
3.90625	71834908.250		
7.8125	128231818.250		
15.625	235089692.250		
31.25	446963520.250		
125	816292584.250		
500	1468702088.250		

Table I.18: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe ferox* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	36182521.000	7457345.960	0.985
3.90625	65465083.500		
7.8125	119640841.500		
15.625	210857923.500		
31.25	400371427.500		
125	704940915.500		
500	1275958339.500		

Table I.19: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe ferox* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	35718535.000	6530248.719	0.983
3.90625	63439579.500		
7.8125	96839693.500		
15.625	204304147.500		
31.25	395241659.500		
125	657383427.500		
500	1090456723.500		

Table I.20: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe ferox* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	34322852.750	7720270.478	0.976
3.90625	64724512.750		
7.8125	117842360.750		
15.625	221653798.750		
31.25	416041818.750		
62.5	725379530.750		
125	1343437834.750		
250	2304375946.750		
500	3532697162.750		

Table I.21: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe ferox* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	34149791.125	11169791.194	0.996
3.90625	64384596.125		
7.8125	116068756.125		
15.625	220836928.125		
31.25	405518216.125		
62.5	742610008.125		
125	1351429160.125		
250	2327475272.125		
500	3508206088.125		

Table I.22: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe ferox* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	26474706.625	6283700.722	0.979
3.90625	48920323.125		
7.8125	89789172.125		
15.625	168889304.125		
31.25	349398136.125		
62.5	570202232.125		
125	1080150168.125		
250	1844535400.125		
500	2896533576.125		

Table I.23: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe muth-muth* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	35384182.000	7540010.090	0.986
3.90625	64147836.000		
7.8125	117269163.000		
15.625	212166183.000		
31.25	397871451.000		
62.5	720545107.000		
250	2213229843.000		
500	3560376979.000		

Table I.24: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe muth-muth* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	11946368.750	3973034.165	0.999
3.90625	24942588.500		
7.8125	40681384.000		
15.625	77639705.000		
31.25	160100435.000		
62.5	254621207.000		
250	937957091.000		
500	2010406131.000		

Table I.25: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe muth-muth* gel

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	3147719.125	593487.326	0.992
3.90625	4101187.125		
7.8125	6169437.125		
15.625	10499249.625		
31.25	19374215.875		
62.5	43921995.375		
250	122225989.375		
500	308827261.375		

Table I.26: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 0.5% w/v *Aloe muth-muth* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	39840943.375	7480924.069	0.981
3.90625	62693558.375		
7.8125	119589773.375		
15.625	217397247.375		
31.25	404566267.375		
62.5	745819051.375		
250	2264792347.375		
500	3493268187.375		

Table I.27: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 1.5% w/v *Aloe muth-muth* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	12014921.875	3142676.786	0.995
3.90625	21785796.375		
7.8125	34883418.375		
15.625	59050167.375		
31.25	132005893.375		
62.5	208889507.375		
250	895677435.375		
500	1512093435.375		

Table I.28: Data obtained for the determination of specificity by proving linearity of FITC-dextran 4400 (FD4) in 3% w/v *Aloe muth-muth* whole leaf extract

FD4 concentration (mg/ml)	Mean fluorescent value	Slope	r ²
1.953125	2484857.375	518341.578	0.999
3.90625	3459933.875		
7.8125	5463426.625		
15.625	9326829.875		
31.25	16733369.125		
62.5	38243999.625		
250	121642595.625		
500	262300359.625		

APPENDIX J

PERMEATION DATA

J.1 Introduction

This Appendix provides all permeation data for controls and experimental groups across both models. This includes FITC-dextran 4400, Lucifer Yellow and rhodamine 123 transport alone across the RPMI 2650 cell line as well as the excised sheep nasal mucosa. The experimental groups follow with FITC-dextran 4400 and rhodamine 123 in the presence capsaicin and piperine as well as FITC-dextran 4400 in the presence of *Aloe vera*, *A. ferox* and *A. muth-muth* gel and whole leaf extract across both models. Data is presented as cumulative percentage transport and apparent permeability (P_{app}) of three transport experiments (i.e. Chambers) over a series of time.

J.2 Marker molecule permeation across the RPMI 2650 cell model

Table J.1: Cumulative percentage transport and apparent permeability (P_{app}) values of Lucifer Yellow across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.482	0.408	0.354	0.415	0.052
10		0.602	0.528	0.403	0.511	0.082
15		0.607	0.537	0.416	0.520	0.079
30		0.619	0.535	0.454	0.536	0.068
60		0.819	0.712	0.568	0.700	0.103
90		1.032	0.847	0.783	0.887	0.105
120		1.396	1.367	1.767	1.510	0.182
180		1.658	1.603	3.134	2.132	0.709
	Cumulative P_{app} ($\times 10^{-6}$)	1.123	1.111	2.197	1.477	0.509

Table J.2: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.024	0.016	0.005	0.015	0.008
10		0.040	0.037	0.035	0.037	0.002
15		0.063	0.053	0.034	0.050	0.012
30		0.141	0.094	0.118	0.118	0.019
60		0.238	0.184	0.225	0.216	0.023
90		0.345	0.314	0.323	0.327	0.013
120		0.449	0.432	0.442	0.441	0.007
180		0.560	0.538	0.580	0.559	0.017
	Cumulative P_{app} ($\times 10^{-7}$)	4.789	4.655	4.997	4.814	0.140

Table J.3: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.015	0.004	0.003	0.007	0.005
10		0.024	0.006	0.004	0.011	0.009
15		0.032	0.013	0.009	0.018	0.010
30		0.059	0.067	0.052	0.060	0.006
60		0.219	0.197	0.156	0.191	0.026
90		0.317	0.317	0.310	0.315	0.004
120		0.503	0.500	0.507	0.503	0.003
180		0.752	0.800	0.800	0.784	0.023
	Cumulative P_{app} ($\times 10^{-7}$)	6.264	6.630	6.668	6.521	0.182

Table J.4: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.051	0.043	0.036	0.043	0.006
10		0.160	0.128	0.087	0.125	0.030
15		0.209	0.174	0.113	0.165	0.039
30		0.304	0.256	0.197	0.252	0.044
60		0.610	0.505	0.417	0.511	0.079
90		1.016	0.805	0.674	0.832	0.141
120		1.504	1.116	0.967	1.196	0.226
180		2.328	1.799	1.594	1.907	0.309
	Cumulative P_{app} ($\times 10^{-6}$)	1.868	1.427	1.269	1.521	0.254

J.3 Marker molecule permeation across excised sheep nasal mucosa

Table J.5: Cumulative percentage transport and apparent permeability (P_{app}) values of Lucifer Yellow across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.293	0.297	0.241	0.277	0.025
10		0.283	0.306	0.238	0.276	0.028
15		0.330	0.337	0.253	0.307	0.038
30		0.394	0.361	0.379	0.378	0.013
60		1.651	0.611	0.664	0.976	0.478
90		2.158	2.098	0.753	1.670	0.649
120		3.243	2.610	1.361	2.404	0.782
180		3.632	3.303	2.935	3.290	0.284
	Cumulative P_{app} ($\times 10^{-6}$)	2.058	1.795	1.309	1.721	0.310

Table J.6: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.006	0.002	0.002	0.003	0.002
10		0.006	0.001	0.002	0.003	0.002
15		0.008	0.002	0.003	0.004	0.003
30		0.023	0.017	0.025	0.022	0.003
60		0.068	0.180	0.101	0.116	0.047
90		0.222	0.297	0.248	0.256	0.031
120		0.505	0.496	0.435	0.479	0.031
180		0.842	0.997	0.886	0.908	0.065
	Cumulative P_{app} ($\times 10^{-6}$)	4.307	4.985	4.384	4.558	0.303

Table J.7: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.025	0.016	0.011	0.018	0.006
10		0.024	0.017	0.007	0.016	0.007
15		0.027	0.037	0.008	0.024	0.012
30		0.040	0.072	0.027	0.046	0.019
60		0.360	0.149	0.051	0.187	0.129
90		0.553	0.340	0.126	0.340	0.174
120		0.750	0.521	0.194	0.488	0.228
180		0.882	0.888	0.440	0.736	0.210
	Cumulative P_{app} ($\times 10^{-7}$)	5.200	4.529	2.091	3.940	1.336

Table J.8: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.070	0.006	0.128	0.068	0.050
10		0.082	0.010	0.154	0.082	0.059
15		0.089	0.010	0.164	0.088	0.063
30		0.130	0.024	0.199	0.118	0.072
60		0.314	0.173	0.486	0.324	0.128
90		0.597	0.658	1.034	0.763	0.193
120		1.209	1.089	1.342	1.213	0.103
180		1.711	1.757	1.719	1.729	0.020
	Cumulative P_{app} ($\times 10^{-7}$)	8.920	9.296	9.322	9.179	0.184

J.4 FITC-dextran 4400 permeation in the presence of capsaicin across the RPMI 2650 cell line

Table J.9: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 50 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.090	0.013	0.016	0.039	0.035
10		0.603	0.057	0.047	0.236	0.260
15		0.612	0.083	0.062	0.252	0.254
30		0.610	0.137	0.174	0.307	0.215
60		0.666	0.228	0.282	0.392	0.195
90		0.832	0.425	0.392	0.550	0.200
120		0.888	0.502	0.570	0.653	0.168
180		1.119	0.735	0.848	0.901	0.161
	Cumulative P_{app} ($\times 10^{-7}$)	7.154	6.063	6.900	6.706	0.466

Table J.10: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 100 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.011	0.014	0.005	0.010	0.004
10		0.037	0.025	0.028	0.030	0.005
15		0.068	0.044	0.046	0.053	0.011
30		0.137	0.127	0.121	0.129	0.007
60		0.242	0.232	0.224	0.232	0.007
90		0.412	0.389	0.300	0.367	0.048
120		0.475	0.562	0.464	0.500	0.044
180		0.768	0.774	0.823	0.788	0.025
	Cumulative P_{app} ($\times 10^{-7}$)	6.234	6.573	6.445	6.418	0.140

Table J.11: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 200 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.019	0.006	0.007	0.011	0.006
10		0.036	0.020	0.024	0.026	0.007
15		0.044	0.035	0.041	0.040	0.003
30		0.114	0.075	0.089	0.093	0.016
60		0.199	0.174	0.175	0.182	0.011
90		0.248	0.238	0.234	0.240	0.006
120		0.405	0.374	0.458	0.412	0.035
180		0.634	0.639	0.634	0.636	0.002
	Cumulative P_{app} ($\times 10^{-7}$)	5.029	5.077	5.260	5.122	0.100

J.5 FITC-dextran 4400 permeation in the presence of capsaicin across excised sheep nasal mucosa

Table J.12: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 50 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.008	0.006	0.006	0.007	0.001
10		0.012	0.008	0.021	0.013	0.006
15		0.016	0.014	0.042	0.024	0.013
30		0.044	0.050	0.130	0.075	0.039
60		0.141	0.159	0.358	0.220	0.098
90		0.256	0.291	0.594	0.380	0.152
120		0.394	0.449	0.877	0.574	0.216
180		0.657	0.744	1.403	0.934	0.333
	Cumulative P_{app} ($\times 10^{-7}$)	3.404	3.880	7.377	4.887	1.771

Table J.13: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 100 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.003	0.001	0.001
10		0.003	0.001	0.005	0.003	0.002
15		0.010	0.006	0.011	0.009	0.002
30		0.051	0.044	0.045	0.047	0.003
60		0.196	0.189	0.194	0.193	0.003
90		0.390	0.376	0.389	0.385	0.007
120		0.569	0.579	0.560	0.569	0.008
180		0.932	0.958	0.948	0.946	0.011
	Cumulative P_{app} ($\times 10^{-7}$)	4.938	5.056	4.976	4.990	0.049

Table J.14: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 200 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.007	0.001	0.004	0.004	0.003
10		0.025	0.005	0.007	0.012	0.009
15		0.049	0.012	0.014	0.025	0.017
30		0.139	0.047	0.055	0.080	0.041
60		0.353	0.166	0.221	0.247	0.078
90		0.543	0.322	0.379	0.415	0.093
120		0.734	0.467	0.559	0.587	0.111
180		1.088	0.755	0.877	0.907	0.138
	Cumulative P_{app} ($\times 10^{-7}$)	5.849	4.004	4.685	4.846	0.762

J.6 Rhodamine 123 permeation in the presence of capsaicin across RPMI 2650 cell line

Table J.15: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.018	0.013	0.010	0.014	0.003
10		0.029	0.019	0.014	0.020	0.006
15		0.042	0.037	0.041	0.040	0.002
30		0.092	0.076	0.070	0.079	0.009
60		0.225	0.197	0.182	0.202	0.018
90		0.443	0.439	0.371	0.418	0.033
120		0.645	0.603	0.556	0.601	0.037
180		1.142	1.051	0.841	1.011	0.126
	Cumulative P_{app} ($\times 10^{-7}$)	9.112	8.498	7.025	8.211	0.876

Table J.16: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.511	0.509	0.506	0.509	0.002
10		0.774	0.806	0.791	0.790	0.013
15		0.776	0.808	0.826	0.803	0.021
30		0.970	0.989	0.956	0.972	0.014
60		1.407	1.484	1.391	1.427	0.040
90		1.890	1.996	1.914	1.933	0.045
120		2.302	2.444	2.396	2.381	0.059
180		3.078	3.227	3.145	3.150	0.061
	Cumulative P_{app} ($\times 10^{-6}$)	2.257	2.390	2.317	2.321	0.054

Table J.17: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 100 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.010	0.013	0.000	0.008	0.006
10		0.013	0.017	0.006	0.012	0.005
15		0.032	0.043	0.015	0.030	0.012
30		0.192	0.143	0.081	0.138	0.045
60		0.426	0.353	0.218	0.333	0.086
90		0.764	0.565	0.371	0.567	0.161
120		1.036	0.849	0.573	0.819	0.190
180		1.590	1.187	0.891	1.223	0.286
	Cumulative P_{app} ($\times 10^{-6}$)	1.342	1.018	0.746	1.035	0.244

Table J.18: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 100 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.236	0.260	0.247	0.248	0.010
10		0.394	0.426	0.387	0.402	0.017
15		0.420	0.442	0.407	0.423	0.015
30		0.836	0.673	0.597	0.702	0.100
60		1.491	1.226	1.019	1.245	0.193
90		2.029	1.663	1.412	1.701	0.253
120		2.468	2.098	1.803	2.123	0.272
180		3.099	2.859	2.372	2.777	0.303
	Cumulative P_{app} ($\times 10^{-6}$)	2.583	2.270	1.880	2.244	0.288

Table J.19: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 200 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.449	0.068	0.069	0.195	0.179
10		1.298	0.363	0.084	0.582	0.519
15		5.674	4.544	1.912	4.043	1.576
30		7.806	5.478	3.641	5.642	1.704
60		7.261	5.166	3.450	5.293	1.558
90		6.811	4.878	3.318	5.002	1.429
120		6.586	4.742	3.226	4.851	1.374
180		6.416	5.366	3.364	5.048	1.266
	Cumulative P_{app} ($\times 10^{-6}$)	4.449	3.755	2.661	3.621	0.736

Table J.20: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 200 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.679	0.665	0.648	0.664	0.013
10		1.058	1.051	1.052	1.054	0.003
15		1.082	1.082	1.107	1.090	0.012
30		1.242	1.209	1.240	1.230	0.015
60		1.547	1.469	1.499	1.505	0.032
90		1.855	1.802	1.765	1.808	0.037
120		2.186	2.155	2.078	2.140	0.045
180		2.842	2.704	2.644	2.730	0.083
	Cumulative P_{app} ($\times 10^{-6}$)	1.879	1.788	1.719	1.795	0.066

J.7 Rhodamine 123 permeation in the presence of capsaicin across excised sheep nasal mucosa

Table J.21: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.064	0.034	0.018	0.038	0.019
10		0.072	0.031	0.016	0.040	0.024
15		0.116	0.035	0.019	0.057	0.043
30		0.197	0.060	0.035	0.098	0.071
60		0.454	0.148	0.127	0.243	0.149
90		0.763	0.306	0.397	0.489	0.197
120		1.161	0.752	0.895	0.936	0.170
180		1.932	1.502	1.579	1.671	0.187
	Cumulative P_{app} ($\times 10^{-7}$)	9.775	7.201	7.942	8.306	1.082

Table J.22: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.918	0.346	0.117	0.460	0.337
10		0.896	0.385	0.091	0.457	0.333
15		0.850	0.435	0.082	0.456	0.314
30		0.862	0.537	0.142	0.514	0.294
60		1.032	0.798	0.378	0.736	0.271
90		1.227	1.184	0.770	1.060	0.206
120		1.538	1.824	1.590	1.651	0.124
180		2.132	2.647	2.529	2.436	0.220
	Cumulative P_{app} ($\times 10^{-6}$)	0.780	1.262	1.281	1.108	0.232

Table J.23: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 100 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.007	0.010	0.068	0.028	0.028
10		0.010	0.008	0.032	0.017	0.010
15		0.013	0.008	0.059	0.027	0.023
30		0.033	0.032	0.226	0.097	0.091
60		0.152	0.128	0.648	0.309	0.240
90		0.373	0.302	1.112	0.596	0.366
120		0.644	0.542	1.643	0.943	0.496
180		1.342	1.144	2.489	1.659	0.593
	Cumulative P_{app} ($\times 10^{-6}$)	0.659	0.558	1.326	0.847	0.341

Table J.24: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 100 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.265	0.216	0.411	0.297	0.083
10		0.289	0.230	0.504	0.341	0.118
15		0.294	0.232	0.562	0.362	0.143
30		0.369	0.314	0.742	0.475	0.190
60		0.522	0.559	1.023	0.701	0.228
90		0.695	0.866	1.186	0.916	0.204
120		0.978	1.158	1.372	1.170	0.161
180		1.451	1.916	1.658	1.675	0.190
	Cumulative P_{app} ($\times 10^{-7}$)	6.570	9.118	7.478	7.22	1.055

Table J.25: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 200 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.021	0.008	0.005	0.011	0.007
10		0.020	0.007	0.004	0.010	0.007
15		0.025	0.011	0.006	0.014	0.008
30		0.080	0.057	0.039	0.059	0.017
60		0.574	0.393	0.306	0.424	0.111
90		1.147	0.937	1.012	1.032	0.087
120		1.735	1.870	1.792	1.799	0.055
180		2.276	2.548	2.453	2.426	0.113
	Cumulative P_{app} ($\times 10^{-6}$)	1.291	1.404	1.363	1.353	0.046

Table J.26: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 200 μM capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.112	0.144	0.119	0.125	0.014
10		0.117	0.117	0.122	0.119	0.002
15		0.122	0.115	0.119	0.118	0.003
30		0.128	0.136	0.153	0.139	0.010
60		0.202	0.181	0.223	0.202	0.017
90		0.375	0.248	0.344	0.322	0.054
120		0.522	0.317	0.430	0.423	0.084
180		0.876	0.446	0.615	0.646	0.177
	Cumulative P_{app} ($\times 10^{-7}$)	4.048	1.900	2.867	2.950	0.894

J.8 FITC-dextran 4400 permeation in the presence of piperine across RPMI 2650 cell line

Table J.27: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 10 μM piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.000	0.000	0.000
10		0.008	0.016	0.010	0.011	0.003
15		0.036	0.033	0.028	0.032	0.003
30		0.169	0.129	0.129	0.142	0.019
60		0.393	0.291	0.285	0.323	0.049
90		0.637	0.492	0.482	0.537	0.071
120		0.840	0.679	0.604	0.708	0.098
180		1.185	0.981	0.874	1.013	0.129
	Cumulative P_{app} ($\times 10^{-6}$)	1.029	0.840	0.745	0.875	0.115

Table J.28: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 25 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.018	0.017	0.015	0.017	0.001
10		0.046	0.051	0.044	0.047	0.003
15		0.068	0.079	0.070	0.073	0.005
30		0.182	0.216	0.169	0.189	0.020
60		0.359	0.366	0.309	0.344	0.026
90		0.525	0.578	0.501	0.535	0.032
120		0.683	0.706	0.661	0.684	0.018
180		0.957	1.056	1.020	1.011	0.041
	Cumulative P_{app} ($\times 10^{-7}$)	8.085	8.748	8.365	8.399	0.272

Table J.29: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 50 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.000	0.000	0.000
10		0.000	0.000	0.000	0.000	0.000
15		0.015	0.015	0.021	0.017	0.003
30		0.090	0.091	0.081	0.087	0.004
60		0.241	0.237	0.222	0.233	0.008
90		0.420	0.414	0.396	0.410	0.010
120		0.958	0.555	0.545	0.686	0.192
180		1.306	0.876	0.840	1.007	0.212
	Cumulative P_{app} ($\times 10^{-6}$)	1.115	0.739	0.712	0.855	0.184

J.9 FITC-dextran 4400 permeation in the presence of piperine across excised sheep nasal mucosa

Table J.30: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 10 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.005	0.004	0.008	0.006	0.001
10		0.015	0.008	0.008	0.010	0.003
15		0.035	0.018	0.010	0.021	0.010
30		0.137	0.077	0.041	0.085	0.039
60		0.378	0.257	0.160	0.265	0.089
90		0.645	0.474	0.319	0.480	0.133
120		0.944	0.705	0.276	0.642	0.276
180		1.566	1.339	0.794	1.233	0.324
	Cumulative P_{app} ($\times 10^{-7}$)	8.181	6.773	3.772	6.242	1.839

Table J.31: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 25 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.008	0.006	0.069	0.028	0.029
10		0.011	0.008	0.078	0.033	0.032
15		0.016	0.013	0.125	0.051	0.052
30		0.151	0.061	0.284	0.166	0.091
60		0.318	0.171	0.395	0.294	0.093
90		0.576	0.420	0.480	0.492	0.064
120		0.847	0.681	0.599	0.709	0.103
180		1.214	1.099	0.822	1.045	0.165
	Cumulative P_{app} ($\times 10^{-7}$)	6.615	5.776	4.148	5.513	1.024

Table J.32: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 50 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.011	0.008	0.018	0.013	0.004
10		0.034	0.022	0.027	0.028	0.005
15		0.018	0.015	0.027	0.020	0.005
30		0.048	0.047	0.066	0.054	0.009
60		0.341	0.173	0.207	0.241	0.072
90		0.525	0.351	0.441	0.439	0.071
120		0.737	0.548	0.725	0.670	0.086
180		0.795	0.964	1.125	0.961	0.135
	Cumulative P_{app} ($\times 10^{-7}$)	4.832	4.933	5.917	5.227	0.489

J.10 Rhodamine 123 permeation in the presence of piperine across RPMI 2650 cell line

Table J.33: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 10 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.009	0.000	0.000	0.003	0.004
10		0.037	0.005	0.000	0.014	0.016
15		0.056	0.014	0.000	0.023	0.024
30		0.131	0.060	0.042	0.077	0.038
60		0.371	0.155	0.112	0.213	0.113
90		0.610	0.298	0.201	0.370	0.175
120		0.798	0.389	0.344	0.510	0.205
180		1.176	0.585	0.592	0.784	0.277
	Cumulative P_{app} ($\times 10^{-7}$)	9.992	5.009	4.813	6.605	2.397

Table J.34: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 10 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.001	0.000	0.000
10		0.007	0.005	0.015	0.009	0.004
15		0.024	0.025	0.041	0.030	0.008
30		0.357	0.246	0.229	0.277	0.057
60		0.836	0.572	0.647	0.685	0.111
90		1.346	1.095	1.116	1.186	0.113
120		1.602	1.418	1.465	1.495	0.078
180		2.023	1.996	2.137	2.052	0.061
	Cumulative P_{app} ($\times 10^{-6}$)	1.852	1.751	1.849	1.818	0.047

Table J.35: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 25 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.016	0.012	0.000	0.009	0.007
10		0.025	0.031	0.008	0.021	0.010
15		0.032	0.044	0.015	0.030	0.012
30		0.174	0.137	0.111	0.141	0.026
60		0.341	0.352	0.304	0.332	0.021
90		0.646	0.645	0.502	0.598	0.068
120		0.912	0.979	0.834	0.909	0.059
180		1.277	1.244	1.111	1.211	0.072
	Cumulative P_{app} ($\times 10^{-6}$)	1.099	1.102	0.973	1.058	0.060

Table J.36: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 25 μ M piperine across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.366	0.362	0.362	0.363	0.002
10		0.580	0.582	0.600	0.587	0.009
15		0.599	0.599	0.608	0.602	0.004
30		0.810	0.742	0.753	0.768	0.030
60		1.309	1.276	1.246	1.277	0.026
90		2.058	1.921	1.857	1.945	0.084
120		2.410	2.295	2.320	2.342	0.049
180		3.434	3.357	3.299	3.363	0.055
	Cumulative P_{app} ($\times 10^{-6}$)	2.658	2.565	2.521	2.581	0.057

Table J.37: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.015	0.003	0.006	0.008	0.005
10		0.014	0.008	0.009	0.010	0.003
15		0.021	0.015	0.022	0.019	0.003
30		0.414	0.205	0.143	0.254	0.116
60		0.814	0.501	0.326	0.547	0.202
90		1.214	0.630	0.523	0.789	0.304
120		1.509	0.932	0.809	1.083	0.305
180		1.968	1.494	1.211	1.558	0.312
	Cumulative P_{app} ($\times 10^{-6}$)	1.759	1.239	1.021	1.339	0.310

Table J.38: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.283	0.285	0.267	0.278	0.008
10		0.462	0.448	0.439	0.450	0.010
15		0.475	0.468	0.464	0.469	0.005
30		0.886	0.765	0.684	0.778	0.083
60		1.558	1.304	1.166	1.343	0.162
90		2.331	1.975	1.816	2.041	0.215
120		2.875	2.339	2.294	2.502	0.264
180		3.838	3.305	3.276	3.473	0.258
	Cumulative P_{app} ($\times 10^{-6}$)	3.146	2.629	2.589	2.788	0.254

J.11 Rhodamine 123 permeation in the presence of piperine across excised sheep nasal mucosa

Table J.39: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 10 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.032	0.029	0.093	0.051	0.030
10		0.039	0.030	0.119	0.062	0.040
15		0.047	0.039	0.153	0.080	0.052
30		0.080	0.074	0.234	0.129	0.074
60		0.156	0.206	0.445	0.269	0.126
90		0.296	0.387	0.650	0.444	0.150
120		0.484	0.597	0.847	0.643	0.152
180		0.863	1.019	0.523	0.802	0.207
	Cumulative P_{app} ($\times 10^{-7}$)	4.247	5.177	3.591	4.338	0.650

Table J.40: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 10 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.711	0.340	0.183	0.412	0.221
10		0.720	0.364	0.200	0.428	0.217
15		0.713	0.359	0.193	0.422	0.217
30		0.771	0.472	0.282	0.508	0.201
60		0.950	0.693	0.621	0.755	0.141
90		1.355	1.203	1.126	1.228	0.095
120		2.360	1.855	1.629	1.948	0.306
180		3.749	4.118	2.689	3.519	0.606
	Cumulative P_{app} ($\times 10^{-6}$)	1.638	1.838	1.336	1.604	0.207

Table J.41: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 25 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.002	0.008	0.008	0.006	0.003
10		0.031	0.021	0.012	0.021	0.008
15		0.051	0.093	0.049	0.064	0.020
30		0.075	0.103	0.040	0.073	0.025
60		0.221	0.173	0.168	0.188	0.024
90		0.543	0.317	0.450	0.437	0.093
120		0.859	0.486	0.764	0.703	0.158
180		1.516	0.954	1.422	1.297	0.246
	Cumulative P_{app} ($\times 10^{-7}$)	7.726	4.602	7.140	6.489	1.356

Table J.42: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 25 μ M piperine across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.025	0.054	0.138	0.072	0.048
10		0.029	0.062	0.196	0.096	0.072
15		0.054	0.078	0.190	0.107	0.059
30		0.092	0.316	0.323	0.244	0.107
60		0.305	0.707	0.501	0.504	0.164
90		0.885	1.102	0.997	0.995	0.089
120		1.450	1.505	1.285	1.413	0.093
180		2.412	2.498	2.179	2.363	0.135
	Cumulative P_{app} ($\times 10^{-6}$)	1.252	1.290	1.069	1.204	0.094

Table J.43: Absorptive cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.025	0.030	0.043	0.033	0.008
10		0.026	0.030	0.048	0.035	0.009
15		0.034	0.036	0.050	0.040	0.007
30		0.081	0.088	0.081	0.083	0.003
60		0.379	0.351	0.195	0.308	0.081
90		0.893	0.766	0.385	0.681	0.216
120		1.376	1.272	0.643	1.097	0.324
180		2.372	2.150	1.093	1.872	0.558
	Cumulative P_{app} ($\times 10^{-6}$)	1.228	1.110	0.547	0.962	0.297

Table J.44: Secretory cumulative percentage transport and apparent permeability (P_{app}) values of rhodamine 123 in the presence of 50 μ M capsaicin across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.897	0.466	0.174	0.322	0.297
10		0.946	0.468	0.180	0.324	0.316
15		0.910	0.472	0.186	0.328	0.298
30		0.954	0.508	0.232	0.384	0.298
60		1.038	0.605	0.387	0.547	0.270
90		1.205	0.882	0.723	0.871	0.200
120		1.400	1.258	1.049	1.228	0.144
180		1.922	1.831	1.699	1.946	0.091
	Cumulative P_{app} ($\times 10^{-7}$)	6.538	7.824	8.198	7.520	0.711

J.12 FITC-dextran 4400 permeation in the presence of aloe species across RPMI 2650 cell line

Table J.45: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe vera* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.009	0.010	0.009	0.009	0.001
10		0.042	0.054	0.044	0.046	0.005
15		0.058	0.082	0.052	0.064	0.013
30		0.098	0.107	0.104	0.103	0.004
60		0.225	0.228	0.198	0.217	0.014
90		0.346	0.375	0.306	0.342	0.028
120		0.476	0.525	0.472	0.491	0.024
180		0.696	0.633	0.593	0.640	0.042
	Cumulative P_{app} ($\times 10^{-7}$)	5.772	5.500	5.084	5.452	0.283

Table J.46: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe vera* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.020	0.026	0.032	0.026	0.005
10		0.046	0.054	0.055	0.051	0.004
15		0.071	0.083	0.076	0.077	0.005
30		0.131	0.164	0.144	0.146	0.014
60		0.283	0.260	0.226	0.256	0.023
90		0.380	0.411	0.314	0.368	0.041
120		0.562	0.554	0.528	0.548	0.015
180		0.770	0.764	0.792	0.775	0.012
	Cumulative P_{app} ($\times 10^{-7}$)	6.429	6.315	6.262	6.335	0.070

Table J.47: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe vera* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.032	0.027	0.027	0.029	0.002
10		0.050	0.052	0.045	0.049	0.003
15		0.083	0.083	0.070	0.078	0.006
30		0.189	0.153	0.141	0.161	0.020
60		0.292	0.240	0.179	0.237	0.046
90		0.386	0.350	0.337	0.358	0.021
120		0.543	0.486	0.453	0.494	0.037
180		0.719	0.638	0.702	0.687	0.035
	Cumulative P_{app} ($\times 10^{-7}$)	5.940	5.287	5.585	5.604	0.267

Table J.48: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe vera* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.009	0.005	0.004	0.006	0.002
10		0.024	0.024	0.028	0.025	0.002
15		0.038	0.043	0.039	0.040	0.002
30		0.076	0.091	0.088	0.085	0.007
60		0.147	0.222	0.212	0.194	0.033
90		0.258	0.377	0.334	0.323	0.049
120		0.329	0.565	0.508	0.467	0.100
180		0.513	0.811	0.738	0.687	0.127
	Cumulative P_{app} ($\times 10^{-7}$)	4.203	6.857	6.183	5.747	1.127

Table J.49: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe vera* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.007	0.006	0.005	0.006	0.001
10		0.031	0.034	0.031	0.032	0.001
15		0.045	0.052	0.041	0.046	0.005
30		0.126	0.129	0.102	0.119	0.012
60		0.353	0.275	0.249	0.292	0.044
90		0.542	0.428	0.356	0.442	0.077
120		0.681	0.548	0.539	0.589	0.065
180		1.028	0.897	0.890	0.938	0.063
	Cumulative P_{app} ($\times 10^{-7}$)	8.699	7.307	7.188	7.732	0.686

Table J.50: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe vera* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.003	0.002	0.005	0.003	0.001
10		0.014	0.016	0.018	0.016	0.002
15		0.028	0.033	0.029	0.030	0.002
30		0.052	0.083	0.070	0.068	0.013
60		0.171	0.210	0.162	0.181	0.021
90		0.269	0.280	0.307	0.285	0.016
120		0.428	0.451	0.573	0.484	0.063
180		0.827	2.404	1.434	1.555	0.650
	Cumulative P_{app} ($\times 10^{-6}$)	0.646	1.580	1.050	1.092	0.382

Table J.51: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe ferox* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.020	0.015	0.010	0.015	0.004
10		0.044	0.040	0.029	0.038	0.006
15		0.053	0.051	0.048	0.051	0.002
30		0.103	0.096	0.081	0.094	0.009
60		0.194	0.182	0.175	0.184	0.008
90		0.353	0.275	0.474	0.367	0.082
120		0.456	0.439	0.593	0.496	0.069
180		0.577	0.523	0.660	0.587	0.056
	Cumulative P_{app} ($\times 10^{-7}$)	4.989	4.528	6.176	5.231	0.694

Table J.52: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe ferox* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.036	0.020	0.023	0.026	0.007
10		0.066	0.037	0.040	0.048	0.013
15		0.092	0.064	0.064	0.073	0.013
30		0.115	0.100	0.119	0.112	0.008
60		0.203	0.156	0.185	0.181	0.019
90		0.300	0.297	0.327	0.308	0.014
120		0.403	0.428	0.445	0.426	0.017
180		0.576	0.639	0.597	0.604	0.026
	Cumulative P_{app} ($\times 10^{-7}$)	4.559	5.172	4.989	4.907	0.257

Table J.53: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe ferox* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.020	0.020	0.024	0.021	0.002
10		0.036	0.046	0.040	0.041	0.004
15		0.067	0.076	0.081	0.075	0.006
30		0.103	0.129	0.115	0.115	0.011
60		0.173	0.190	0.209	0.190	0.015
90		0.330	0.393	0.370	0.364	0.026
120		0.452	0.487	0.546	0.495	0.039
180		0.713	0.698	0.748	0.720	0.021
	Cumulative P_{app} ($\times 10^{-7}$)	5.726	5.776	6.228	5.910	0.226

Table J.54: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe ferox* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.036	0.020	0.015	0.024	0.009
10		0.063	0.046	0.033	0.047	0.013
15		0.113	0.077	0.073	0.088	0.018
30		0.265	0.147	0.217	0.210	0.048
60		0.357	0.246	0.255	0.286	0.051
90		0.499	0.395	0.368	0.420	0.057
120		0.715	0.555	0.513	0.594	0.087
180		0.911	0.791	0.761	0.821	0.065
	Cumulative P_{app} ($\times 10^{-7}$)	7.594	6.518	6.129	6.747	0.620

Table J.55: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe ferox* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.008	0.012	0.013	0.011	0.003
10		0.053	0.043	0.027	0.041	0.011
15		0.158	0.050	0.046	0.085	0.052
30		0.308	0.098	0.092	0.166	0.101
60		0.385	0.188	0.202	0.258	0.090
90		0.487	0.280	0.265	0.344	0.101
120		0.611	0.392	0.430	0.478	0.096
180		0.760	0.556	0.618	0.645	0.085
	Cumulative P_{app} ($\times 10^{-7}$)	6.319	4.588	5.098	5.335	0.277

Table J.56: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe ferox* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.003	0.008	0.007	0.006	0.002
10		0.020	0.029	0.018	0.023	0.005
15		0.153	0.038	0.026	0.072	0.057
30		0.294	0.090	0.076	0.153	0.100
60		0.375	0.197	0.220	0.264	0.079
90		0.463	0.227	0.749	0.480	0.214
120		0.581	0.429	0.914	0.641	0.203
180		0.693	0.617	1.039	0.783	0.184
	Cumulative P_{app} ($\times 10^{-7}$)	5.904	5.049	9.911	6.955	2.119

Table J.57: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe muth-muth* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.013	0.010	0.015	0.012	0.002
10		0.034	0.031	0.032	0.032	0.001
15		0.064	0.072	0.068	0.068	0.004
30		0.116	0.101	0.111	0.109	0.006
60		0.266	0.234	0.202	0.234	0.026
90		0.367	0.371	0.346	0.361	0.011
120		0.498	0.532	0.495	0.509	0.017
180		0.513	0.550	1.093	0.719	0.265
	Cumulative P_{app} ($\times 10^{-7}$)	4.774	5.112	8.135	6.007	1.511

Table J.58: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe muth-muth* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.008	0.012	0.018	0.013	0.004
10		0.033	0.042	0.040	0.038	0.004
15		0.065	0.090	0.086	0.080	0.011
30		0.116	0.152	0.166	0.144	0.021
60		0.290	0.269	0.235	0.265	0.023
90		0.430	0.530	0.481	0.480	0.041
120		0.586	0.706	0.643	0.645	0.049
180		0.597	0.765	0.893	0.752	0.121
	Cumulative P_{app} ($\times 10^{-7}$)	5.654	7.035	7.492	6.727	0.782

Table J.59: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe muth-muth* gel across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.000	0.000	0.000
10		0.017	0.000	0.001	0.006	0.008
15		0.026	0.023	0.035	0.028	0.005
30		0.137	0.103	0.122	0.121	0.014
60		0.350	0.373	0.330	0.351	0.017
90		0.614	0.458	0.381	0.484	0.097
120		0.732	0.725	0.550	0.669	0.084
180		1.079	1.060	0.937	1.025	0.063
	Cumulative P_{app} ($\times 10^{-7}$)	9.343	9.049	7.615	8.667	0.755

Table J.60: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe muth-muth* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.130	0.043	0.061
10		0.001	0.009	0.241	0.084	0.112
15		0.024	0.032	0.240	0.099	0.100
30		0.135	0.094	0.263	0.164	0.072
60		0.322	0.234	0.360	0.305	0.053
90		0.473	0.341	0.432	0.415	0.055
120		0.573	0.458	0.561	0.531	0.051
180		0.971	0.798	0.795	0.854	0.082
	Cumulative P_{app} ($\times 10^{-7}$)	8.035	6.463	5.451	6.650	1.063

Table J.61: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe muth-muth* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.011	0.012	0.015	0.012	0.002
10		0.053	0.051	0.051	0.052	0.001
15		0.085	0.079	0.076	0.080	0.004
30		0.162	0.134	0.163	0.153	0.014
60		0.305	0.304	0.316	0.309	0.006
90		0.551	0.494	0.456	0.500	0.039
120		0.726	0.667	0.638	0.677	0.036
180		0.862	0.807	0.909	0.859	0.042
	Cumulative P_{app} ($\times 10^{-7}$)	7.679	7.133	7.553	7.455	0.234

Table J.62: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe muth-muth* whole leaf extract across the RPMI 2650 cell line

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.009	0.014	0.019	0.014	0.004
10		0.044	0.055	0.056	0.052	0.005
15		0.080	0.085	0.085	0.083	0.002
30		0.174	0.148	0.168	0.163	0.011
60		0.319	0.310	0.327	0.319	0.007
90		0.506	0.494	0.496	0.498	0.005
120		0.784	0.664	0.689	0.712	0.052
180		1.232	0.995	1.112	1.113	0.097
	Cumulative P_{app} ($\times 10^{-7}$)	9.984	8.191	8.920	9.032	0.736

J.13 FITC-dextran 4400 permeation in the presence of aloe species across excised sheep nasal mucosa

Table J.63: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe vera* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.041	0.027	0.043	0.037	0.007
10		0.045	0.051	0.057	0.051	0.005
15		0.052	0.066	0.064	0.061	0.006
30		0.094	0.142	0.129	0.122	0.020
60		0.222	0.314	0.286	0.274	0.038
90		0.417	0.513	0.553	0.494	0.057
120		0.665	0.741	0.862	0.756	0.081
180		1.242	1.220	1.279	1.247	0.024
	Cumulative P_{app} ($\times 10^{-7}$)	6.100	6.230	6.699	6.343	0.257

Table J.64: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe vera* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.016	0.011	0.010	0.012	0.003
10		0.026	0.015	0.015	0.018	0.005
15		0.040	0.019	0.020	0.026	0.010
30		0.123	0.053	0.055	0.077	0.032
60		0.392	0.161	0.170	0.241	0.107
90		0.733	0.293	0.308	0.445	0.204
120		1.151	0.440	0.468	0.686	0.329
180		2.132	0.702	0.763	1.199	0.660
	Cumulative P_{app} ($\times 10^{-6}$)	1.076	0.368	0.398	0.614	0.327

Table J.65: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe vera* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.008	0.005	0.104	0.000	0.000
10		0.008	0.146	0.130	0.039	0.046
15		0.008	0.019	0.108	0.095	0.061
30		0.013	0.013	0.120	0.045	0.045
60		0.049	0.019	0.220	0.049	0.050
90		0.202	2.887	0.520	0.096	0.089
120		0.299	3.034	0.736	1.203	1.198
180		0.463	2.838	1.070	1.356	1.200
	Cumulative P_{app} ($\times 10^{-6}$)	0.247	1.913	0.539	0.900	0.726

Table J.66: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe vera* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.017	0.000	0.001	0.006	0.008
10		0.018	0.000	0.007	0.008	0.007
15		0.021	0.006	0.036	0.021	0.012
30		0.057	0.040	0.048	0.048	0.007
60		0.173	0.154	0.151	0.159	0.010
90		0.307	0.334	0.325	0.322	0.011
120		0.468	0.472	0.495	0.478	0.012
180		0.788	0.766	0.735	0.763	0.022
	Cumulative P_{app} ($\times 10^{-7}$)	4.053	4.089	3.950	4.031	0.059

Table J.67: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe vera* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.317	0.278	0.397	0.331	0.050
10		0.343	0.473	0.426	0.414	0.054
15		0.337	0.458	0.420	0.405	0.050
30		0.385	0.618	0.482	0.495	0.096
60		0.509	0.627	0.538	0.558	0.050
90		0.665	0.768	0.632	0.688	0.058
120		0.798	0.898	0.673	0.790	0.092
180		1.028	1.133	0.753	0.972	0.160
	Cumulative P_{app} ($\times 10^{-7}$)	4.385	4.624	2.659	3.889	0.876

Table J.68: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe vera* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.252	0.184	0.206	0.214	0.028
10		0.263	0.208	0.222	0.231	0.023
15		0.252	0.193	0.223	0.222	0.024
30		0.325	0.264	0.310	0.300	0.026
60		0.419	0.367	0.504	0.430	0.056
90		0.651	0.666	0.770	0.696	0.053
120		0.873	1.026	1.029	0.976	0.073
180		1.266	1.379	1.518	1.387	0.103
	Cumulative P_{app} ($\times 10^{-7}$)	5.768	6.804	7.322	6.631	0.646

Table J.69: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe ferox* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.019	0.008	0.023	0.017	0.007
10		0.040	0.012	0.050	0.034	0.016
15		0.058	0.025	0.079	0.054	0.022
30		0.117	0.127	0.128	0.124	0.005
60		0.213	0.269	0.205	0.229	0.028
90		0.321	0.467	0.289	0.359	0.078
120		0.434	2.347	0.453	1.078	0.898
180		0.655	1.316	0.554	0.842	0.338
	Cumulative P_{app} ($\times 10^{-7}$)	3.378	9.776	2.946	5.367	3.123

Table J.70: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe ferox* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.004	0.002	0.003	0.003	0.001
10		0.007	0.002	0.068	0.026	0.030
15		0.012	0.002	0.041	0.018	0.017
30		0.051	0.032	0.096	0.060	0.027
60		0.201	0.091	0.163	0.152	0.046
90		0.362	0.191	0.444	0.332	0.106
120		0.506	0.302	0.613	0.474	0.129
180		0.717	0.523	0.886	0.709	0.148
	Cumulative P_{app} ($\times 10^{-7}$)	3.973	2.706	4.731	3.803	0.835

Table J.71: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe ferox* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.001	0.026	0.000	0.009	0.012
10		0.004	0.040	0.000	0.015	0.018
15		0.011	0.051	0.006	0.023	0.020
30		0.132	0.098	0.064	0.098	0.028
60		0.516	0.187	0.239	0.314	0.144
90		0.914	0.364	0.594	0.624	0.226
120		1.295	0.679	1.113	1.029	0.259
180		1.652	1.107	1.422	1.394	0.223
	Cumulative P_{app} ($\times 10^{-7}$)	9.523	5.594	8.026	7.714	1.619

Table J.72: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe ferox* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.012	0.002	0.008	0.007	0.004
10		0.014	0.002	0.011	0.009	0.005
15		0.017	0.003	0.009	0.010	0.006
30		0.054	0.026	0.047	0.042	0.012
60		0.175	0.110	0.224	0.170	0.046
90		0.439	0.296	0.376	0.370	0.058
120		0.779	0.510	0.552	0.614	0.118
180		1.086	1.032	0.758	0.959	0.144
	Cumulative P_{app} ($\times 10^{-7}$)	5.922	5.124	4.225	5.090	0.693

Table J.73: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe ferox* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.021	0.062	0.000	0.028	0.026
10		0.046	0.002	0.018	0.022	0.018
15		0.146	0.020	0.009	0.058	0.062
30		0.302	0.059	0.083	0.148	0.109
60		0.602	0.246	0.335	0.394	0.151
90		0.819	0.454	0.648	0.640	0.149
120		1.260	0.736	0.860	0.952	0.224
180		1.367	1.054	1.279	1.233	0.132
	Cumulative P_{app} ($\times 10^{-7}$)	7.902	5.670	7.028	6.867	0.918

Table J.74: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe ferox* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.010	0.002	0.000	0.004	0.004
10		0.005	0.003	0.000	0.003	0.002
15		0.013	0.004	0.002	0.006	0.005
30		0.235	0.393	0.565	0.398	0.135
60		0.929	0.627	0.705	0.754	0.128
90		1.359	0.902	0.819	1.027	0.237
120		1.564	1.052	0.941	1.185	0.271
180		2.012	1.344	1.493	1.616	0.286
	Cumulative P_{app} ($\times 10^{-6}$)	1.177	0.770	0.784	0.910	0.189

Table J.75: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe muth-muth* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.007	0.005	0.003	0.005	0.001
10		0.008	0.008	0.008	0.008	0.000
15		0.009	0.013	0.005	0.009	0.003
30		0.025	0.089	0.047	0.054	0.027
60		0.188	0.206	0.248	0.214	0.025
90		0.388	0.356	0.392	0.379	0.016
120		0.544	0.611	0.602	0.586	0.029
180		0.812	0.906	0.837	0.852	0.040
	Cumulative P_{app} ($\times 10^{-7}$)	4.446	4.840	4.651	4.646	0.161

Table J.76: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe muth-muth* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.000	0.000	0.000
10		0.000	0.000	0.000	0.000	0.000
15		0.001	0.000	0.001	0.001	0.001
30		0.025	0.172	0.023	0.073	0.070
60		0.096	0.281	0.241	0.206	0.079
90		0.204	0.480	0.492	0.392	0.133
120		0.346	0.609	0.675	0.543	0.142
180		0.587	0.874	1.586	1.016	0.420
	Cumulative P_{app} ($\times 10^{-7}$)	3.055	4.819	7.735	5.203	1.930

Table J.77: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe muth-muth* gel across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.005	0.000	0.002	0.002
10		0.000	0.025	0.000	0.008	0.012
15		0.000	0.053	0.008	0.020	0.024
30		0.047	0.164	0.074	0.095	0.050
60		0.160	0.401	0.236	0.266	0.101
90		0.330	0.667	0.418	0.472	0.143
120		0.581	0.955	0.638	0.725	0.164
180		0.988	1.444	0.971	1.134	0.219
	Cumulative P_{app} ($\times 10^{-7}$)	5.128	7.710	5.236	6.025	1.192

Table J.78: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 0.5% w/v *Aloe muth-muth* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.003	0.004	0.002	0.002
10		0.000	0.009	0.001	0.003	0.004
15		0.002	0.019	0.007	0.010	0.007
30		0.030	0.091	0.056	0.059	0.025
60		0.094	0.261	0.173	0.176	0.068
90		0.231	0.447	0.375	0.351	0.090
120		0.378	0.684	0.620	0.561	0.132
180		0.553	1.000	0.959	0.837	0.202
	Cumulative P_{app} ($\times 10^{-7}$)	3.014	5.424	5.113	4.517	1.071

Table J.79: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 1.5% w/v *Aloe muth-muth* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.011	0.008	0.051	0.023	0.020
10		0.025	0.012	0.065	0.034	0.023
15		0.023	0.024	0.073	0.040	0.023
30		0.065	0.093	0.101	0.086	0.015
60		0.107	0.156	0.160	0.141	0.024
90		0.260	0.477	0.270	0.336	0.100
120		0.370	0.702	0.372	0.481	0.156
180		0.570	1.089	0.630	0.763	0.232
	Cumulative P_{app} ($\times 10^{-7}$)	2.985	5.789	3.018	3.931	1.314

Table J.80: Cumulative percentage transport and apparent permeability (P_{app}) values of FITC-dextran 4400 in the presence of 3% w/v *Aloe muth-muth* whole leaf extract across excised sheep nasal mucosa

Time (min)		Chamber 1	Chamber 2	Chamber 3	Average	Standard deviation
5	Percentage transport	0.000	0.000	0.000	0.000	0.000
10		0.000	0.000	0.000	0.000	0.000
15		0.002	0.000	0.000	0.001	0.001
30		0.028	0.041	0.010	0.026	0.013
60		0.190	0.258	0.169	0.206	0.038
90		0.917	0.409	0.399	0.575	0.242
120		1.348	0.795	0.823	0.989	0.254
180		1.508	0.976	1.006	1.163	0.244
	Cumulative P_{app} ($\times 10^{-7}$)	9.160	5.588	5.762	6.837	1.645

APPENDIX K

VISCOSITY OF ALOE SUSPENSIONS

K.1 Introduction

Viscosity of Krebs-Ringer bicarbonate buffer as well as *Aloe vera*, *A. ferox* and *A. muth-muth* gel and whole leaf extract was determined using an TA Instruments ARES-G2 (Serial nr: 4010-0677) across all three concentrations deployed (0.5% w/v, 1.5% w/v and 3% w/v) in order to elucidate the effects it could possibly have on FITC-dextran 4400 molecules suspended in it when applied for permeation studies. Data is present as viscosity (Pa·s) against a progressively increasing shear rate (1/s).

K.2 Viscosity values

Table K.1: Viscosity values of Krebs-Ringer bicarbonate buffer samples

Shear rate (1/s)	Viscosity (Pa·s)
	Krebs-Ringer bicarbonate buffer
0.100	1.283E-02
0.117	1.487E-02
0.136	1.262E-02
0.159	1.412E-02
0.185	1.734E-02
0.215	2.047E-02
0.251	2.073E-02
0.293	2.341E-02
0.342	2.623E-02
0.398	2.675E-02
0.464	2.866E-02
0.541	2.818E-02
0.631	2.936E-02
0.736	2.727E-02
0.858	2.442E-02
1.000	2.220E-02
1.166	1.939E-02
1.360	1.712E-02
1.585	1.305E-02
1.848	1.440E-02
2.155	1.660E-02
2.512	1.289E-02
2.929	1.648E-02
3.415	1.486E-02
3.982	1.339E-02
4.642	1.444E-02
5.412	1.142E-02
Average	-0.001
Regression (r ²)	0.254

Table K.2: Viscosity values of *Aloe vera* gel samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	4.382E-03	7.177E-03	3.165E-02
0.117	3.597E-03	8.736E-03	3.647E-02
0.136	5.307E-03	8.781E-03	4.020E-02
0.159	8.200E-03	9.929E-03	4.083E-02
0.185	8.008E-03	1.045E-02	4.359E-02
0.215	6.778E-03	1.070E-02	4.336E-02
0.251	5.753E-03	1.130E-02	4.483E-02
0.293	3.897E-03	1.116E-02	4.761E-02
0.341	4.961E-03	1.138E-02	4.985E-02
0.398	5.110E-03	1.153E-02	5.104E-02
0.464	6.302E-03	1.514E-02	5.095E-02
0.541	7.056E-03	1.301E-02	5.442E-02
0.631	9.127E-03	1.326E-02	5.619E-02
0.736	6.276E-03	1.523E-02	6.005E-02
0.858	7.658E-03	1.382E-02	6.116E-02
1.000	6.784E-03	1.713E-02	6.584E-02
1.166	8.282E-03	1.715E-02	6.980E-02
1.360	8.726E-03	1.770E-02	7.283E-02
1.585	7.146E-03	1.897E-02	7.831E-02
1.848	8.478E-03	2.173E-02	8.256E-02
2.155	8.382E-03	2.201E-02	8.768E-02
2.512	1.387E-02	2.377E-02	9.026E-02
2.929	1.174E-02	2.815E-02	9.647E-02
3.415	1.095E-02	3.145E-02	1.033E-01
3.982	1.163E-02	3.136E-02	1.131E-01
4.642	1.431E-02	3.396E-02	1.241E-01
5.412	1.500E-02	3.761E-02	1.322E-01
Average	1.832E-03	5.572E-03	1.809E-02
Regression (r^2)	0.787	0.971	0.968

Table K.3: Viscosity values of *Aloe vera* whole leaf extract samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	2.341E-03	7.555E-04	-4.407E-04
0.117	1.502E-03	1.113E-03	-6.566E-05
0.136	3.234E-03	2.589E-03	4.868E-04
0.159	5.267E-03	3.387E-03	1.891E-03
0.185	3.698E-03	3.331E-03	2.363E-03
0.215	1.870E-03	3.819E-03	3.783E-03
0.251	2.327E-03	5.675E-03	3.881E-03
0.293	1.537E-03	3.676E-03	5.013E-03
0.341	1.805E-03	2.693E-03	7.887E-03
0.398	4.446E-03	1.948E-03	9.806E-03
0.464	5.184E-03	1.611E-03	9.502E-03
0.541	2.073E-03	8.184E-04	9.629E-03
0.631	2.990E-03	1.527E-03	1.004E-02
0.736	3.683E-03	1.217E-03	9.224E-03
0.858	4.762E-03	5.106E-03	8.638E-03
1.000	7.188E-03	4.010E-03	8.671E-03
1.166	5.089E-03	2.674E-03	6.386E-03
1.360	5.190E-03	3.897E-03	8.977E-03
1.585	4.851E-03	4.168E-03	7.455E-03
1.848	5.584E-03	3.385E-03	5.577E-03
2.155	5.432E-03	4.288E-03	3.756E-03
2.512	6.320E-03	5.140E-03	7.334E-03
2.929	5.017E-03	7.852E-03	8.247E-03
3.415	5.290E-03	8.466E-03	1.237E-02
3.982	3.853E-03	5.443E-03	1.237E-02
4.642	5.454E-03	7.340E-03	1.173E-02
5.412	5.586E-03	7.356E-03	1.331E-02
Average	5.629E-04	1.119E-03	1.689E-03
Regression (r^2)	0.279	0.597	0.421

Table K.4: Viscosity values of *Aloe ferox* gel samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	1.550E-03	8.893E-03	8.476E-03
0.117	3.031E-03	7.036E-03	5.897E-03
0.136	2.240E-03	8.547E-03	8.133E-03
0.159	4.151E-03	1.013E-02	7.930E-03
0.185	4.186E-03	1.101E-02	1.026E-02
0.215	4.836E-03	1.353E-02	1.106E-02
0.251	6.187E-03	1.429E-02	9.808E-03
0.293	6.626E-03	1.464E-02	1.059E-02
0.341	6.314E-03	1.799E-02	1.313E-02
0.398	7.936E-03	1.664E-02	1.298E-02
0.464	8.072E-03	1.545E-02	1.572E-02
0.541	8.677E-03	1.888E-02	1.237E-02
0.631	9.638E-03	1.777E-02	1.306E-02
0.736	9.946E-03	1.620E-02	1.200E-02
0.858	9.347E-03	1.820E-02	1.136E-02
1.000	9.937E-03	1.920E-02	1.032E-02
1.166	9.614E-03	1.980E-02	1.132E-02
1.360	1.131E-02	1.724E-02	9.855E-03
1.585	9.158E-03	1.547E-02	1.274E-02
1.848	9.154E-03	1.519E-02	1.291E-02
2.155	1.003E-02	1.656E-02	1.334E-02
2.512	9.682E-03	1.503E-02	1.429E-02
2.929	9.964E-03	1.679E-02	1.364E-02
3.415	1.132E-02	1.643E-02	1.451E-02
3.982	1.069E-02	1.622E-02	1.452E-02
4.642	9.334E-03	1.832E-02	1.451E-02
5.412	9.966E-03	1.782E-02	1.659E-02
Average	1.161E-03	9.572E-04	1.168E-03
Regression (r^2)	0.378	0.178	0.477

Table K.5: Viscosity values of *Aloe ferox* whole leaf extract samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	-4.651E-03	9.384E-03	3.939E-02
0.117	-4.495E-03	1.066E-02	3.101E-02
0.136	-4.224E-03	9.101E-03	3.014E-02
0.159	-1.385E-03	1.199E-02	3.198E-02
0.185	-1.221E-03	1.170E-02	2.489E-02
0.215	-2.838E-03	1.143E-02	2.707E-02
0.251	-3.987E-03	1.240E-02	2.710E-02
0.293	-2.536E-04	1.246E-02	2.847E-02
0.341	-1.316E-03	1.303E-02	2.806E-02
0.398	2.989E-03	1.272E-02	2.669E-02
0.464	-1.392E-03	1.306E-02	2.865E-02
0.541	-1.520E-05	1.654E-02	2.679E-02
0.631	1.761E-03	1.683E-02	2.413E-02
0.736	4.039E-04	1.654E-02	2.806E-02
0.858	7.308E-04	1.378E-02	2.600E-02
1.000	-3.456E-04	1.302E-02	2.368E-02
1.166	-8.948E-05	1.316E-02	2.695E-02
1.360	-6.974E-04	1.355E-02	2.796E-02
1.585	-2.628E-04	1.283E-02	3.120E-02
1.848	-2.135E-03	1.195E-02	3.530E-02
2.155	-1.353E-03	1.150E-02	3.695E-02
2.512	2.871E-05	1.475E-02	3.887E-02
2.929	2.955E-03	1.681E-02	4.144E-02
3.415	1.111E-03	1.964E-02	4.959E-02
3.982	1.300E-03	2.181E-02	5.077E-02
4.642	1.682E-03	2.054E-02	5.453E-02
5.412	2.232E-03	1.919E-02	6.152E-02
Average	8.283E-04	1.731E-03	6.026E-03
Regression (r^2)	0.333	0.616	0.816

Table K.6: Viscosity values of *Aloe muth-muth* gel samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	3.168E-02	3.312E-03	2.993E-03
0.117	3.489E-02	2.597E-04	3.551E-03
0.136	3.700E-02	1.272E-03	4.393E-03
0.159	3.310E-02	9.345E-04	3.344E-03
0.185	3.234E-02	1.591E-03	2.629E-03
0.215	3.346E-02	2.682E-03	3.210E-03
0.251	3.575E-02	1.179E-03	4.109E-03
0.293	3.658E-02	2.273E-03	4.202E-03
0.341	3.866E-02	-1.984E-04	7.842E-03
0.398	3.855E-02	2.014E-03	7.977E-03
0.464	4.034E-02	1.357E-03	7.071E-03
0.541	4.341E-02	2.008E-03	8.971E-03
0.631	4.193E-02	4.589E-03	9.835E-03
0.736	4.333E-02	4.717E-03	1.087E-02
0.858	4.326E-02	3.392E-03	1.253E-02
1.000	4.477E-02	3.725E-03	1.479E-02
1.166	4.762E-02	4.375E-03	1.673E-02
1.360	4.546E-02	5.105E-03	2.138E-02
1.585	4.580E-02	5.823E-03	2.482E-02
1.848	4.480E-02	8.210E-03	2.869E-02
2.155	4.462E-02	1.118E-02	3.340E-02
2.512	4.254E-02	1.225E-02	3.770E-02
2.929	4.109E-02	1.194E-02	4.462E-02
3.415	4.628E-02	1.791E-02	4.948E-02
3.982	5.101E-02	1.791E-02	5.798E-02
4.642	5.273E-02	2.097E-02	6.511E-02
5.412	5.538E-02	2.169E-02	7.754E-02
Average	3.424E-03	4.286E-03	1.417E-02
Regression (r^2)	0.690	0.963	0.998

Table K.7: Viscosity values of *Aloe muth-muth* whole leaf extract samples

Shear rate (1/s)	Viscosity (Pa·s)		
	0.5% w/v	1.5% w/v	3% w/v
0.100	5.331E-03	3.443E-04	3.820E-03
0.117	9.483E-03	-1.853E-03	2.696E-03
0.136	1.084E-02	-8.944E-04	3.199E-03
0.159	1.303E-02	4.412E-04	2.797E-03
0.185	1.673E-02	8.827E-04	4.567E-03
0.215	1.960E-02	2.150E-03	4.864E-03
0.251	2.253E-02	8.273E-04	8.824E-03
0.293	2.552E-02	2.067E-03	9.115E-03
0.341	2.732E-02	3.093E-03	8.215E-03
0.398	2.897E-02	2.551E-03	8.631E-03
0.464	2.889E-02	2.832E-03	1.023E-02
0.541	2.800E-02	4.256E-03	1.237E-02
0.631	3.058E-02	3.638E-03	1.337E-02
0.736	2.681E-02	2.977E-03	1.419E-02
0.858	2.632E-02	6.125E-03	1.641E-02
1.000	2.302E-02	4.052E-03	1.688E-02
1.166	2.397E-02	4.940E-03	2.010E-02
1.360	2.657E-02	6.594E-03	2.423E-02
1.585	3.414E-02	7.874E-03	3.075E-02
1.848	3.537E-02	8.847E-03	3.251E-02
2.155	4.282E-02	1.030E-02	3.496E-02
2.512	3.850E-02	1.042E-02	3.891E-02
2.929	3.656E-02	1.182E-02	4.320E-02
3.415	4.311E-02	1.327E-02	5.162E-02
3.982	4.045E-02	1.601E-02	5.597E-02
4.642	3.559E-02	1.921E-02	6.085E-02
5.412	4.254E-02	2.063E-02	7.178E-02
Average	5.324E-03	3.916E-03	1.311E-02
Regression (r^2)	0.595	0.967	0.985

APPENDIX L

TOXICITY STUDIES

L.1 Introduction

Toxicity studies were conducted on the RPMI 2650 cell line after exposure to experimental natural compounds. For pepper extracts an MTT was used, while a neutral red assay was used for aloe plant materials. Different toxicity assays were used in order to prevent the antioxidant effects of aloe to present with false-positive results in the MTT assay. Results are given as percentage cell viability (i.e. percentage surviving cells) calculated from mean absorbance values pertained over a concentrating range of experimental compounds.

L.2 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity assays with pepper extracts

Table L.1: Absorbance values of MTT assay to determine RPMI 2650 cell viability after exposure to capsaicin at different concentrations

	Absorbance					Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4	5			
Untreated Cells	1.274	1.289	1.307	1.310	1.267	1.289	100.000	5.710
25 μM	1.170	1.151	1.110	1.153	1.216	1.160	89.921	11.033
50 μM	1.204	1.185	1.147	1.154	1.191	1.176	91.199	1.335
100 μM	1.143	1.118	1.132	1.166	1.208	1.153	89.408	10.821
200 μM	0.933	0.966	0.969	0.980	0.995	0.968	75.012	10.207
300 μM	0.449	0.447	0.447	0.456	0.452	0.450	34.662	5.660
350 μM	0.487	0.487	0.497	0.516	0.536	0.505	38.913	6.990
400 μM	0.344	0.349	0.355	0.365	0.378	0.358	27.496	6.424
500 μM	0.051	0.053	0.054	0.057	0.059	0.055	3.866	0.589
Dead cells	0.033	0.035	0.035	0.038	0.039	0.036	2.417	0.508

Table L.2: Absorbance values of MTT assay to determine RPMI 2650 cell viability after exposure to piperine at different concentrations

	Absorbance					Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4	5			
Untreated Cells	1.274	1.289	1.307	1.310	1.267	1.289	100.000	5.710
50 μM	1.327	1.293	1.271	1.251	1.233	1.275	98.882	15.134
100 μM	1.420	1.383	1.342	1.350	1.371	1.373	106.536	4.115
500 μM	1.037	1.008	1.021	1.052	1.082	1.040	80.577	15.152
750 μM	1.016	1.021	1.024	1.031	1.042	1.027	79.555	7.666
1000 μM	0.879	0.844	0.825	0.838	0.855	0.848	65.625	6.168
1250 μM	0.655	0.628	0.633	0.654	0.685	0.651	50.308	3.761
1500 μM	0.710	0.719	0.722	0.714	0.707	0.714	55.236	11.013
2000 μM	0.498	0.487	0.474	0.461	0.459	0.476	36.660	11.247
Dead cells	0.033	0.035	0.035	0.038	0.039	0.036	2.417	0.508

L.3 Neutral red toxicity assays with aloe materials

Table L.3: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe vera* gel at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.562	0.571	0.552	0.543	0.557	100.000	4.206
0.25% w/v	0.448	0.439	0.424	0.403	0.428	76.246	3.633
0.5% w/v	0.352	0.334	0.348	0.362	0.349	61.573	2.154
1% w/v	0.244	0.251	0.214	0.277	0.247	42.743	4.796
2% w/v	0.123	0.102	0.114	0.141	0.120	19.396	2.991
2.5% w/v	0.172	0.142	0.154	0.169	0.159	26.669	2.605
3% w/v	0.168	0.112	0.126	0.126	0.133	21.786	4.492
4% w/v	0.113	0.116	0.126	0.133	0.122	19.802	1.713
5% w/v	0.113	0.118	0.108	0.106	0.111	17.766	0.980
6% w/v	0.139	0.135	0.121	0.096	0.123	19.886	3.532
Dead cells	0.031	0.032	0.033	0.035	0.033	3.301	0.621

Table L.4: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe vera* whole leaf extract at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.438	0.460	0.443	0.347	0.422	100.000	13.230
0.25% w/v	0.408	0.394	0.408	0.436	0.412	97.447	4.258
0.5% w/v	0.369	0.410	0.404	0.395	0.395	93.332	4.393
1% w/v	0.374	0.375	0.403	0.405	0.389	92.045	4.137
2% w/v	0.280	0.294	0.328	0.324	0.306	71.962	5.686
2.5% w/v	0.307	0.279	0.274	0.306	0.291	68.344	4.197
3% w/v	0.237	0.178	0.248	0.278	0.235	54.765	10.136
4% w/v	0.149	0.107	0.122	0.124	0.126	28.134	4.175
5% w/v	0.050	0.069	0.087	0.062	0.067	13.957	3.730
6% w/v	0.095	0.106	0.120	0.124	0.112	24.713	3.279
Dead cells	0.081	0.077	0.100	0.060	0.079	16.947	5.280

Table L.5: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe ferox* gel at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.552	0.574	0.521	0.481	0.532	100.000	8.822
0.25% w/v	0.496	0.562	0.621	0.536	0.554	104.154	10.086
0.5% w/v	0.464	0.497	0.561	0.570	0.523	98.204	9.751
1% w/v	0.449	0.491	0.575	0.530	0.511	95.997	10.308
2% w/v	0.513	0.512	0.521	0.538	0.521	97.880	2.323
2.5% w/v	0.492	0.473	0.503	0.508	0.494	92.691	2.965
3% w/v	0.576	0.460	0.516	0.465	0.504	94.637	10.344
4% w/v	0.663	0.413	0.482	0.489	0.512	96.135	20.391
5% w/v	0.443	0.357	0.393	0.433	0.406	75.927	7.552
6% w/v	0.390	0.358	0.313	0.341	0.351	65.239	6.177
Dead cells	0.100	0.115	0.090	0.094	0.100	17.153	2.498

Table L.6: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe ferox* whole leaf extract at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.552	0.574	0.521	0.481	0.532	100.000	8.822
0.25% w/v	0.552	0.524	0.462	0.476	0.503	94.484	8.038
0.5% w/v	0.568	0.504	0.465	0.426	0.491	92.077	11.555
1% w/v	0.444	0.469	0.480	0.481	0.468	87.806	3.263
2% w/v	0.377	0.392	0.388	0.360	0.379	70.722	2.729
2.5% w/v	0.386	0.416	0.350	0.350	0.376	70.026	6.069
3% w/v	0.352	0.362	0.374	0.340	0.357	66.398	2.775
4% w/v	0.393	0.365	0.287	0.221	0.317	58.719	14.908
5% w/v	0.224	0.240	0.244	0.209	0.229	41.992	3.059
6% w/v	0.227	0.203	0.286	0.246	0.241	44.138	6.736
Dead cells	0.100	0.115	0.090	0.094	0.100	17.153	2.498

Table L.7: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe muth-muth* gel at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.388	0.409	0.400	0.377	0.394	100.000	4.025
0.25% w/v	0.397	0.348	0.310	0.317	0.313	86.854	10.206
0.5% w/v	0.365	0.384	0.316	0.321	0.346	87.802	8.550
1% w/v	0.379	0.354	0.359	0.305	0.349	88.542	8.192
2% w/v	0.323	0.322	0.284	0.261	0.298	75.139	7.894
2.5% w/v	0.332	0.299	0.258	0.290	0.295	74.397	7.824
3% w/v	0.362	0.312	0.255	0.275	0.301	75.980	12.163
4% w/v	0.185	0.272	0.251	0.188	0.224	56.088	11.434
5% w/v	0.119	0.124	0.137	0.098	0.119	29.073	4.185
6% w/v	0.054	0.097	0.060	0.066	0.070	16.162	4.936
Dead cells	0.027	0.031	0.049	0.054	0.040	8.582	3.157

Table L.8: Absorbance values of Neutral Red assay to determine RPMI 2650 cell viability after exposure to *Aloe muth-muth* whole leaf extract at different concentrations

	Absorbance				Mean absorbance	% Cell viability	Standard Deviation
	1	2	3	4			
Untreated Cells	0.562	0.571	0.552	0.543	0.557	100.000	4.206
0.25% w/v	0.489	0.413	0.484	0.439	0.456	81.368	6.812
0.5% w/v	0.413	0.435	0.411	0.411	0.417	74.208	2.215
1% w/v	0.402	0.418	0.407	0.390	0.404	71.816	2.138
2% w/v	0.363	0.350	0.357	0.372	0.360	63.699	1.746
2.5% w/v	0.364	0.382	0.344	0.364	0.363	64.273	2.865
3% w/v	0.280	0.337	0.384	0.313	0.329	57.835	8.016
4% w/v	0.300	0.287	0.279	0.274	0.285	49.788	2.110
5% w/v	0.293	0.393	0.289	0.270	0.311	54.667	10.237
6% w/v	0.280	0.302	0.247	0.243	0.268	46.722	5.142
Dead cells	0.031	0.032	0.033	0.035	0.033	3.301	0.621

APPENDIX M

STATISTICAL ANALYSIS

M.1 Introduction

Statistical analysis was performed to determine whether the permeation enhancement and efflux modulation caused by pepper extracts and aloe leaf materials was statistically significantly higher than that of the control (i.e. FITC-dextran 4400 and rhodamine 123 alone). Multiple comparisons of the p-values were performed using the Kruskal-Wallis test followed by the Dunn's post hoc test for all natural compound and across both models. Data is presented in p-values, with a group being statistically significantly higher than that of its individual control when $p \leq 0.05$.

M.2 Statistical analysis of permeation in the presence of pepper extracts across the RPMI 2650 cell line

Table M.1: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of capsaicin at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: $H(3, N=12) = 9.461538$ $p = .0237$				
	FD4 alone	FD4 in capsaicin 50 μM	FD4 in capsaicin 100 μM	FD4 in capsaicin 200 μM
FD4 alone		0.039470	0.104503	1.000000
FD4 in capsaicin 50 μM	0.039470		1.000000	0.536576
FD4 in capsaicin 100 μM	0.104503	1.000000		1.000000
FD4 in capsaicin 200 μM	1.000000	0.536576	1.000000	

Table M.2: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of piperine at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	FD4 alone	FD4 in piperine 50 μ M	FD4 in piperine 100 μ M	FD4 in piperine 200 μ M
FD4 alone		0.141240	0.188703	0.536576
FD4 in piperine 50 μ M	0.141240		1.000000	1.000000
FD4 in piperine 100 μ M	0.188703	1.000000		1.000000
FD4 in piperine 200 μ M	0.536576	1.000000	1.000000	

Table M.3: Multiple comparisons of p-values of absorptive rhodamine 123 (R123) permeation in the presence of capsaicin at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in capsaicin 50 μ M	R123 in capsaicin 100 μ M	R123 in capsaicin 200 μ M
R123 alone		1.000000	0.420248	0.013407
R123 in capsaicin 50 μ M	1.000000		1.000000	0.420248
R123 in capsaicin 100 μ M	0.420248	1.000000		1.000000
R123 in capsaicin 200 μ M	0.013407	0.420248	1.000000	

Table M.4: Multiple comparisons of p-values of secretory rhodamine 123 (R123) permeation in the presence of capsaicin at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in capsaicin 50 μM	R123 in capsaicin 100 μM	R123 in capsaicin 200 μM
R123 alone		0.104503	0.141240	1.000000
R123 in capsaicin 50 μM	0.104503		1.000000	0.420248
R123 in capsaicin 100 μM	0.141240	1.000000		0.536576
R123 in capsaicin 200 μM	1.000000	0.420248	0.536576	

Table M.5: Multiple comparisons of p-values of absorptive rhodamine 123 (R123) permeation in the presence of piperine at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in piperine 50 μM	R123 in piperine 100 μM	R123 in piperine 200 μM
R123 alone		1.000000	0.846190	0.188703
R123 in piperine 50 μM	1.000000		0.536576	0.104503
R123 in piperine 100 μM	0.846190	0.536576		1.000000
R123 in piperine 200 μM	0.188703	0.104503	1.000000	

Table M.6: Multiple comparisons of p-values of secretory rhodamine 123 (R123) permeation in the presence of piperine at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in piperine 50 μM	R123 in piperine 100 μM	R123 in piperine 200 μM
R123 alone		1.000000	0.325473	0.076428
R123 in piperine 50 μM	1.000000		0.677542	0.188703
R123 in piperine 100 μM	0.325473	0.677542		1.000000
R123 in piperine 200 μM	0.076428	0.188703	1.000000	

M.3 Statistical analysis of permeation in the presence of pepper extracts across excised sheep nasal mucosa

Table M.7: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of capsaicin at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	FD4 alone	FD4 in capsaicin 50 μM	FD4 in capsaicin 100 μM	FD4 in capsaicin 200 μM
FD4 alone		1.000000	1.000000	1.000000
FD4 in capsaicin 50 μM	1.000000		1.000000	1.000000
FD4 in capsaicin 100 μM	1.000000	1.000000		1.000000
FD4 in capsaicin 200 μM	1.000000	1.000000	1.000000	

Table M.8: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of piperine at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	FD4 alone	FD4 in piperine 50 μ M	FD4 in piperine 100 μ M	FD4 in piperine 200 μ M
FD4 alone		1.000000	1.000000	1.000000
FD4 in piperine 50 μ M	1.000000		1.000000	1.000000
FD4 in piperine 100 μ M	1.000000	1.000000		1.000000
FD4 in piperine 200 μ M	1.000000	1.000000	1.000000	

Table M.9: Multiple comparisons of p-values of absorptive rhodamine 123 (R123) permeation in the presence of capsaicin at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in capsaicin 50 μ M	R123 in capsaicin 100 μ M	R123 in capsaicin 200 μ M
R123 alone		0.536578	0.846190	0.019445
R123 in capsaicin 50 μ M	0.536576		1.000000	1.000000
R123 in capsaicin 100 μ M	0.846190	1.000000		0.846190
R123 in capsaicin 200 μ M	0.019445	1.000000	0.846190	

Table M.10: Multiple comparisons of p-values of secretory rhodamine 123 (R123) permeation in the presence of capsaicin at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in capsaicin 50 μM	R123 in capsaicin 100 μM	R123 in capsaicin 200 μM
R123 alone		1.000000	1.000000	0.141240
R123 in capsaicin 50 μM	1.000000		1.000000	0.055247
R123 in capsaicin 100 μM	1.000000	1.000000		1.000000
R123 in capsaicin 200 μM	0.141240	0.055247	1.000000	

Table M.11: Multiple comparisons of p-values of absorptive rhodamine 123 (R123) permeation in the presence of piperine at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in piperine 50 μM	R123 in piperine 100 μM	R123 in piperine 200 μM
R123 alone		1.000000	1.000000	0.188703
R123 in piperine 50 μM	1.000000		0.846190	0.141240
R123 in piperine 100 μM	1.000000	0.846190		1.000000
R123 in piperine 200 μM	0.188703	0.141240	1.000000	

Table M.12: Multiple comparisons of p-values of secretory rhodamine 123 (R123) permeation in the presence of piperine at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis test: H (3, N= 12) =9.461538 p =.0237				
	R123 alone	R123 in piperine 50 µM	R123 in piperine 100 µM	R123 in piperine 200 µM
R123 alone		0.249240	1.000000	1.000000
R123 in piperine 50 µM	0.249240		1.000000	0.013407
R123 in piperine 100 µM	1.000000	1.000000		0.249240
R123 in piperine 200 µM	1.000000	0.013407	0.249240	

M.4 Statistical analysis of permeation in the presence of aloe species in the RPMI 2650 cell line

Table M.13: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe vera* gel (AVG) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

Multiple Comparisons p values Kruskal-Wallis chi-squared = 9.4615, df = 3 p = 0.02			
	FD4 alone	FD4 in AVG 0.5% w/v	FD4 in AVG 1.5% w/v
FD4 in AVG 0.5% w/v	0.0871		
FD4 in AVG 1.5% w/v	0.0011	0.0447	
FD4 in AVG 3% w/v	0.0447	0.3670	0.0871

Table M.14: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe vera* whole leaf extract (AVWL) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 7.6667, df = 3 p = 0.05		
	FD4 alone	FD4 in AVWL 0.5% w/v	FD4 in AVWL 1.5% w/v
FD4 in AVWL 0.5% w/v	0.3253		
FD4 in AVWL 1.5% w/v	0.0208	0.0565	
FD4 in AVWL 3% w/v	0.0118	0.0350	0.4104

Table M.15: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe ferox* gel (AFG) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 4.6959, df = 3 p = 0.2		
	FD4 alone	FD4 in AFG 0.5% w/v	FD4 in AFG 1.5% w/v
FD4 in AFG 0.5% w/v	0.3457		
FD4 in AFG 1.5% w/v	0.4325	0.4103	
FD4 in AFG 3% w/v	0.0269	0.0629	0.0394

Table M.16: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe ferox* whole leaf extract (AFWL) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 5.9744, df = 3 p = 0.11		
	FD4 alone	FD4 in AFWL 0.5% w/v	FD4 in AFWL 1.5% w/v
FD4 in AFWL 0.5% w/v	0.0118		
FD4 in AFWL 1.5% w/v	0.2140	0.0705	
FD4 in AFWL 3% w/v	0.0447	0.2856	0.1825

Table M.17: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe muth-muth* gel (AMMG) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 7.6154, df = 3 p = 0.05		
	FD4 alone	FD4 in AMMG 0.5% w/v	FD4 in AMMG 1.5% w/v
FD4 in AMMG 0.5% w/v	0.1541		
FD4 in AMMG 1.5% w/v	0.0705	0.3253	
FD4 in AMMG 3% w/v	0.0033	0.0447	0.1065

Table M.18: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe muth-muth* whole leaf extract (AMMWL) at different concentrations across the RPMI 2650 cell line with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 9.4615, df = 3 p = 0.02		
	FD4 alone	FD4 in AMMWL 0.5% w/v	FD4 in AMMWL 1.5% w/v
FD4 in AMMWL 0.5% w/v	0.0871		
FD4 in AMMWL 1.5% w/v	0.0447	0.3670	
FD4 in AMMWL 3% w/v	0.0011	0.0447	0.0871

M.5 Statistical analysis of permeation in the presence of aloe species in the excised sheep nasal mucosa

Table M.19: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe vera* gel (AVG) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 2.2821, df = 3 p = 0.52		
	FD4 alone	FD4 in AVG 0.5% w/v	FD4 in AVG 1.5% w/v
FD4 in AVG 0.5% w/v	0.0871		
FD4 in AVG 1.5% w/v	0.4549	0.1065	
FD4 in AVG 3% w/v	0.2856	0.2140	0.3253

Table M.20: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe vera* whole leaf extract (AVWL) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 7.8205, df = 3 p = 0.05		
	FD4 alone	FD4 in AVWL 0.5% w/v	FD4 in AVWL 1.5% w/v
FD4 in AVWL 0.5% w/v	0.1065		
FD4 in AVWL 1.5% w/v	0.3253	0.2140	
FD4 in AVWL 3% w/v	0.0705	0.0033	0.0271

Table M.21: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe ferox* gel (AFG) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 4.4359, df = 3 p = 0.22		
	FD4 alone	FD4 in AFG 0.5% w/v	FD4 in AFG 1.5% w/v
FD4 in AFG 0.5% w/v	0.4104		
FD4 in AFG 1.5% w/v	0.2140	0.2856	
FD4 in AFG 3% w/v	0.1065	0.0705	0.0208

Table M.22: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe ferox* whole leaf extract (AFWL) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 8.0769, df = 3 p = 0.04		
	FD4 alone	FD4 in AFWL 0.5% w/v	FD4 in AFWL 1.5% w/v
FD4 in AFWL 0.5% w/v	0.3253		
FD4 in AFWL 1.5% w/v	0.0350	0.0871	
FD4 in AFWL 3% w/v	0.0064	0.0208	0.2485

Table M.23: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe muth-muth* gel (AMMG) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values Kruskal-Wallis chi-squared = 4.2308, df = 3 p = 0.24		
	FD4 alone	FD4 in AMMG 0.5% w/v	FD4 in AMMG 1.5% w/v
FD4 in AMMG 0.5% w/v	0.3670		
FD4 in AMMG 1.5% w/v	0.2485	0.3670	
FD4 in AMMG 3% w/v	0.0271	0.0565	0.1065

Table M.24: Multiple comparisons of p-values of FITC-dextran 4400 (FD4) permeation in the presence of *Aloe muth-muth* whole leaf extract (AMMWL) at different concentrations across excised sheep nasal mucosa with statistical significance indicated at p-values < 0.05

	Multiple Comparisons p values (2-tailed): Conc Kruskal-Wallis chi-squared = 4.5897, df = 3 p = 0.2		
	FD4 alone	FD4 in AMMWL 0.5% w/v	FD4 in AMMWL 1.5% w/v
FD4 in AMMWL 0.5% w/v	0.4104		
FD4 in AMMWL 1.5% w/v	0.5000	0.4104	
FD4 in AMMWL 3% w/v	0.0350	0.0565	0.0350