Development and characterization of a transdermal formula for an extract of the medicinal plant *Harpagophytum procumbens*

by

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ABBREVIATIONS

AA	Arachadonic acid
BAW	Butanol, acetic acid and water
COX-2	Cyclooxygenase-2
EMW	Ethyl Acetate, methanol, water
DMSO	Dimethsulphoxide
HPLC	High pressure liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LPS	Lipopolysaccharide
NSAID	Non-steroidal anti-inflammatory drugs
PBS	phosphate buffer solution
SC	Stratum corneum
SDS	Sodium dodecyl sulphate
TLC	Thin layer chromatography
TMPD	N,N,N',N'– tetramethyl-*p*-phenylenediamine
TNF	Tumour necrosis factor
UV	Ultra violet

Development and characterization of a transdermal formula for an extract of the medicinal plant *Harpagophytum procumbens* (Burch.) DC. ex Meisn.

ABSTRACT

The skin is the largest and most readily accessible organ of the body. The stratum corneum forms the outermost layer of the skin, which functions as a barrier to the external environment and regulates the passage of molecules across the skin. Drug delivery across the skin offers advantages over other routes of administration, bypassing the hepatic first pass metabolism, maintaining therapeutically effective drug levels, and improved patient compliance. Currently, there is a strong trend in the use of plant materials and extracts for medicinal purposes. Devil's Claw (*Harpagophytum procumbens*) is a medicinal plant located in many regions throughout Southern and South Africa. It is used for its anti-inflammatory and analgesic properties with the harpagoside and harpagide components present reported to be responsible for these properties. Its activity has been reported to be as a result of the direct and indirect inhibition of Cyclooxygenase- 2 enzyme (COX-2).

Efforts to improve the permeation of synthetic conventional compounds are constantly investigated, and great improvements of these formulations have led to very effective formulation of transdermal dosage forms such as anti-inflammatory drugs. The same should be possible for medicinal plants.

Optimal permeation across the skin requires a drug to possess lipophilic as well as hydrophilic properties. Research suggests that a drug should have an aqueous solubility of more than 1 mg/ml and an octanol-water partition coefficient (log P) between 1 and 2 to optimally penetrate the skin (higher values indicating increased lipophilicity). Compounds not possessing these characteristics may be facilitated across the skin by the introduction of permeation enhancers in the formulation that include Azone® and sodium dodecyl sulphate

(SDS).

The main aims of this study were to make an extract of *Harpagophytum procumbens* and to determine the direct COX-2 inhibition activity of this extract and further to formulate this extract into a pharmaceutical gel, with permeation enhancers that maintains its COX-2 enzyme inhibition qualities after transdermal penetration.

The analytical techniques verified the existence of a harpagide and harpagoside components in the crude *Harpagophytum procumbens* extract and they were quantified in the extract at 3% and 1% respectively.

COX-2 inhibition by the *Harpagophytum procumbens* extract was determined by a direct enzyme inhibition study and quantified by means of measuring the production of the product formed by the enzyme over a time interval in the presence of excess enzyme substrate.

Crude *Harpagophytum procumbens* extract demonstrated a greater COX-2 enzyme inhibition than pure harpagide and harpagoside individually and combined. This indicated the existence of compounds in the extract contributing to this synergistic effect. This was reflected in the IC_{50} values indicating that the *Harpagophytum procumbens* crude extract had the lowest IC_{50} values concentration when compared to the harpagide and harpagoside.

Octanol-PBS partition coefficient (log D) experiments were performed with various permeation enhancers and gels with varying combinations in order to determine the changing partitioning properties of harpagide and harpagoside.

The octanol-PBS partition coefficient (log D) experiments indicated that harpagoside had a higher log D value than harpagide.

The addition of permeation enhancers resulted in changes in the partition co-efficient of the marker compounds. The permeation enhancer, Azone®, resulted in the smallest reduction of Log D for harpagoside and the largest increase in Log D compared to other permeation enhancers tested for harpagide.

When the 2 marker compounds were formulated into different gel formulations (hydroxypropyl cellulose, Carbopol Ultrez 21® and Pluronic®), harpagide had the largest increase in Log D with the Carbopol Ultrez 21® gel. Harpagoside had the largest increase in the hydroxypropyl cellulose gel.

Combinations of gel formulations with permeation enhancers were tested. The 2 best performing permeation enhancers in hydroxypropyl cellulose gel were SDS and Azone®. These compounds resulted in the largest increase in lipophilic partitioning for harpagide and harpagoside compound and higher values were achieved with SDS.

Following the partition co-efficient determination, permeation studies with synthetic membranes were performed with the selected enhancer/gel formulations using Franz diffusional cells.

The permeation (flux) across the hydrophobic synthetic membrane (Sil-Tec®) indicated flux of 27.1µg.cm^{-2}.h^{-1} for harpagoside and 156.0 µg.cm^{-2}.h^{-1} for harpagide in the hydroxypropyl cellulose gel. Incorporation of Azone® in the hydroxypropyl cellulose formulation resulted in an enhancement ratio (ER) of 14 for harpagoside. The hydroxypropyl cellulose gel with the permeation enhancers (Azone® and SDS) did not result in enhancement of permeation of the harpagide.

Permeation of harpagoside and harpagide across the hydrophilic synthetic membrane (Tuffyrn®) yielded flux values of 537.1 µg.cm^{-2}.h^{-1} and 101.1µg.cm^{-2}.h^{-1} respectively in the Gel formulations. The Gel formulation containing Azone® and SDS, resulted in similar flux across this membrane compared to the formulation consisting of the Gel only, with the Azone® containing formulation resulting in lower flux for harpagide.

Transdermal permeation was measured through excised female human abdominal skin using Franz diffusion cells.

Flux values for harpagoside and harpagide were 18.4 µg.cm^{-2}.h^{-1} and 5.0 µg.cm^{-2}.h^{-1} respectively when the crude extract was in the hydroxypropyl cellulose gel tested on human skin. Formulation of the crude extract in hydroxypropyl cellulose gel including Azone® and SDS permeation enhancers resulted in enhancement of 8 and 7 respectively for harpagide. Azone® increased the flux of harpagoside 14 times in the same hydroxypropyl cellulose gel formulation. The SDS had very little effect. The enhancement ratios achieved with the human skin was higher than that of the synthetic membranes.

Post transdermal COX-2 inhibition activity studies were performed with the Azone® Gel formulation after it permeated across the human skin membrane to determine to what extent inhibitory activity of the crude extract was maintained. The crude drug retained its direct COX-2 inhibitory activity after permeation across the human skin with 77% inhibitory activity.

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THESIS OVERVIEW

Standardization – Identification and extraction of *Harpagophytum procumbens* extract.

Identification and purity testing of marker compounds in crude plant extract.

Standardization of extraction technique.

- **Chapter Four**

Determination of direct Cyclooxygenase- 2 (COX-2) inhibition.

After the marker compounds were indentified and quantified in the crude extract these marker compounds (harpagide and harpagoside) were tested to determine their ability to directly inhibit COX-2 enzymes. These studies were also performed with the *Harpagophytum procumbens* crude extract.

- **Chapter Five**

Physiochemical properties and solubility.

Physiochemical properties of compounds play a fundamental role in the ability of compounds to permeate across membranes. It was therefore necessary to determine what the physiochemical parameters of the marker compunds were such as solubilty, partition co-efficient and molecular weight.

- **Chapter Six**

Gel Formulation

In order to perform permeation studies (synthetic and human membranes) various gel formulations in combination with permeation enhancers were tested for their influence on altering the partition co-efficient to achieve values close to the suggested range of log D between 1-2. The best performing gel and enhancers were selected for synthetic membrane permeation experiments.

- **Chapter Six**

Synthetic membranes

This was performed in order to determine the release of harpagoside and harpagide from gel formulations (with and without enhancers). This indicated the effect that the permeation enhancers, Azone® and SDS, had on the permeation pattern of harpagoside and harpagide.

- **Chapter Seven**

Transdermal permeation

The selected gel formulation with permeation enhancers were tested for its transdermal permeation ability. The gel formulation and enhancer combination with the highest flux were selected for post COX-2 enzyme inhibition activity.

- **Chapter Seven**

Post-permeation COX-2 enzyme inhibition activity.

This was performed to determine to what extent the the crude *Harpagophytum procumbens* plant extract retained its ability to directly inhibit the COX-2 enzyme after permeation across the human skin.

- **Chapter Eight**

CHAPTER ONE: Introduction and problem statement.

1.1 INTRODUCTION

Devil's Claw (*Harpagophytum procumbens*) is a medicinal plant geographically located in many regions throughout Southern Africa. Its typical habitat is open savannahs and arid regions. It has been used for a range of medicinal properties and historically been used to treat various ailments. The root of the plant has been used mainly for its anti-inflammatory, antipyretic, osteoarthritic pain and analgesic properties (Van Wyk *et al.,* 1997). Devil's Claw contains iridiode glycosides, sugars, triterpenoids, sterols, carbohydrates and waxes (Wichtl, 2004). The harpagosides (iridiode glycosides) is believed to be the main compound imparting medicinal properties (Van Wyk *et al.,* 1997). *Harpagophytum procumbens* extract as well as some of its components such as harpagoside and harpagide has demonstrated anti-inflammatory properties by indirect inhibition Cyclooxygenase -2 enzyme (COX-2) (Zhang *et al.,* 2011; Gyurkovska *et al.,* 2011; Fiebich *et al.,* 2001). COX-2 catalyzes reactions that produce prostaglandins, which are involved in inflammation processes.

Traditional dosage forms of *Harpagophytum procumbens* include infusions and decoctions, which are taken orally as well as ointments, which are applied topically (Van Wyk *et al.,* 1997). More conventional dosage forms include tablets, teas, tinctures, liquid extracts and creams. These crude forms usually contain between 1-2% of the harpagoside extract (Wichtl, 2004).

Standardization is important to ensure consistency of active ingredients in all batches of crude drugs or extracts and ultimately the dosage forms. Quality and purity assays can be performed by thin layer chromatography (TLC), High-pressure liquid chromatography (HPLC), and Gas liquid chromatography (GC) techniques.

Efforts to improve the dermal permeation of pharmacologically active compounds are constantly investigated, and great improvements of formulations have led to very effective formulation of transdermal dosage forms such as those containing anti-inflammatory drugs. Ibuprofen and diclofenac sodium have successfully been formulated into transdermal dosage forms. The same is possible for medicinal plants and the formulation process should be facilitated through the development of a standardized membrane permeation model.

Transdermal drug delivery has advantages over other conventional delivery routes such as ease of administration, bypassing first pass hepatic metabolism, avoidance of variable rates of absorption and metabolism as well as the elimination of gastrointestinal irritation (El-Kattan *et al.,* 2000; Naik *et al.,* 2000; Godwin *et al.,* 1997). Due to these advantages there is a trend in reformulating existing formulation into transdermal drug delivery systems.

The stratum corneum (SC) forms the outermost layer of the skin and is a multilamellar lipid milieu with protein-filled coenocytes, which augment membrane integrity, and increases membrane tortuosity. This intrinsic tortuosity coupled with the lipophilic nature of the SC ensures a barrier against the entry of drug molecules with the exception of highly lipophilic substances (Kalia and Guy, 2001). When a drug is released from a transdermal device it should diffuse through the SC, as well as the hydrophilic viable epidermis and dermal layer (Barry, 2007). An ideal drug candidate for systemic transdermal drug delivery should have sufficient lipophilicity to penetrate the SC, as well as sufficient hydrophobicity to enable the second partitioning step into the viable epidermis and eventually the systemic circulation (Kalia and Guy, 2001).

For most drug compounds the rate-limiting step for drug transport across the skin is transit across the stratum corneum (Nolan *et al.,* 2003). There are various techniques to measure drug diffusion across the skin, including human and animal *in vitro* studies.

Transdermal membrane models such as synthetic polymeric membranes e.g. Carbosil, Silistic, and Sil- Tec® (Feldstein *et al.,* 1998; Megrab *et al.,* 1995; Schwarb *et al.,* 1999) may be used to measure in vitro drug diffusion. The advantage of these models over skin is their availability, uniformity, tensile strength and chemical purity. It aims to mimic the permeation activity of the skin (*in vitro* and *in vivo*) and usually is the first step in determining drug permeation across the SC. Membrane choice is important when studying trans membrane movement of drugs as with Devils Claw, which contain numerous compounds with different lipophilic and hydrophilic characteristics.

Human skin (normally difficult to obtain) may be obtained from cadavers or surgical and cosmetic procedures. Variables such as age, gender, anatomical site and general health of the donor, lead to variability in drug permeation (Feldstein *et al.,* 1998). This is the preferred membrane to use in permeation studies.

When formulating a topical transdermal drug delivery dosage form, the main complication is usually enhancement and controlling the rate of permeation. The incorporation of permeation enhancers resulting in manipulation of the partition coefficient may be used to overcome these complications (Rowat *et al.,* 2006; Masson *et al.,*, 1999; Megrab *et al.,*, 1995).

Medicinal plant contain a complexity of compounds with varying physical and physiochemical properties many of which are unknown of which *Harpagophytum procumbens* is no exception. The formulation of a topical transdermal dosage form consisting of crude plant extracts such as *Harpagophytum procumbens* extract would require consideration as to how these compounds could affect the physical and physio chemical properties of the dosage form. These properties could influence the release profile of compounds in the crude extract and permeation across the human skin.

If successful transdermal permeation of crude medicinal plant components is achieved in a formulated topical dosage form, it would be essential to establish whether the activity of

these crude plant extracts has been maintained after the permeation process. In the case of *Harpagophytum procumbens* crude extract, it would mean determining if this medicinal plant can directly inhibit COX-2 enzyme activity and then establish to what extent the crude extract has maintained the COX -2 inhibition ability after transdermal permeation.

This could be the first step towards the successful creation of a therapeutically effective transdermal preparation of *Harpagophytum procumbens.*

1.2 AIM AND OBJECTIVES

To prepare and standardize an extract of *Harpagophytum procumbens* and to formulate the extract into a transdermal pharmaceutical preparation. This preparation should have the ability to effectively permeate the skin without losing its COX-2 inhibition activity. The preparation should have the potential to be utilized as an effective natural anti-inflammatory transdermal dosage form.

In order to meet these goals the following objectives were set:

- Develop a standardized extraction method to produce a constant crude *Harpagophytum procumbens* extract.
- Perform quality, purity and identity tests by means of Thin layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) on the *Harpagophytum procumbens* crude extract.
- Develop a method and experimentally measure the direct COX–2 enzyme inhibitory activity of the crude extract of *Harpagophytum procumbens*, harpagoside and harpagide glycosides.
- Experimentally perform partition coefficient studies with selected permeation enhancers and gel formulation combinations of the *Harpagophytum procumbens* crude extract containing harpagoside and harpagide.
- To formulate the crude extract into a transdermal topical gel preparation.
- Experimentally determine the permeation (flux) of harpagoside and harpagide compounds from the crude extract in gel formulations across synthetic membranes.

- Experimentally determine the permeation (flux) of harpagoside and harpagide compounds from the crude extract in gel formulations across the human skin (transdermal).
- Experimentally determine the permeation flux of harpagoside and harpagide with various permeation enhancers.
- Determine the relationship between the flux values of the synthetic membrane and human skin.
- Determine the extent to which the gel formulation retained the ability to directly inhibit COX–2 enzyme after transdermal permeation.

The COX-2 percentage inhibition, partition co-efficient and flux values data generated were evaluated statistically using one way analysis of variance (ANOVA). Tukey's multiple comparisons test was applied (if ANOVA indicated significant difference) to find the source of possible differences between the various data obtained. Differences were considered statistically significant for a P-value ≤0.05. Analysis of data was performed using GraphPad Prism® 5.

CHAPTER TWO: Plant Medicine

2.1 Introduction to plant medicine

Plants have always formed the basis of traditional medicine and continue to reveal new remedies. The first records of medicinal properties of plants were from Mesopotamia (2600 BC); among the substances used were oils of *Cedrus* species (Cedar), *Glycyrrhiza glabra* (licorice), *Commiphora* species (Myrrh) and Papaver somniferum (Poppy juice) all of which are still used today for ailments ranging from coughs, infections to inflammation (Gurib-Fakim, 2006).

Traditional African medicine is the oldest and possibly the most diverse of all traditional medicines systems due to its rich cultural and biodiversity (Gurib-Fakim, 2006). Well known African medicinal plants include *Agathosma betulina* (Buchu), *Acacia Senegal* (Gum Arabic), *Aspalanthus linearis* (Rooibos tea), *Artemesia afra* (African wormwood) and *Harpagophytum procumbens* (Devil's claw).

Modern allopathic medicine has its roots in ancient medicine, and it is likely that new remedies will be discovered and commercialized following leads provided by traditional knowledge. It is estimated that 80% of the world's population, mostly developing countries, are dependent on traditional medicines for primary healthcare and that 25% of all prescribed medicines contain some ingredients derived from plants (Mander and Le Brenton, 2006).

In the past couple of decades there has once again been a shift in the use of traditional plant medicines, sales figures in the USA increasing each year over the last 5 years (Blumenthal *et al.,* 2011) suggesting that it may be an attractive area of research. Newer technologies in equipment, separation techniques and structure elucidations have resulted in making it easier to determine the mechanisms of activity of plant compounds with better understanding their role in disease states and discovery of new chemical entities (Mukherjee and Houghton, 2009).

What is also evident is that newer versions of monographs and pharmacopeias such as British pharmacopeia, European pharmacopeia and European Scientific Cooperative on Phytotherapy (ESCOP) monographs have included monographs for more plant compounds with more sophisticated methods for identification, purity and assay procedures.

The interest in plant based medicine is apparent in that globally, there were more than 90 registered clinical trails involving various plant compounds between 2001 and 2007 for the treatment of conditions such as central nervous system (CNS) related diseases, cancer therapy and inflammatory conditions (Saklani and Kutty, 2008).

Medicinal plant products can be categorized as follows (Mander and Le Brenton, 2006):

Herbal remedies – these are processed plant based medicinal products whose therapeutic claims do not have formal legal recognition. They are based on traditional therapies, which may be single products to multi-species complex blends.

Crude plant based traditional medicine - frequently little processing is required before administration, and at most, the plant material is ground or boiled and sometimes mixed with other plant components. These medicines have a long history of use and their healing powers are closely linked with cultural beliefs.

Plant derived allopathic medicine – these products have a degree of legal recognition and are sometimes referred to as phytochemicals. They usually consist of single plant species, which are incorporated into conventional pharmaceutical techniques and traded on the basis of proven medical efficacy.

Unlike formulated conventional active components, which are mostly synthesized and usually free from contamination and batch-to-batch variation, plants components are naturally sourced compounds. This affects extract composition, potency and the extent of unwanted compounds such as contaminants (Mukherjee and Houghton, 2009).

The traditional modes of administration for medicinal plant components are usually the oral route for decoctions, infusions/teas and tinctures; the nasal route for snuffing; and topical route from cream, ointment and gels (Van Wyk and Wink, 2004).

2.2 Medicinal crude plant extracts

Medicinal plants usually contain a vast variety of compounds, which may act individually, through polyvalence or synergistically for its desired therapeutic effect (Houghton, 2009). Traditional plant medicines are usually administered as a crude extract as opposed to individual isolated components (Van Wyk and Wink, 2004; Mills and Bone, 2000). This is in line with traditional therapy aiming to restore body function through a more holistic approach, which include whole extracts or mixtures of plant substances. There may be other advantages of using the crude extract compared to using individual components. The co-effectors may impact the solubility, stability or half-life of the active compound and the presence of unknown components in the crude extract may play a part in the overall effects of crude extracts which may offer better stability of various active compounds (Williamson, 2001; Houghton, 2009).

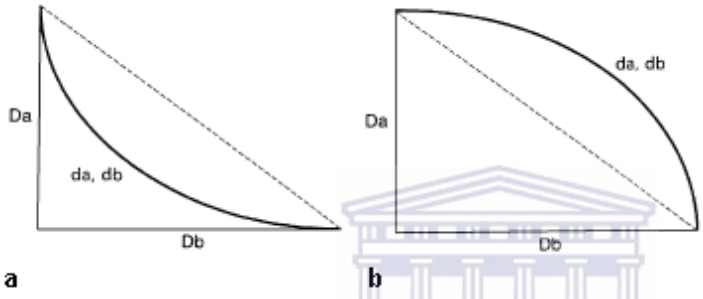
2.2.1 Synergy

Synergy may be understood as an effect seen by a combination of substances being greater than would have been expected from a consideration of individual contributions (Williamson, 2001). Many effective phytomedicines are present in the form of whole extracts and it suggested that it is as a result of synergistic interactions between the components of the individual or a mixture of plant extracts. Synergy is not unique in plant extracts or between various plant species as it has been demonstrated to be present in various conventional medicines and between conventional and plant medicines (Pelizzaro-Rocha *et al.,* 2010; Veras 2011; Nitteranon *et al.,* 2011; Fiebich *et al.,* 2011).

The isobole method (Berenbaum, 1989) is the most appropriate method for describing synergism in plant material as it is independent of the mechanism of action and makes no assumptions about the behavior of each component and it is appropriate for multi-component mixtures.

This is demonstrated in figure 2.1 (Williamson, 2001) where the combination of compounds (da and db) is represented on the curve, the axis being the dose-axes of these individual compounds (Da and Db). If there is no interaction between these compounds then a straight isobole is the resultant (dashed line). If there were synergistic activity present the combination of the components required would be less than for the sum of the individual compounds and a concave curve is a result (Fig. 2.1.b) the converse of that would be antagonism (Fig. 2.1.a).

Figure 2.1 Synergism and antagonism of crude plant extracts.



Until recently, there were not many clinical studies proving the concept of synergy in plant material, which was often dismissed by skeptics. This view was supported by the fact that the mechanisms of actions of the activity of many medicinal plants cannot be explained. However, the clinical and in vitro effects of synergy in plants components are reported more frequently. Gyurkovska *et al. (*2011) tested the inhibition of COX-2 expression by crude extracts and components of *Harpagophytum procumbens*. It yielded strong inhibitory effects and suggested that this high activity is as a result of synergistic activity between the various components of the crude extract. Nitteranon *et al.,* (2011) demonstrated the synergistic effects of the components of the extract *Morinda Citrifolia* where low concentrations of quercetin and scopoletin resulted in suppression of inducible nitric oxide synthase (iNOS) and COX-2 expression which are components in inflammatory responses. Synergy has also been reported with other plant extracts such as *Ginkgo biloba*, *Cannabis sativa, Croton gratissimus* and *Lippia sidoides* (Williamson, 2001; van Vuuren and Viljoen 2008; Veras *et al.,* 2011).

2.2.2 Polyvalence

Polyvalence can be defined as the range of biological activities that an extract may exhibit which contribute to the overall effect observed clinically or *in vivo* (Houghton, 2009). In certain instances as with *Harpagophytum procumbens*, the crude extract may contain a range of active compounds which all contribute to its efficacy. This may not necessarily indicate synergy (Williamson, 2001). What should be considered is that the additive effects of the individual components of a crude extract contribute to the total effects produced by the crude extract. This is represented by Mulyaningsih *et al.,* (2010) investigating the synergistic properties of the terpenoids aromadendrene and 1,8 –cineole of *Eucalyptus globulus* who indicated that activity of the eucalyptus extract components could be the result of additive effects of its other components rather than synergy.

2.3 Formulation of plant extracts into conventional dosage forms.

The shift to renewed interest through a more structured approach in identification and regulation of plant medicines serves as an opportunity to develop a good connection between crude extracts of traditional plant medicine with the standardized and regulated practices used in the evaluation and formulation of allopathic medicines.

Although traditional medicine has been used for many centuries, scientific research and development in phytotherapy have suffered through lack of patent protection and the relative small scale of the industries involved when compared to conventional pharmaceutical industries (Mukherjee, 2009).

Formulation processes with crude extracts become more difficult due to the need to consider more than one active compound unlike conventional allopathic formulations, which in most cases, only consist of one synthesized active compound. In plant extracts there are several known and unknown compounds such as active compounds, waxes and sugars present. This could affect factors such as solubility, partitioning coefficient and pH of the formulation making formulation of plant extracts into conventional dosage forms such as gels more complicated.

In recent years, more traditional plant compounds (crude extracts and isolated compounds) have been formulated into more conventional dosages forms. These include tablets, capsules and topical dosage forms. The benefit with this approach is that the formulation and qualitative tests generated could be useful for improved quality control, delivery and shelf-life (Vyas *et al.,* 2011*;* Georgetti *et al., 2008*; Endale *et al.,* 2005). Casagrande *et al.,* (2007) formulated the plant derived flavonoid, quercetin, into a topical dosage form indicated that quercetin was released from the dosage form and displayed activity after release from the topical dosage form. Soybean extract has also been formulated into topical dosage

forms consisting of emulsifying and gelling agents; these topical preparations demonstrated physiochemical stability and anti-oxidant properties. (Georgetti *et al.,* 2008; Georgetti *et al., 2006)*.

2.4 Current therapy for inflammatory conditions.

Medicinal treatment for inflammatory conditions such as acute and chronic arthritic pain includes the administration of corticosteroids and anti-inflammatory agents. The anti-inflammatory agents comprise the non-steroidal anti-inflammatory drugs (NSAIDs) and the selective COX-2 inhibitors drugs.

2.4.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Most NSAIDs inhibit both COX-1 and COX-2 when administered at effective doses in the reduction of inflammation and pain with the majority displaying COX-1 selectivity (Motilva *et al.,* 2005; Hawkey, 1999). Despite their toxicity, NSAIDs is a commonly prescribed group of drugs; this is a reflection of the widespread prevalence and chronic nature of arthritic and musculoskeletal disease (Hawkey, 1999). However the main limitation of the use of NSAIDs is gastrointestinal injury (Laine, 2002).

2.4.2 Selective COX-2 inhibitors

The selective COX-2 inhibitors have been developed for a better gastrointestinal safety profile (Motilva *et al.,* 2005). COX-2 selective inhibitors, such as celecoxib and refocoxib demonstrate the same anti-inflammatory, antipyretic and analgesic effects as the non-selective NSAIDs but may result in 50% to 60% lower gastrointestinal side effects (Laine, 2002). However, the selective COX-2 anti-inflammatory compounds do produce side effects, most notably the increased cardiovascular risks which have resulted in the commercially available drug Vioxx® (refocoxib) being removed from the market (Laine *et al.,* 2008; Baron

et al., 2008; Juni *et al.,* 2004). The consequence of this is that it has likely resulted in continued use of the NSAIDs in spite of their gastrointestinal side effects.

Medicinal plant compounds could be considered a possible alternative to NSAIDS and selective COX-2 inhibitors in the treatment of various inflammatory conditions. The medicinal plant *Harpagophytum procumbens* extract with its iridiode glycosides harpagoside and harpagide has proven anti-inflammatory activity.

2.2.3 Iridiodes

They are a subclass of the monoterpenes (10 carbon structure), which includes more than 200 structures, which are distributed in the plant families, *Apocynaceae, Gentiannaceae, Lamiaceae, Loganiacceae, Menyanthacceae, Plantaginacceae, Rubiaceae, Scrophulariacceae, Valerianacceae and Verbanacceae* (Van Wyk and Wink, 2004). Various iridiode glycosides including harpagoside and harpagide iridiodes are present in different plant species such as *Harpagophytum procumbens (Pedaliaceae), Scrophularia ningpoensis (Scrophulariacceae)* and *Scrophularia scorodonia* (Ebrahim and Uebel, 2011; Benito *et al.,* 2000*;* Li *et al.,* 1999). The iridiodes have anti-inflammatory properties and are used as analgesics and anti-rheumatics. The lactone ring of the iridiode glycoside generates a reactive aldehyde function, which can form covalent bonds with amino groups of proteins. This action could explain the anti-inflammatory properties of iridiode compounds where the alkylation could lead to the inhibition of cyclooxygenase interfering with inflammation promoters such as prostaglandins (Van Wyk and Wink, 2004).

2.5 *Harpagophytum procumbens* (Devil's Claw)

The typical habitat of Devil's Claw (*Harpagophytum procumbens*) is open savannahs and arid regions geographically located in many regions throughout Southern Africa that include South Africa, Namibia Zambia and Zimbabwe.

2.5.1 Macroscopic description

The plant consists of long flat branches (up to 1,5 meters) spread on the ground, lobed leaves, reddish violet flowers covered with hook-shaped projections and active components are suggested to be contained in the secondary storage roots (figure 2.2 and figure 2.3).

Figure 2.2 *Harpagophytum procumbens* plant.

Figure 2.3 *Harpagophytum procumbens* root.

2.5.2 Uses

Traditionally in South Africa, it is used in digestive disorders, anti-inflammatory conditions, analgesia and the treatment of allergies (Wichtl, 2004).

2.5.3 Chemical composition

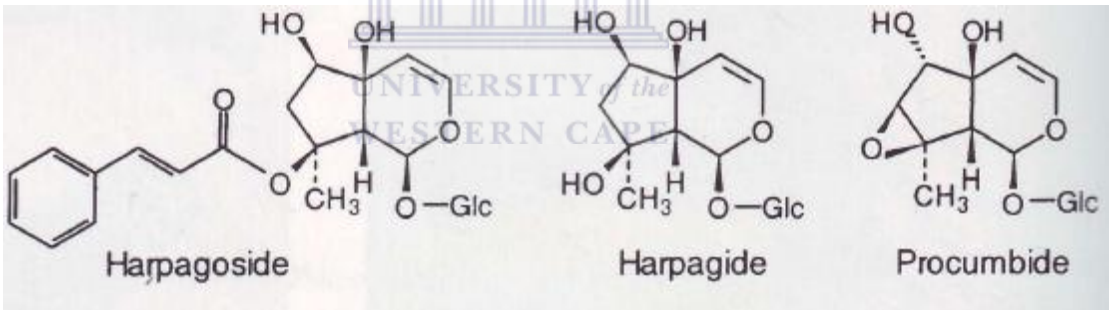
Harpagophytum procumbens contains sugars; phytosterols; phenolic acid; triterpenes; sterols; fats and iridiode glycosides (Wichtl, 2004). The iridiode glycosides consist of

harpagoside, harpagide, procumbide and procumboside (Bruneton, 1999). Harpagoside, the main compound in the fresh and dried root and harpagide (possibly a degradation product of harpagoside and procumbide) (figure 2.4) are believed to be the main components to have medicinal properties (Van Wyk *et al.,*, 1997).

2.5.4 Harpagoside

The iridiode glycoside harpagoside is the main compound in the fresh and dried root (Van Wyk *et al.,*, 1997) According to the European Pharmacopoeia, the crude extract should not contain less than 1.2% harpagoside. It has a molecular weight of 494 Dalton and is soluble in acetonitrile, ethanol and water. It exhibits a maxima UV wavelength absorbance at 280 nm ± 2 nm.

Figure 2.4: Chemical structures of harpagoside, harpagide and procumbide.



2.5.5 Harpagide

The iridiode glycoside harpagide is a possible degradation product of harpagoside with a molecular weight of 364 Dalton, soluble in water and methanol. This compound does not possess absorbance spectra in the UV absorbance range.

2.5.6 Preparations

Harpagophytum procumbens is most commonly administered orally as a tea (infusion) and more recently as dry extract preparations in the form of capsule, powder and tablet (Van Wyk, 1997). The tea is made by pouring 300ml of water over 4.5 gram of finely cut root or coarse powder, steeped for 8 hours at room temperature and then strained. The teas are taken three times per day. In the case of the dry extract preparation it usually contains between 200 mg to 480 mg per gram of preparation.

2.5.7 Clinical studies

The clinical efficacy of *Harpagophytum procumbens* for inflammatory conditions has been reported. These clinical tests predominantly use the whole *Harpagophytum procumbens* extract, mainly investigating its effects on osteoarthritis and lower back pain (Gagnier *et al.,*, 2004; Chrubasik *et al.,* 2002). A study conducted by *Chantre et al.,* (2000) indicated that Harpadol capsules which is a formulation containing 435 mg of *Harpagophytum procumbens* extract is comparable to 100 mg/ml diacerein in efficacy for the treatment of osteoarthritis. The study also revealed that patients using Harpadol required less frequent usage of the NSAID, diclofenac.

Gagnier *et al.,* (2004) suggests that the *Harpagophytum procumbens* extracts should contain between 50mg and 100mg of harpagoside to have moderate to higher efficacy for clinical conditions such as osteoarthritis and chronic lower back pain with the nature of the solvent used in the extraction procedure also contributing to the concentration required.

Variances exist with respect to extent of the clinical efficacy of *Harpagophytum procumbens* extracts. This may be related to different clinical assessment criteria used and type of extract used in the studies (McGregor *et al.,* 2005; Gagnier *et al.,* 2004).

2.5.8 Animal studies

Studies involving COX-2 inhibition by *Harpagophytum procumbens* extract in animals indicate a reduction in pain and inflammation during experimental test procedures. This is demonstrated by Lanhers *et al.,* 1992 who reported that the crude extract produced significant dose dependent anti-inflammatory effects in response to acute inflammatory induction (carrageenan-induced rat paw oedema test). Similarly, Anderson *et al.,* 2004 reported the anti-inflammatory effects with extracts of *Harpagophytum procumbens* in rats with Freund's adjuvant induced arthritis in the acute and chronic phase of inflammation by measuring reduced oedema. However, animal studies tend to indicate that isolated components such as harpagoside do not demonstrate anti-inflammatory properties (McGregor *et al.,* 2005; Lanhers *et al.,* 1992).

2.5.9 *In Vitro* studies

2.5.9.1 Cyclooxygenase 2

Cyclooxygenase is a membrane bound enzyme responsible for the oxidation of arachadonic acid to prostaglandin and the subsequent reduction of prostaglandin G_2 to prostaglandin H2 (PGH_2). Cyclooxygenase enzymes are expressed in at least two main isoforms, Cyclooxygenase-1 (COX-1) and Cylclooxygenase-2 (COX-2). These two isoforms display about 50% homology and have similar catalytic activity but they are physiologically distinct (Eldeen and Van Staden, 2008). The COX-1 is a constitutive enzyme present in most cells, which act as homeostatic regulators, involved with functions such as gastric cytoprotection and platelet aggregation (Rang *et al.,* 2003; Ouellet, 2001).

COX-2, an inducible enzyme, is not normally present in cells but it is strongly induced in inflammatory cells – in response to tissue injury or pro-inflammatory agents, with the primary inflammatory cytokines, interleukin and tumour necrosis factor (TNF)-α playing an important

role (Motilva *et al.,* 2005; Rang *et al.,* 2003). COX-2 is the principal isoform that participates in inflammation and its induction is responsible for the production of prostaglandins at the site of inflammation (Murias *et al.,* 2004).

The process of producing anti-inflammatory effects by compounds could involve various mechanisms. These include the measurement of the inhibition of enzyme activity through reduced enzyme expression (McGregor *et al.,* 2005); measurement of direct inhibition of the enzyme (Corazzi *et al., 2005)* or measuring other biochemical activities due to reduced cyclooxygenase enzyme output (Kundu *et al.,* 2005).

The anti-inflammatory properties of *Harpagophytum procumbens* extract and some of its components have been attributed to the inhibition of the enzyme activity related to COX-1 (Jang *et al.,* 2003) and COX-2 (Ouitas and Heard, 2008), the latter being more frequently investigated.

As a result of varying methodologies for assessing COX-2 activity, the literature indicates varying degrees of efficacy of *Harpagophytum procumbens* and its components in possessing COX-2 inhibitory effects. A study by Zhang *et al.,* (2011) investigating the effect of harpagoside and harpagide on the inhibition of the COX-2 enzyme by measuring prostaglandin concentration revealed that these two compounds did not have inhibitory activity at concentrations of up to 100µM. However, Abdelouahab and Heard, 2008 reported that the harpagide component had pro-inflammatory activity as a result of an increase in COX-2 activity.

The study by Gyurkovska *et al.,* 2011 investigated the COX-2 inhibitory activity of *Harpagophytum procumbens* crude extract and various isolated components through various mechanisms of action. They demonstrated a decrease in the concentration of nitric oxide, which is a powerful pro-inflammatory mediator with the crude extract as well as its individual components. The same study also performed tests to observe the effect compounds would have on the TNF-α and IL-6 pro- inflammatory mediator concentration.

The results indicate that the crude extract displayed COX-2 inhibitory effects but harpagoside and harpagide did not produce significant COX-2 inhibitory effects. The inhibitory effect of the crude extract is similar to a study by Fiebich *et al.,* (2001) who indicated a decrease in LPS induced TNF-α and IL-6 release. Conversely, the Fiebich study did produce inhibitory action attributed to harpagoside.

When observing the different methods and techniques used to evaluate the COX-2 inhibition and anti-inflammatory effects of *Harpagophytum procumbens* it does indicate that generally, the crude extracts display anti-inflammatory characteristics and that opposing results with respect to efficacy is mainly attributed to the individual components of the crude *Harpagophytum procumbens* extract such as harpagoside. This may be related to differences in the method of inhibition assessment and plant extract composition (McGregor *et al.,* 2005; Loew *et al.*, 2001). The *Harpagophytum procumbens* extract with all its constituents seems to be the most effective in the inhibition of COX-2 compared to its individual constituents, which may be related to synergy (Abdelouahab and Heard, 2008.)

The studies mentioned above demonstrate the COX-2 inhibition by *Harpagophytum procumbens* extract through the assessment of indirect COX-2 enzyme inhibition rather than direct inhibition of the COX-2 enzyme.

The present study will demonstrate the direct inhibition of COX-2 enzyme (in contrast to others which focus on indirect inhibition) by an extract of *Harpagophytum procumbens* as well as harpagoside and harpagide. DuP-697 was used as the reference inhibitor.

2.5.9.2 DuP-697

DuP-697 [5-bromo-2- (4-flourophenyl)-3-(4-(methylsulfonyl)-thiophene)] is a member of the diaryl heterocyclic group of selective COX- 2 inhibitors, which includes MK-966 recoxib and celocoxib (Burdan *et al.,* 2003). Dup-697 is a selective, time dependent and essentially

irreversible COX-2 inhibitor (Pinto *et al.,* 1996). It has greater inhibition over COX-2 than COX-1 enzymes (50 times). It is soluble in organic solvents such as ethanol, DMSO and DMF. DuP-697 is not used clinically due to its long plasma half-life of 242 hours in humans (Pinto *et al.,* 1996). It is, however, used in laboratory experimental work as a reference for COX-2 inhibitory studies as it demonstrates significant COX-2 inhibition (Copeland *et al.,* 1994).

CHAPTER THREE: Transdermal Drug Permeation

3.1 Introduction

The skin offers an excellent barrier to the external environment therefore the rational for using the transdermal route of drug delivery needs to be carefully considered. In instances where the most convenient drug delivery routes such as the oral route is not viable for drug administration then alternatives must be sought. The intravenous route may be considered as an option as it has advantages of bypassing gastrointestinal and hepatic metabolism but it is an invasive and apprehensive method of drug delivery. Further, most anatomical orifices have been investigated for the ability to serve as a route for drug administration. Nevertheless, the transdermal route of drug administration offers advantages as a route of delivery in addition to bypassing gastrointestinal and hepatic metabolism (Naik *et al.,* 2000).

The skin has been regarded as a favourable route for the administration of compounds with patient compliance and the ease of administration making it an appealing route for delivery (El-Kattan *et al.,* 2000). The main advantages of the transdermal drug delivery over other conventional delivery modes such as oral administration are bypassing first pass hepatic and intestinal metabolism, avoidance of variable rates of absorption and metabolism as well as the elimination of gastrointestinal irritation as a result of various compounds in dosage forms (Godwin *et al.,* 1997).

The skin is the largest organ of the body; it covers an area over about 2 m^2 and weighs about between 4.5 and 5.0 kg with the thickness ranging from 0.5mm to 4mm depending on the location on the body (Wickett and Visscher, 2006; Tortora and Gabowski, 1993).

Structurally, the skin consists of an outer portion, the epidermis, which is connected to an inner thicker connective tissue, the dermis. Beneath the dermis is the hypodermis, which consists of areolar and adipose tissue. (Tortora and Gabowski, 1993)

The skin serves as a two way barrier where it controls the loss of water, electrolytes and other body constituents as well as preventing entry of unwanted solutes from the environment (Lane and Mclean 2008; Hadgraft 2001). This formidable barrier by function therefore restricts the permeation of many compounds; this contributes to the limitation of the transdermal delivery as a route of administration.

The choice of compound used in transdermal delivery is determined by a number of factors, which include physiochemical properties such a lipophilicity, solubility, molecular weight and interactions with membranes (Naik *et al.,* 2000; Malan *et al.,* 2002; Kalia and Guy 2001).

Topical drug delivery targets five main groups, namely the skin surface, horny layer, viable epidermis and upper dermis, skin glands and systemic circulation. An ideal compound for transdermal drug delivery would have sufficient lipophilicity to partition in the stratum corneum and also have good aqueous solubility allowing it to partition into the viable dermis, epidermis and systemic circulation (Kalia and Guy, 2001).

3.2 The skin structure

The cross section of the skin reveals four layers, namely the non-viable epidermis stratum corneum, viable epidermis, dermis and subcutaneous layer.

3.2.1 Stratum corneum

The stratum corneum is thought to be the main barrier to skin penetration and is comprised of 20 to 25 layers of flat dead cells surrounded by an intercellular matrix of lipids and aqueous layers (Barry, 2001; Barbato *et al.,* 1998; Tortora and Gabowski, 1993). It is mainly a hydrophobic layer made up of coenocytes, which are embedded, in continuous intercellular lipids. The corneocytes occur in lipid lamellar layers, which may be described as having a brick wall formation with the corneocytes being the bricks and the intercellular lipids representing the wall (Bouwstra and Ponec, 2006). The tough structure of corneocytes provides a form of mechanical support for the intercellular lipid lamellae (Lee *et al.,* 2008).

The uptake of penetrants into the stratum corneum and its passive diffusion are thought to be the rate-limiting step in transdermal penetration (Kalia and Guy, 2001; Barbato *et al.,* 1998).

3.2.2 Viable epidermis

The viable epidermis is found between the stratum corneum and the dermis. The epidermis is about 50-100 μm thick and is a dynamic, constantly self-renewing tissue where cells lost at the stratum corneum is balanced by cell growth in the lower basal epidermis layer (Bouwstra and Ponec, 2006). It consists of various layers, namely, (from the inside to the outside) stratum basale, stratum spinosum and the stratum granulsum (Wickett and Visscher, 2006). The predominant cell type in the epidermis is the keratinocyte. They exist from the stratum basale to the stratum granulosum at which they transform into the stratum

corneum (Wickett and Visscher, 2006). The viable dermis is a more aqueous soluble layer than the stratum corneum (Naik *et al.,* 2000). This could be related to its higher water content (Morganti *et al.,* 2001).

3.2.3 Dermis and Hypodermis

The dermis is rich in blood vessels, nerve endings and lymphatic vessels with an extensive network, which connects to the systemic circulation where the lymphatic vessel has the function of draining excess extracellular fluid and clearing antigenic material (Murthy and Shivakumar, 2010). The hypodermis is attached to underlying organs such as bone and muscle and is the deepest layer of the skin, which consists of the subcutaneous tissue filled with fat cells, macrophages and fibroblasts (Murthy and Shivakumar, 2010; Tortora and Gabowski, 1993).

3.2.4 Skin appendages

In addition to the different layers, the skin consists of sweat glands, hair follicles and sebaceous glands. The sweat glands exists in two forms, the larger apocrine glands which are exclusively associated in the groin and axillae, and the smaller eccrine glands which is a simple coiled structure located in the reticular dermis with an opening directly onto the dermis (Bristow and Turner, 2002). The follicular area account for 0.1% and eccrine 10^{-3}% of the total surface area of the skin (Hadgraft, 2001).

3.3 Percutaneous absorption

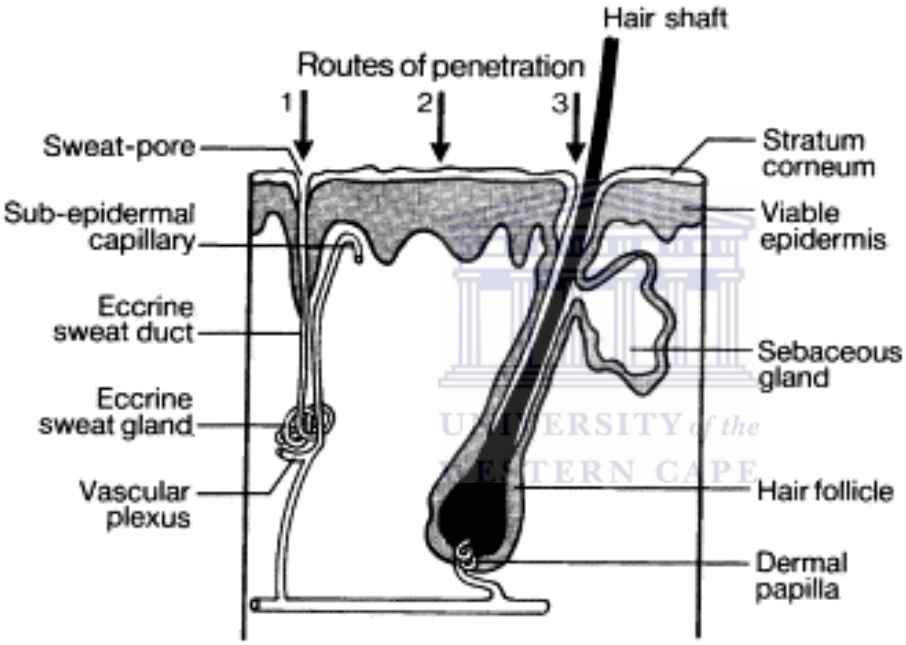
Percutaneous absorption is a passive process (Hadgraft, 2001) and may be described as the uptake of a compound into systemic circulation after dermal application. The process may be described as follows (Kalia and Guy, 2001; Flynn and Stewart, 1988):

- Partitioning to the outermost layer of the skin, the stratum corneum
- Diffusion through the stratum corneum, mainly via the intercellular pathway
- Partitioning from the stratum corneum into the aqueous viable dermis
- Diffusion through the viable epidermis into the upper dermis
- Uptake into the local capillary network to systemic circulation

3.4 Routes of transdermal delivery

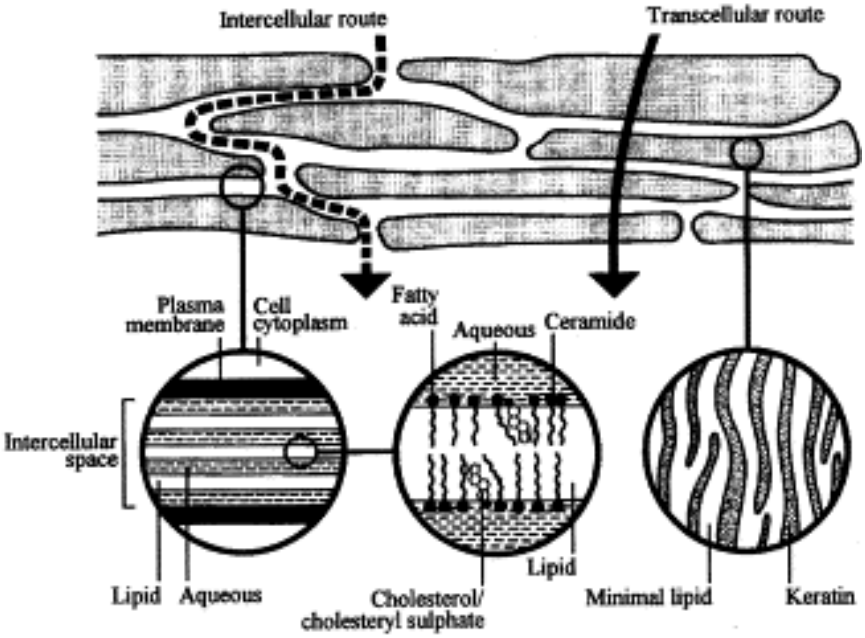
When active molecules come into contact with the surface of the skin they are able to penetrate via three potential pathways. This may be through the sebaceous and the sweat glands, the hair follicles (the two are together called the transappendageal route) or directly across the stratum corneum (figure 3.1) (Benson, 2005).

Figure 3.1 Routes of transdermal delivery.



Penetration across the stratum corneum involves two potential pathways for the absorption of penetrants, the transcellular route (across corneocytes and the lipid matrix) and the intercellular route (via the lipid domains between the corneocytes (Suhonen *et al.,* 1999). The intercellular route is the primary route of permeation.

Figure 3.2 Absorption across the stratum corneum.



3.4.1 Transcellular and Intercellular pathway

Compounds in the transcellular pathway pass directly though the hydrated keratin corneocyte layer as seen in figure 3.2 (Barry, 2001). The intercellular route of penetration is reported to be the primary means of permeation across the stratum corneum (Kalia and Guy, 2001; Bach and Lippold, 1998). It is a longer and more tortuous route across the corneum stratum when compared to the transcellular route. The diffusional pathway is therefore longer than the simple path length of the thickness of the stratum corneum and has been estimated to be as long as 500µm. This route contains structured lipids and a diffusing molecule has to cross lipophilic and hydrophilic domains before reaching the junction between the stratum corneum and viable epidermis (Hadgraft, 2004).

3.4.2 Transappendageal route

Under normal conditions the appendageal route is not thought to be significant in the permeation process, which could be related to the low surface area covered by the appendages (Hadgraft, 2001). Sebaceous glands release sebum which creates an

environment rich in neutral, non-polar lipids in the region of the hair follicle which may be a prerequisite for the follicular uptake of some lipophilic drugs. However, sebum may impede the transport of hydrophilic compounds (Meidan *et al.,* 2005).

3.5 Factors affecting transdermal permeation

3.5.1 Biological factors

3.5.1.1 Disease and skin conditions

The skin provides the body with a barrier to the external environment but function impairment often arises as a result of disease or from direct damage followed by a breach in the stratum corneum, These diseases include ichthyosis, psoriasis, atopic dermatitis as well as cuts and abrasions (Rawlings *et al.,* 2008; Barry, 2007; Cork *et al.,* 2006). This impairment may be demonstrated by the altered integrity of the stratum corneum and may lead to an increase in transepidermal water loss (Rawlings *et al.,* 2008).

3.5.1.2 Skin age

It is suggested that there is no structural difference in the skin of infants and adults (Barry, 2007). However, the skin like all organs will undergo morphological and functional decrement with increased age which results in the thinning of the epidermis by 10% to 50% and the slow replacement of lipids which leads to disturbed barrier function (Zouboulis and Makrantonaki, 2011).

3.5.1.3 Skin hydration

Skin hydration can result in an increase in permeability by causing swelling of the stratum corneum though perspiration, water diffusion from underlying epidermal layers or occlusive vehicles (Barry, 2007; Wickett and Visscher, 2006). The state of hydration of the stratum

corneum is one of the most important factors in the determination of the rate of percutaneous absorption of a compound, while the level of hydration is a function of the water concentration gradient between the dermis and the surface of the skin (Morganti *et al.,* 2001).

3.5.1.4 Skin region

Skin permeability rates vary for specific compounds passing through identical skin sites of different healthy subjects and permeability various at various skin sites on the body (Barry, 2007). Variance may be related to age, gender, race and health (Flynn, 2002). The thickness of the skin varies at different sites in the, for example, the feet and hands consist of double the epidermis thickness compared to other sites (Draelos, 2006). There is an inverse relationship between the diffusion path length and flux of penetrants across membranes. It could therefore be expected that a decrease in permeability would exist with thicker regions of the stratum corneum. The female abdominal skin is the most commonly used region for *in vitro* human transdermal studies due to it not being as thick (compared to other regions) and it's is availability as a result of cosmetic surgical procedures.

3.5.1.5 Temperature

The body maintains an isothermal temperature of 37º C. Raised temperature increases blood flow through the skin, this could facilitate the removal of compounds from the dermis resulting in an increase in the concentration gradient across the skin. This, however, is not clinically important (Barry, 2007; Flynn, 2002).

3.5.1.6 Skin metabolism

The skin consists of various layers, components and appendages; which may contribute to the metabolism of compounds permeating through it and affect the bioavailability of topical

compounds (Muller *et al.,* 2003). Metabolism is particularly important for compounds exhibiting local pharmacological effects (Bando *et al.,* 1997). The exact location in the skin's layers and anatomical distributions of metabolic enzyme systems remain uncertain, although the epidermis is considered the main site for metabolic activity (Streinstrasser and Merkle, 1995).

The metabolizing enzymes present in the skin, particularly the viable dermis, may affect the absorption of compounds by changing the structure of the compounds and therefore its physiochemical properties (Haberland *et al.,* 2006). This could affect the lag time in a permeation process (Seko *et al.,* 1999). Bioconversion of lipophilic drugs to a hydrophilic form plays an important role in percutaneous permeation (Bando *et al.,* 1997).

However, skin metabolism is not an important consideration as the skin has much less capacity to metabolize compounds than in the hepatic or gastrointestinal region (Flynn and Stewart, 1988).

3.6 Physiochemical properties

Due to the good barrier properties of the stratum corneum, permeation will be a function of the physiological properties of compounds and its role needs to be understood in light of the mechanisms of permeation across the stratum corneum (Hadgraft, 2004). The diffusion of molecules across a barrier is dependent on its physiochemical properties such as partition coefficient, solubility and molecular weight (Salgado *et al.,* 2010).

3.6.1 Drug Solubility in the stratum corneum

The stratum corneum layer is mainly hydrophobic (Godin and Touitou, 2007). As earlier suggested, the permeant should possess both lipophilic and hydrophilic solubilities to allow for permeation across the various skin layers. If it were too hydrophilic, the compound would not be able to transfer into the stratum corneum and if too lipophilic the compound will have a tendency to remain in the stratum corneum (Naik *et al.,* 2000). Therefore, compounds with good solubility in oil and water should permeate well with an octanol partition value of approximately 1-3 (Hadgraft, 2004).

3.6.1.1 Aqueous solubility

Pharmacological activity is dependent on the solubilization of a drug substance in physiological fluids; therefore, compounds must possess some aqueous solubility for absorption and therapeutic response. The pH of a formulation is an important factor to consider as it affects the solubility of weakly acidic and basic compounds. pH may be used to increase the solubility of ionisable compounds, for compounds without the presence of ionisable groups the solubility may be increased by the application of surfactants to aqueous solutions. Aqueous solubility of compounds is also of importance for permeation across the skin, with an aqueous solubility of more than 1 mg/ml recommended (Gerber *et al.,* 2006). Even though compounds in its ionized form offer better aqueous solubility, it should be noted

that increased permeation through the stratum corneum is achieved with compounds forming ion pairs of opposite charges to temporarily form neutral compounds (Barry, 2001). These compounds dissociate into charged species upon reaching the aqueous epidermis.

3.6.2 Partition coefficient

If a third substance is added to a system containing 2 immiscible liquids, this substance will distribute between the liquid phases until the ratio of its concentration in each phase attains a certain value known as the partition coefficient (Berthod and Carda-Broch, 2004). This value is a measure of the lipophilicity of compounds, which may be used as a prediction of how well it will be able to move across membranes. The experimentally determined partition coefficient in aqueous and octanol phases is called log P (*n*-octanol-water coefficient). In most instances, the aqueous phase would be water and the organic phase is *n*-octanol. *n*-Octanol is chosen as the solvent for the organic (oil) phase as it displays properties similar to that of biological membranes (Barry, 2007).

The pathway of a drug permeating through the skin involves permeation through the more lipophilic stratum corneum and the viable epidermis, which is mainly hydrophilic before reaching the dermis. Therefore, an ideal candidate for permeation should have sufficient lipophilicity to partition into the stratum corneum and also adequate hydrophilicity to enable partitioning into the viable dermal layer of the skin (Kalia and Guy, 2001). It is suggested that compounds with a high lipophilicity will have difficulty leaving the stratum corneum and in contrast, compounds with very high hydrophilicity may have difficulty permeating into this layer. Therefore, the recommended Log D (*n*-octanol-PBS coefficient) for a compound considered for transdermal permeation is in the range of 1-3 (Gerber *et al.,* 2006, Hadgraft, 2004).

The *n*-octanol-water partition constant (as referred to as partition co-efficient) has been known to be a physical property that correlates best with biological activity. It can be used as

a measure of lipophilicity and therefore, suitable for characterizing the interaction between a chemical substance and biological systems (Danielson and Zhang 1996).

3.6.3 Molecular weight and volume

The ability of a drug to pass through a membrane is a function of its size as well as its volume where the molecular volume is dependent on weight conformation (Mahato, 2007; Malan *et al.,* 2002). Molecules with compact conformation will form a lower molecular volume and therefore, a higher diffusivity and permeability (Mahato, 2007). Molecular weight of compounds for transdermal permeation should have a molecular weight of under 500 Dalton as increasing molecular weights cause a rapid decline in absorption of compounds (Bos and Meinardi, 2000).

3.6.4 Diffusion

The main component involved in the permeation of compounds through the skin is simple passive diffusion. Fick's law of diffusion can be used to analyze permeation data and used predictively (Yamashita and Hashida 2003). Fick's first law may be used to describe steady state diffusion and may be simplified to (equation 1) (Hadgraft, 2001)

$$J = \frac{DK\Delta c}{h} \qquad (1)$$

J= flux per unit area

D= diffusion coefficient in the skin

Δc= concentration difference across skin

h= diffusional path length

K= skin vehicle partition coefficient

Under normal circumstances the applied quantity of active compound on the surface of the skin (c_{app}) is higher than the concentration under the skin and therefore, equation1 is simplified to:

$$J = k_p.c_{app} \quad (2)$$

Where k_p is permeability constant. The driving force for diffusion may be considered to be the concentration gradient. Fick's Laws of diffusion demonstrates that the flux (J) should increase linearly until c_{app} reaches the solubility limit (equation 2). So any concentration above the solubility limit in the same vehicle will result in the same flux value. However, if the conditions are such that the compound can be supersaturated and these unstable conditions maintained it will result in flux values higher than that in the usual saturated form. Further, in some instances the thermodynamic properties of an active compound may change due to constituents in the formulation, this may result in different formulations containing the same concentration of active compounds having reduced flux values (Hadgraft, 2001).

Upon application to a membrane, little or no drug penetrates the membrane that it comes into contact with the skin in the first moments upon its application (equation 3), this is referred to as the lag time (LT) (Yamashita and Hashida 2003):

$$LT = \frac{L2}{6D} \quad (3)$$

LT = Lag time

L= thickness of the skin (membrane)

D= diffusion coefficient

After the first drug compounds permeate the membrane, the flux of the drug increases until it reaches a steady state portion represented by the linear segment of a cumulative concentration versus time curve.

3.6.5 Flux

Flux is the rate of change of the cumulative mass of drug that passes per unit area and time through the membrane (Xiong *et al.,* 1996). Steady state flux (J_{ss}) is determined from Fick's law of diffusion (equation 4):

$$J_s = dQ_r/Adt \quad (4)$$

Where J_s is the steady state membrane flux, dQ_r is the change in quantity of material passing the membrane into the receptor compartment; A is the active diffusion area and *dt* is the change in time. The steady state flux of the various formulations through the membranes is calculated from the slope of the linear portion of the cumulative amount permeated through a membrane per unit area versus time (Niazy, 1996)

3.6.6 Enhancement Factor

In order to express the extent of enhancement of the flux due to the presence of permeation enhancers a parameter called the enhancement factor (equation 5) [also known as enhancement ratio (ER)] is calculated as follows (Xiong *et al.,* 1996; Diez-Sales *et al.,* 1996):

$$\mathrm{EF} = \frac{\text{Flux with enhancer}}{\text{FLux without enhancer}} \quad (5)$$

This is the ratio of flux with the penetration enhancer to the flux without the enhancer.

3.7 Gels

3.7.1. Pluronic® F-127 gel

Pluronic® is a non-ionic surfactant that consists of polyethylene oxide (PEG) and polypropylene oxide (PPO) co-polymers. At given concentration it remains as individual (non-associated) co-polymers at temperature below the critical micelle temperature (CMT).

Above the CMT the molecules become more lipophilic with PPO groups positioned at the core of the micelle (Antunes *et al.,* 2011).

The high solubilizing capacity and its non-toxic properties make it suitable for drug delivery (Pisal *et al.,* 2004). It consists by weight of 70% ethylene oxide and 30% propylene oxide and is non-toxic (Lee *et al.,* 2004; DiBiase and Rhodes, 1996). Pluronic® F-127 has been used with various active compounds as a vehicle in its gel matrix in ear, nasal and ophthalmic dosage forms as well as transdermal drug delivery studies (Ma *et al.,* 2008; Lee *et al.,* 2004; Pisal *et al.,* 2004; DiBiase and Rhodes, 1996). It has thermo reversible properties where an aqueous solution of Pluronic®-127 forms a non-chemically cross-linked hydrogel upon warming to ambient temperatures and body temperature (Ma *et al.,* 2008; Pisal *et al.,* 2004).

3.7.2 Carbopol Ultrez 21®

Carbomer resins are allylpentaerythritol-cross linked, acrylic acid-based polymers, which have a high molecular weight. Many types of carbomer resins are available with varying degrees of viscosity (Allen, 1998). Carbopol Ultrez 21® polymer performs effectively over a wide range of pH range. It may also be used to incorporate substance with high oil content and botanical ingredients (Lubrizol, 2008)

3.7.3 Hydroxypropyl cellulose

Hydroxypropyl cellulose is a thermoplastic polymer, which may be used as thickening agent in gels. It is soluble in water and compatible organic solvents such as ethanol and propylene glycol (APhA and PSGB, 1986). Janathraprapapa and Stagni (2007) formulated a hydroxypropyl cellulose gel for permeation studies consisting of ethanol, propylene glycol and meloxicam. This gel formulation successfully incorporated various permeation enhancers when used as a topical application for permeation studies.

3.8 Permeation enhancers

Permeation enhancers are chemical components of a formulation that facilitate absorption of compounds when applied to the surface of the skin. They increase permeation by either “pushing” the drug into the skin by the thermodynamic activity of the penetrant being increased in the formulation or by pulling” the drug into the skin by permeating the skin and increasing the solubility of the penetrant in the skin (Sloan *et al.,* 2006). Penetration enhancers should ideally be non-toxic, non-allergic, pharmacologically inert, compatible with penetrants and formulation excipients, tasteless odorless, colourless and have good solvent properties (Sinha and Maninder 2000). In addition, the permeation enhancer should not result in the loss of bodily fluids and electrolytes and the skin should be allowed to regain its barrier properties upon the removal of the enhancer (Barry, 2007).

No permeation enhancer possesses all these properties required for the ideal enhancer; also enhancers perform differently based on various physiochemical properties of the penetrant. Currently, there is plethora of studies investigating permeation enhancer activity and mechanisms, *in vivo* and *in vitro*. Thus, there is sufficient data available for the general classification of the permeation whether it favors hydrophilic or hydrophobic compounds for improved permeation. The mechanism of action of these permeation enhancers may be act by one or more mechanisms from the 3 categories below based on the lipid-protein-partition concept (Barry, 2001):

Protein modification - Surfactants that are ionic in nature such as dimethsulphoxide (DMSO) are able to interact well with the keratin in corneocytes, which results in dense protein structures opening up resulting in them becoming more permeable. These ionic surfactants may also modify the peptide/protein material in the bilayer.

Lipid action - The enhancer disrupts the stratum corneum lipid formation therefore resulting in it becoming more permeable. The accelerant molecules move into the lipid bilayer,

rotating, vibrating and trans-location. This results in the formation of micro cavities and increasing free volume available for drug diffusion.

Partitioning promotion - Solvents enter the stratum corneum, changes the solution properties by altering the chemical environment; this would then result in an increase in the partitioning of a second molecule into the horny layer of the skin that may be a penetrant. These may also be co-solvents such as water, propylene glycol and ethanol.

Many permeation enhancers may display more than one of the above-mentioned mechanisms. The in-use concentration of permeation enhancers should also be considered as a high concentration may reduce activity of the enhancer. Furthermore, these elevated concentrations may also have disruptive and irritant properties at elevated concentrations (Williams and Barry, 2004; Diez-Sales *et al.,* 1996).

The stratum corneum is known to offer diffusional resistance and is constituted by complex interaction of lipid and proteinatious components, which creates distinct hydrophilic and hydrophobic pathways (Walker and Smith, 1996).

3.8.1 Azone®

Azone® (1-dodecylazacycloheeptan-2-one or laurocapron) was the first molecule specifically designed as skin penetration enhancer. It can be considered chemically to be a hybrid of a cyclic amide with an alkylsulphoxide group. It is odorless, possesses a smooth yet oily feel and is highly lipophilic with a log P value of about 6.2 (Williams and Barry, 2004).

Azone® appears to perform its activity by the reduction of the diffusional barrier of the stratum corneum by inserting itself into structured lipids which are located in the intercellular channels resulting in the reduction of the fluidity of the microenvironment of the lipids leading to an increase in diffusion (Degim *et al.,* 1999). This compound is soluble in alcohols and

propylene glycol and has been reported to have the ability to promote flux of hydrophobic and hydrophilic compounds (Williams and Barry, 2004).

The efficacy of Azone® is concentration dependent with the lower values being favoured (Williams and Barry, 2004; Sinha and Kaur, 2000; Degim *et al.,* 1999; Niazy, 1996). Xiong *et al.,* (1996) demonstrated that Azone® incorporated into a Carbopol Ultrez 21® gel formulation displayed higher permeation of the permeant across the skin with a 5% concentration of Azone® compared to a 10% concentration. A high concentration could lead to a decrease in the permeation enhancement ratio, which is related to the lipophilicity of the penetrant used (Diez-Sales, 1996).

Azone® is considered to be a good permeation enhancer with studies showing that it has higher properties of improving flux across various membrane types including the human stratum corneum when compared to other enhancer types (Chatterjee *et al.,* 1997; Xiong *et al.,* 1996). Xiong *et al.,* (1996) investigated the effect of different enhancers on heparin across the human epidermis. The highest steady state flux values were achieved with the system containing Azone® as well as producing an enhancement ratio of 5.6. Azone® also produced an enhancement ratio of 11.4 for pentoxifylline from its gel formulation through full thickness rat skin (Yan *et al.,* 2007).

In addition to Azone® demonstrating activity on its own, the choice of a suitable solvent in the formulation may further increase the permeation of permeants across membranes. A solvent with good complimentary effects with Azone® would be propylene glycol. Chatterjee *et al.,* (1997) indicated that Azone® and propylene glycol combination resulted in higher flux and lower lag values when compared to other enhancer and solvent combinations. It is suggested that reason for this increased permeation activity is that Azone® disrupts the lipid structure of the epidermis where the propylene glycol plays the role of solvating the keratin in the horny cell, displacing bound water and competing with the permeant/drug for

hydrogen bonding sites. The Azone® therefore, enhances intercellular drug transport and the propylene glycol may enhance intracellular transport (Goodman and Barry,1988).

3.8.2 Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate (SDS) is an anionic, amphophilic surfactant with widespread topical application. Its activity has been related to a disruptive effect on the stratum corneum causing impairment of the skin barrier function as a result of the removal of intercellular hydrophobic lipids, which leads to an increase in transdermal water loss (Borra-Blasco *et al.,* 2004).

SDS has the ability to insert itself between bilayers of lipids in the stratum corneum (Ribuad *et al.,* 1994). This results in an increase in the flux value of the permeant. Borra-Blasco *et al.,* (1997) reported when assessing the permeation of four different compounds with varying lipophicities that the effect of SDS decreases as the lipophilicity of the compounds increases. Similarly, Copovi (2006) indicated that SDS had no effect on compounds with a log P value of more than 3 and the most hydrophilic compound (log P -0.95) tested 5-flourouracil, displayed the largest enhancement ratio. It follows the same permeability-lipophilicity correlation as Azone® with various compounds with lipophilicity ranging from hydrophilic to hydrophobic (Borra-Blasco *et al.,* 2004).

3.8.3 Water

When considering safety and effectiveness, water is the best permeation enhancer because most substances penetrate better through a hydrated stratum corneum than through dry tissue (Barry, 2001). This would be for hydrophilic and hydrophobic penetrants. The water content of the stratum corneum layer is between 15% and 20% of the dry weight, additional water in this layer could increase the solubility of the permeant leading to a modification of the partitioning from the vehicle to the stratum corneum (Benson, 2005). Benson (2005) also

suggested that hydration could cause swelling and opening of the stratum corneum resulting in increased permeation. Water used as a co-solvent to facilitate permeation may be due to its polar nature, interacting with the polar head groups of lipids in the stratum corneum and upsetting the packing nature of the polar arrangement (Suhonen *et al.,* 1999). Williams and Barry (2004) suggested that “free” water within the tissue could cause an alteration of the solubility of a permeant in the stratum corneum resulting in modification in the partitioning from the permeant vehicle into the stratum corneum. They further suggested that a mechanism like this would explain the increase in drug flux of hydrophilic drugs due to increased hydration but does not explain how hydration causes an increase in permeation for lipophilic compounds.

3.8.4 Ethanol

Ethanol is the most commonly used alcohol for transdermal permeation enhancement (Heard *et al.,* 2006; Sinha and Kaur, 2000). Concentrations of 30% may increase the lipid fluidity of the stratum corneum at polar interfaces (Suhonen *et al.,* 1999), which could result in increased flux across membranes. Ethanol is an effective solvent for polar and non polar groups of structures and it is feasible that small quantities are capable of leaching significant quantities of non-covalently bound amphophilic stratum corneum lipids which would affect the skin barrier (Heard *et al.,* 2006). It also increases the number of free sulphydryl groups of keratin in the proteins of the stratum corneum (Sinha and Kaur 2000). Higher concentrations of ethanol seem to promote permeation. Jantharaprapapa and Stagni (2007) reported the highest flux values with formulations consisting of ethanol at a concentration of 33%. Further, Thomas and Panchagula (2003) reported that the flux of AZT across the rat epidermis was highest in a formulation consisting of 66% ethanol compared to lower ethanol concentrations.

When incorporating ethanol as an excipient or co-solvent, its volatility should be considered as it may vaporize from the formulation and affect the solubility of the remaining excipients in a formulation resulting in changed permeation potential over time (Morimoto *et al.,* 2002).

3.8.5 Propylene glycol

The mechanism of action propylene glycol is suggested to be similar to that of ethanol, whereby the solvent partitions into the tissue facilitating the uptake of the drug into the skin with the possibility of small disturbances to intercellular packing of the lipids in the stratum corneum (Williams and Barry, 2004)

Though, generally it seems that it has only moderate enhancement properties on its own (Williams and Barry, 2004).

When Propylene glycol is incorporated into a formulation, it is usually selected primarily for its solvent properties, which also happen to have permeation enhancement properties (Thomas and Panchagula 2003; Kim *et al.,* 1993). It appears that the propylene glycol demonstrates enhanced activity, which aids other compounds to increase permeation across membranes (Williams and Barry, 2004; Wang *et al.,* 2004). This was demonstrated by Yan *et al.,* (2007) where various combinations of enhancers used with propylene glycol were investigated with the aim to increase the flux of pentoxifylline. Their study found that the combination containing propylene glycol obtained the highest flux value. However, in the absence of permeation enhancers in formulations, it has been reported that propylene glycol lacked the ability to increase permeation across membranes (Chatterjee *et al.,* 1997; Goodman and Barry, 1988)

However, higher concentrations of propylene glycol resulted in lower flux values. This was related to the dehydration of the epidermis leading to interlocking of polar head groups producing an increase in the barrier nature of the stratum corneum. Similarly, Thomas and

Panchagula (2003) revealed that the presence of high concentrations (above 50%) resulted in a decreased hydration of the epidermis.

3.8.6 Dimethsulphoxide (DMSO)

Dimethsulphoxide (DMSO) is one of the most widely used and earliest studied permeation enhancers. It is colourless and hygroscopic and has been effective to promote enhancement of hydrophilic and hydrophobic permeants (Williams and Barry, 2004) Various mechanisms of activity have been suggested, which include denaturation of the intercellular structural proteins of the stratum corneum as well as increasing the lipid fluidity through disruption of the ordered structure of the lipid chains (Anigbogu *et al.,* 1995). Even though it is commonly used there are concerns about its effects at high concentrations as it may result in denaturation of proteins and erythema in the stratum corneum. Side effects with the use of DMSO have been reported (Williams and Barry, 2004).

3.8.7 1,8 Cineole

Terpenes are found in essential oils and consist of hydrogen, carbon and oxygen atoms. The principal terpene element within eucalyptus oil is 1,8 cineole. 1.8 Cineole has the ability to promote the enhancement of hydrophilic and hydrophobic compounds (Rajan and Vasudevan, 2012). Anjos *et al.,* (2007) reported that this compound may reach the center of a membrane bilayer where it increases the local mobility and volume of the fluid state of the membrane. 1,8 cineole has been used at concentrations ranging from 1% to 10% with permeation enhancement achieved with lipophilic and hydrophilic penetrants across human and animal membrane systems (Gao and Singh1997; Xiong *et al.,* 1996; Ogiso *et al.,* 1995).

However, it is suggested that the activity of terpenes are dependent on the physiochemical properties of the penetrant and that the introduction of cineole may in certain cases, result in

a decrease in the permeation of the penetrant (Sinha and Kaur, 2000; Gao and Singh, 1997).

3.9 Synthetic membranes

Synthetic membranes are used to elucidate information regarding mechanisms of permeation and enhancement properties of excipients from dosage forms and may serve as a precursor to testing before transdermal studies (Hauck *et al.,* 2007; Al-Saidan, 2004; Clement *et al.,* 2000; Schwarb *et al.,* 1999).

Human transdermal permeation experiments are a more realistic reflection of the permeation potential of a compound or delivery system but the availability of skin and the risk related with its use is a motivation to use preliminary studies with synthetic membranes (Clement P, *et al.,* 2000). Synthetic membranes have demonstrated to share a common solubility-diffusion mechanism of drug transport with the skin resulting in it being used for quantitative prediction of transdermal drug delivery and as a skin-imitating membrane in *in vitro* drug delivery evaluation (Dureja *et al.,* 2001; Feldstein *et al.,* 1998).

The success of using of using synthetic membranes vary, Feldstein *et al.,* (1998) reported that a synthetic carbosil membrane displayed similar responses to the permeability of a variety of compounds compared to human skin. Conversely, Al-Saidan (2004) indicated no relation between a hydrophobic synthetic membrane and human skin with ibuprofen. Synthetic membrane models merely act as passive diffusional barriers and do not take into account the complexity of the human epidermis (Barbato *et al.,* 1998).

Synthetic membranes are generally classified as demonstrating hydrophilic or hydrophobic properties. The permeation profiles of permeants are dependent on the properties of synthetic membranes and formulations (Wang *et al.,* 2006).

3.9.1 Sil-Tec®

This silicone membrane with hydrophobic properties has been used with gel formulations containing propylene glycol: ethanol solutions for *in vitro* permeation testing (Salgado *et al.,* 2010; Schwarb et al., 1999). Sil-Tec® has also been used in the assessment of the *in vitro* permeation of the medicinal plant extracts *S. Lutea* from topical emulsions (Isaac *et al.,* 2012).

3.9.2 Tuffryn®

Tuffryn® is a polysulfone membrane with hydrophilic properties (Thakker and Chern, 2003). It has been used in *in vitro* diffusional studies for determining the reliability and reproducibility in the rate release profiles of topical dosage forms (Hauck *et al.,* 2007). It has also been used in studies comparing the flux values of hydrophobic membranes and human skin (Megrab *et al.,* 1995).

Synthetic membranes may be used to elucidate information relating to drug release profiles from dosage forms, enhancement properties of excipients and these membranes are used as precursors to transdermal permeation studies.

3.10 Other membrane systems

Various other membrane systems have been explored to simulate the skin to observe the behavior of penetrants, permeation enhancers and other permeation promoting techniques. These membrane systems include natural membranes such as skin of fruit, snakeskin and other animal skin models (Ansari *et al.,* 2006; Thomas and Panchagula 2003; Haigh *et al.,* 1998). Laboratory design membranes include liposomal suspensions containing lipids and chitosan layered membranes (Dureja *et al.,* 2001; Abraham and Downing, 1989). The disadvantage of non-skin membrane systems is that they do not contain active components which the skin possess and therefore, it is difficult to replicate permeation across the skin even though permeation is a passive process. Therefore, animal skin models are probably the closest models that may be used as a substitute for human skin with porcine skin, guinea pig and rat skin model commonly used (Abdelouahab and Heard, 2008; Morris *et al.,* 2009; Karende *et al.,* 2007; Anjos *et al.,* 2007; Wang *et al.,* 2004).

CHAPTER FOUR: Identification and Quantification of compounds in *Harpagophytum procumbens* extract.

Harpagoside and harpagide glycosides are present in the crude *Harpagophytum procumbens* extract and were used as marker compounds in the extract. These markers were identified and quantified using thin layer chromatography (TLC), UV spectroscopy, high pressure liquid chromatography (HPLC) and densitometry.

4.1 Methods

4.1.1 Extraction of *Harpagophytum procumbens*.

Although the dried *Harpagophytum procumbens* (A. White Chemist, South Africa) was not authenticated by a taxonomist at the University of the Western Cape, identification of the marker compounds in the plant extract was verified against certified standards with certificates of analysis. The plant root was cut into small manageable pieces and powdered using a hammer mill (Cullati, Germany) with decreasing aperture sizes. The resultant powder was collected and weighed. 250g of the powder was placed into 2.5 L containers and methanol (Merck, South Africa) added for extraction. The containers were not filled beyond two thirds of its volume in order to promote agitation and subsequent extraction. This mixture was agitated with a shaker (Labcon, USA) for 6 hours followed by centrifugation (Beckman J2-21, USA) for 30 minutes at 10000 rpm. After centrifuging the supernatant was carefully removed, filtered with filter paper (Whatman, No.1) and collected. The filtered supernatant was transferred in aliquots of 200ml into round bottom flasks, sealed, foiled wrapped and stored at -20° C.

4.1.2 Rotary Evaporation

Solvent removal was performed using a Rotavapour (Buchi R II, Germany) under reduced pressure while heated to a temperature not exceeding 40°C (European Pharmacopoeia, 2004). This process of methanol reduction was maintained until a resinous extract was formed with no further solvent loss and constant mass maintained. The resinous crude extract was, sealed, foiled wrapped and stored at -20° C.

4.1.3 Thin layer chromatography

4.1.3.1 Comprehensive Two-dimensional chromatography

Thin layer chromatography (TLC) including two dimensional thin layer chromatography was used for the identification and purification of the marker compounds harpagoside and harpagide in the *Harpagophytum procumbens* extract using TLC plates (silica gel 60, $F_{254,}$ Merck, South Africa). The chromatograms were examined under UV-light at 254 nm for the detection of individual compounds. The mobile phases used were solvent systems containing butanol, acetic acid and water (12:3:5) (BAW) and ethyl acetate, methanol, water (77:15:8) (EMW). All solvents were obtained from (Merck, South Africa). Samples were spotted for first dimension chromatography, dried, rotated 90° and again chromatographed in the second dimension with the second solvent system.

4.1.3.2 Identification of marker compounds.

4.1.3.2.1 Harpagide and Harpagoside standard preparation.

Stock solutions of harpagoside and harpagide standards (PhytoPlan®, Germany) were prepared with methanol. The stock solutions were stored at less than4°C. All experimental TLC plates included the presence of standards.

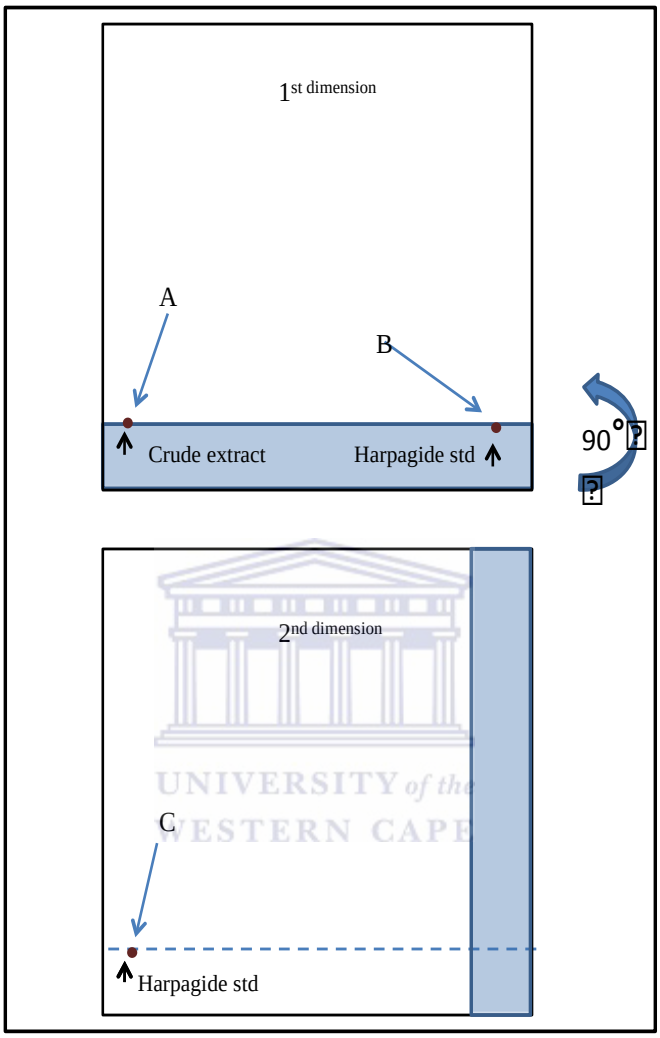
4.1.3.2.2 TLC Spraying colouring reagent.

The TLC spraying reagent contained 1% (w/v) vanillin (Sigma-Aldrich, South Africa), 1% methanol, 5% sulfuric acid, (Merck, South Africa). The spraying reagent was prepared by adding sulphuric acid to methanol followed by the addition of vanillin and made up to the appropriate volume with ethanol. This was gently agitated until a clear solution was formed. The reagent was applied to TLC plates by means of an aerosol-spraying device.

4.1.3.2.3 Harpagide identification.

Thin layer chromatography analysis was performed with crude extract at a concentration of 400 mg/ml. Two-dimensional TLC chromatography was implemented as illustrated in figure 4.1 based on the method as described by Ciesla and Waksmundzka-Hajnos, (2009). Five microlitres of the crude extract (A) as well as the pure harpagide standard (B) were spotted on the TLC plates and then chromatographed over a plate length of 8 centimeters (1st dimension) with BAW (12:3:5) as eluent. The TLC plates were air dried and observed under UV light at a wavelength of 254 nm. The visible chromatographed spots under the UV light were marked with a pencil. The TLC plate was rotated 90° anticlockwise. At this position, 5µl harpagide standard (C) was spotted and chromatographed in the second dimension with a solvent system consisting of EMW (77:15:8) as illustrated in figure 4.1. The TLC plate was air dried, viewed under UV light and sprayed with the TLC colouring reagent. It was then heated for 5 minutes at 80ºC in an oven yielding identifiable visible spots (Wichtl, 2004).

Figure 4.1 Two-dimensional TLC of *Harpagophytum procumbens* crude extract for harpagide identification with 2 solvent systems.

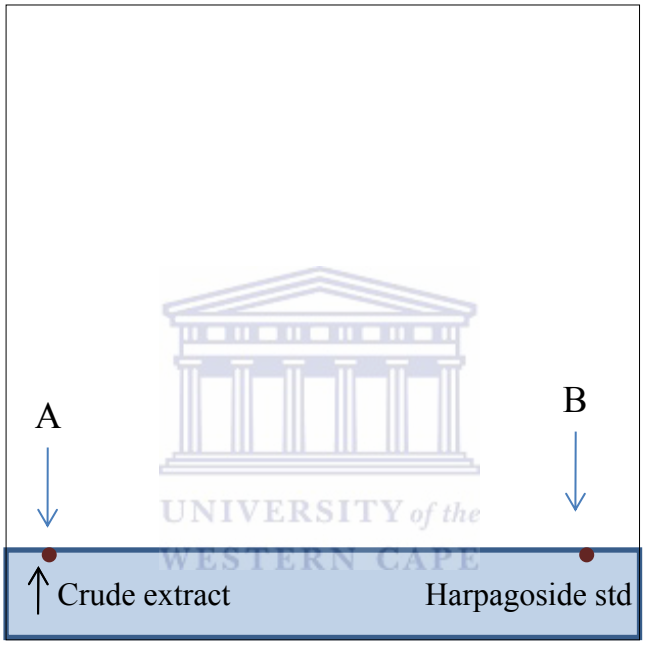


4.1.3.2.4 Harpagoside identification.

Thin layer chromatography was performed with crude extract at a concentration of 400 mg/ml. Five microlitres of the extract (A) as well as the harpagoside standard (B) was applied to the TLC plate and chromatographed over a plate length of 8 centimeters with the BAW solvent system (figure 4.2). The TLC plate was air-dried, observed under UV light at a wavelength of 254 nm. The visible spots under the UV light were marked with a pencil. The

TLC plate was sprayed with the colouring reagent. The TLC plates were heated for 5 minutes at 80ºC in an oven yielding identifiable visible spots (Wichtl, 2004).

Figure 4.2 TLC plate of *Harpagophytum procumbens* crude extract for harpagoside identification.



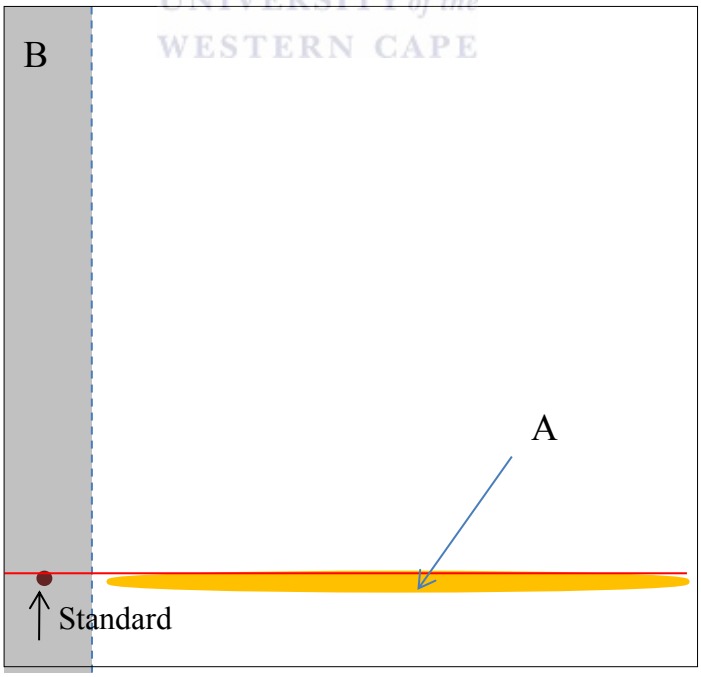
4.1.4 Densitometry

After the TLC plates were sprayed with the colouring reagent to identify the compounds, it was scanned using a scanner connected to a laptop computer with specialized Quantiscan® software version 3 (Biosoft®, England) for the purpose of further identification and quantification as described by Nikolova and Velickovic (2004). The scanned TLC images were digitally converted with the software and each spot was individually quantified. The quantification results were represented in units that correspond to colour intensity.

4.1.5 Purification of marker compounds from crude extract.

The crude drug (400 mg/ml) was carefully streaked across the glass TLC plate with a glass pipette (figure 4.3). The tip of a glass pipette was modified for slow movement of the crude sample from the pipette to ensure a narrow streaked area A of crude extract. The prepared standards of harpagide or harpagoside were spotted on the TLC plate (segment B in figure 4.3). The TLC plate was placed in a (BAW) solvent saturated TLC chamber, chromatographed and air-dried. The plates were then covered with a glass sheet except for segment B. This exposed area was sprayed with the TLC colour reagent spray and dried as previously described.

Figure 4.3 Purification of marker compounds from crude resinous extract with a streaking technique.



After chromatography, the resultant harpagoside or harpagide bands from the crude extract on the TLC plates were marked according to the corresponding R_f values of the standards in segment B of the plate. The marked area was removed by scrapping the silica from the TLC plate with a narrow spatula and suspended in 1 ml methanol. After agitation (Infors HT, Switzerland) for 10 minutes, it was then vortexed for 5 minutes. Thereafter, it was centrifuged at 5000 rpm for 20 minutes (Hëttich®, Germany) and the supernatant (methanol phase) was carefully removed from the pellet. This process was repeated for the purpose of sample enrichment.

4.1.6 Removal of solvent from supernatant.

After centrifugation, the supernatant was concentrated by the removal of the methanol. This was done by transferring it to 8 ml Greiner® vials and placed in a digital dry bath (Labcon®, United Kingdom) set at a temperature of 37º C under a gentle stream of nitrogen via a manifold. This evapouration process took about 2 hours resulting in a resinous extracts of harpagoside and harpagide. These purified extracts were stored at -20º C.

4.1.7 UV-spectroscopy for Harpagoside identification.

Confirmative UV-spectroscopy on the purified harpagoside was performed. The UV spectrum of the TLC purified harpagoside was determined by UV-spectroscopy (Cintra® 202, Australia) in phosphate buffered saline (PBS) at pH 7. A wavelength scan range was set between 190– 500 nm, which was within the λ_{max} for harpagoside. The TLC purified sample was dissolved in PBS then filtered with a 0.45 µm filter. Absorbance was measured against phosphate buffered saline (PBS) blank. The sample was diluted by serial dilutions in order to obtain an absorbance range of between 0-1 for increased accuracy.

4.1.8 High Pressure Liquid Chromatography

High pressure liquid chromatography system consisted of a Hewlett Packard® Agilent® 1200 series system. A Phenomenex® (Luna C-18, 20 x 4.5 mm, 5 µm) with a pre-guard filter. Agilent® Chemstation® software package was used for analysis of data.

Two HPLC methods were used. The first system consisted of a flow rate of 1.5 ml/ minute at a wavelength of 210 nm with a gradient mobile phase Methanol: Water (30%- 60% gradient) over 10 minutes. Chlorocresol was used as an internal standard.

The second method consisted of an isocratic mobile phase system. For harpagide detection, a flow rate of 1.5 ml/minute at a wavelength of 210 nm with a 20% methanol/ buffer (50 mM K_2HPO_4 -KH_2PO_4) mobile phase was used. For the harpagoside detection, a flow rate of 1.5ml/minute was used at a wavelength of 278 nm with a 60% methanol/ buffer (50 mM K_2HPO_4 -KH_2PO_4 buffer) mobile phase.

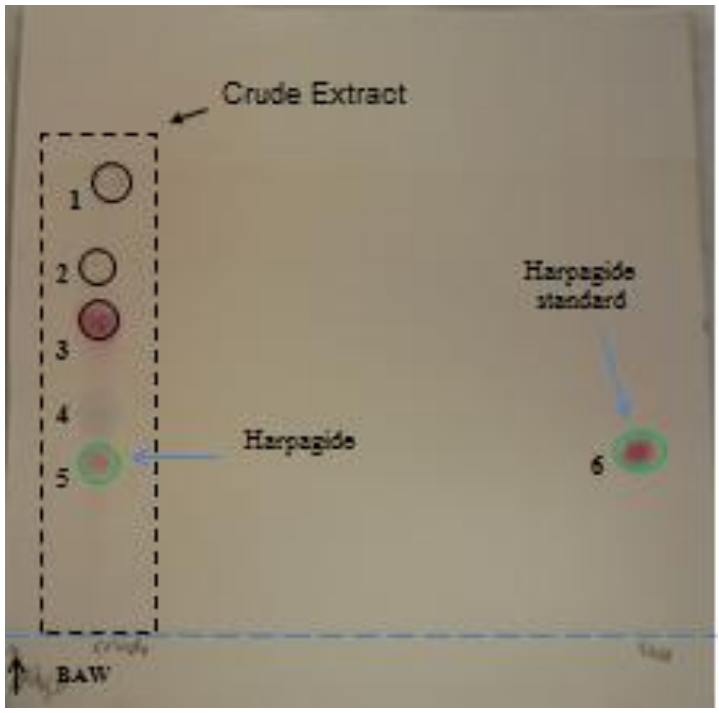
4.2. Results and Discussion

4.2.1 Thin layer chromatography identification of Harpagide and Harpagoside.

4.2.1.1 Harpagide identification

For harpagide identification, two-dimensional TLC was used. The first dimension of the TLC plates developed over a distance of 8 centimeters (figure 4.4). When observed under UV light at a wavelength of 254 nm, 3 identifiable bands were visible (black circles). These were bands 1, 2 and 3 with their respective R_f values listed in table 4.1. As expected, the spotted harpagide standard was not visible under UV light. After the TLC plate was sprayed with the colouring reagent spray, 3 additional spots appeared, numbered 4, 5 and 6.

Figure 4.4 Thin layer chromatography of *Harpagophytum procumbens* extract. Butanol, acetic acid and water (BAW) (12:3:5) solvent system.



This included the harpagide standard (spot 6) revealing an R_f value of 0.37. This value corresponded to the R_f value of band 5 of the spotted crude *Harpagophytum procumbens* extract. In addition, these two spots where not visible under UV light. When sprayed with the colouring agent, they appeared the same violet-red colour. The colour was the same as described by Wichtl (2004) in identifying harpagide with the same spraying solution. Spot number 5 had the same colour as spot 6 but more faint, this may be as a result of its lower concentration compared to the harpagide standard (spot number 6.)

Table 4.1. R_f values of compounds in *Harpagophytum procumbens* extract with butanol, acetic acid and water (BAW) solvent system (1st dimension).

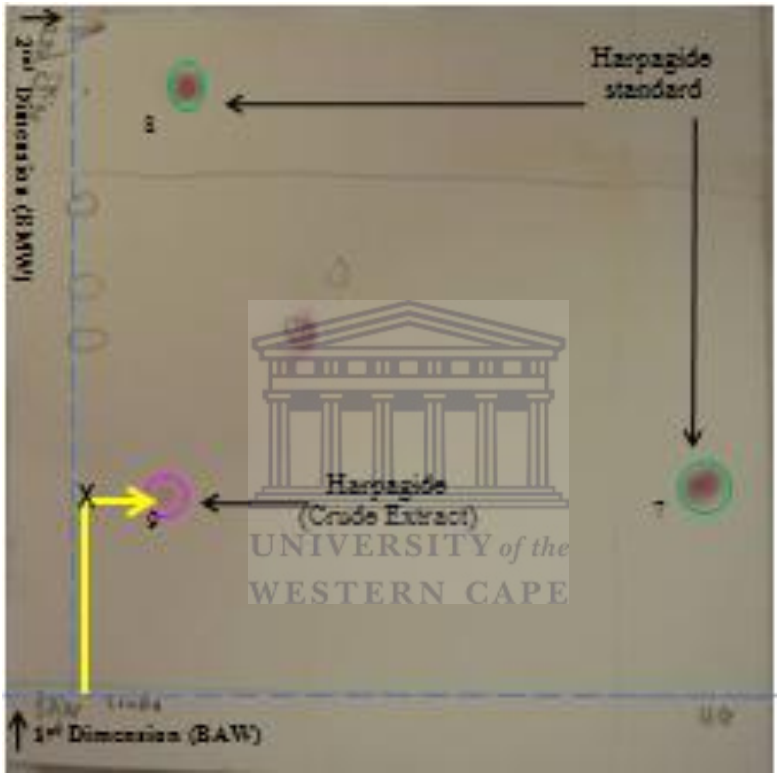
TLC spot number	R_f Value	Spot Colour after spraying
1	0.93	**Colourless**
2	0.82	**Colourless**
3	0.68	**dark red**
4	0.42	**grey/purple**
5	0.37	**dark red**
6	**0.37**	**dark red**

To confirm spot 5 as the harpagide present in the crude extract, a two-dimensional TLC solvent system was implemented.

Figure 4.5 presents the TLC plate chromatographed and developed over the second dimension. The second system consisted of EMW followed by spraying with the colouring reagent spray. In the second dimension, the solvent front was 8 cm. Spot number 5 from the

first dimension (indicated by X) migrated to form spot number 9. The yellow arrow indicates the migration pattern of spot 5 over the two dimensions.

Figure 4.5 Thin layer chromatography of *Harpagophytum procumbens* extract. Ethyl acetate, methanol and water (EMW) (77:15:8) solvent system.



Spot 7 was the harpagide standard from the first dimension. The second harpagide standard spotted in the second dimension migrated to form a dark red spot (spot 8) with R_f value of 0.19 (table 4.2). This corresponded in colour and R_f value to band number 9. Spot 9 corresponded horizontally and vertically to the harpagide standards in the first and second dimension (spot 7 and 8) respectively with respect to R_f values.

Table 4.2. R_f values of compounds in *Harpagophytum procumbens* extract with ethyl acetate, methanol and water solvent system (EMW) (2^{nd} dimension).

TLC spot number	R_f Value	Spot Colour after spraying
7	0.37	**dark red**
8	0.19	**dark red**
9	0.19	**dark red**

This in addition to the colour of spot 9 being the same as the harpagide standards and that none of these spots were visible under UV light 254 nm, confirms that spot 9 is the harpagide component present in the *Harpagophytum procumbens* extract.

4.2.1.2 Harpagoside identification

TLC plates were chromatographed using the mobile phase consisting of BAW and developed over a distance of 8 centimeters (figure 4.6). When observed under UV light at a wavelength of 254 nm, four identifiable spots were observed. These were bands labeled 1, 2, 3 and 6, with their respective R_f listed in table 4.3. Spot 3 with an Rf value of 0.68 corresponded to the R_f value of the pure harpagoside standard (spot 6). Two-dimensional chromatography was not performed with harpagoside.

Figure 4.6 Thin layer chromatography of *Harpagophytum procumbens* extract. Butanol, acetic acid and water (BAW) (12:3:5) solvent system.

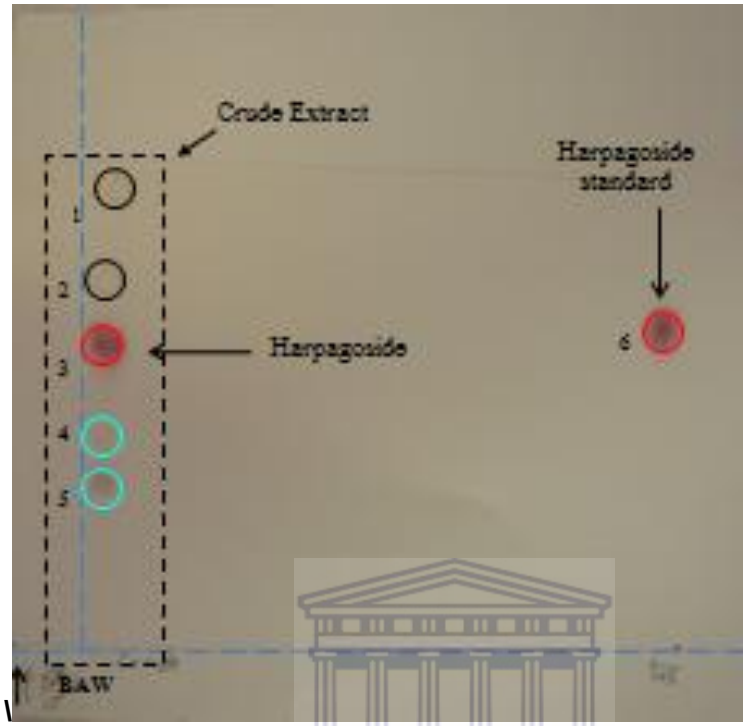


Table 4.3. R_f values of compounds in *Harpagophytum procumbens* extract with butanol, acetic acid and water solvent system (BAW).

TLC spot number	R_f Value	Spot Colour after spraying
1	0.93	**colourless**
2	0.82	**colourless**
3	0.68	**dark red**
4	0.42	**grey/purple**
5	0.34	**dark red**
6	0.68	**dark red**

After the TLC plate was sprayed with the colouring reagent spray, 2 additional spots appeared, numbered 4 and 5 with R_f values of 0.42 and 0.34 respectively.

Spots number 3 and 6 which had the same R_f value, were visible under UV light was also coloured, appearing as violet red bands. The colour, which developed, is the same as described by Wichtl (2004) in identifying harpagoside with the same spraying solution. Spot 5 had the same colour but was less intense probably as a result, its lower concentration. Spot 4 appeared as a faint grey/purple spot (does not appear clearly on the photograph).

To confirm spot five as harpagoside, it was collected and purified from preparative plates and analyzed spectrophotometrically to observe its UV spectrum. The UV spectra of the purified compound (figure 4.7) were identical to that of the harpagoside standard (figure 4.8) with a ratio of peak maxima (280 nm) to minima (233 nm) of 1.87.

Figure 4.7. UV spectrum of TLC purified harpagoside in *Harpagophytum procumbens* extract dissolved in PBS.

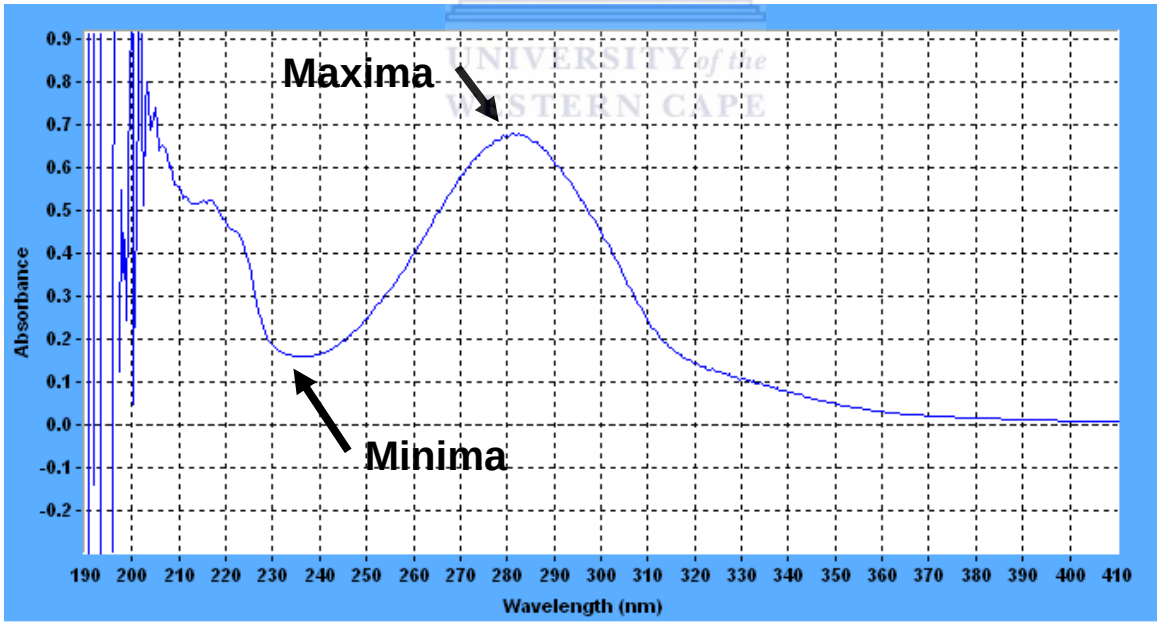
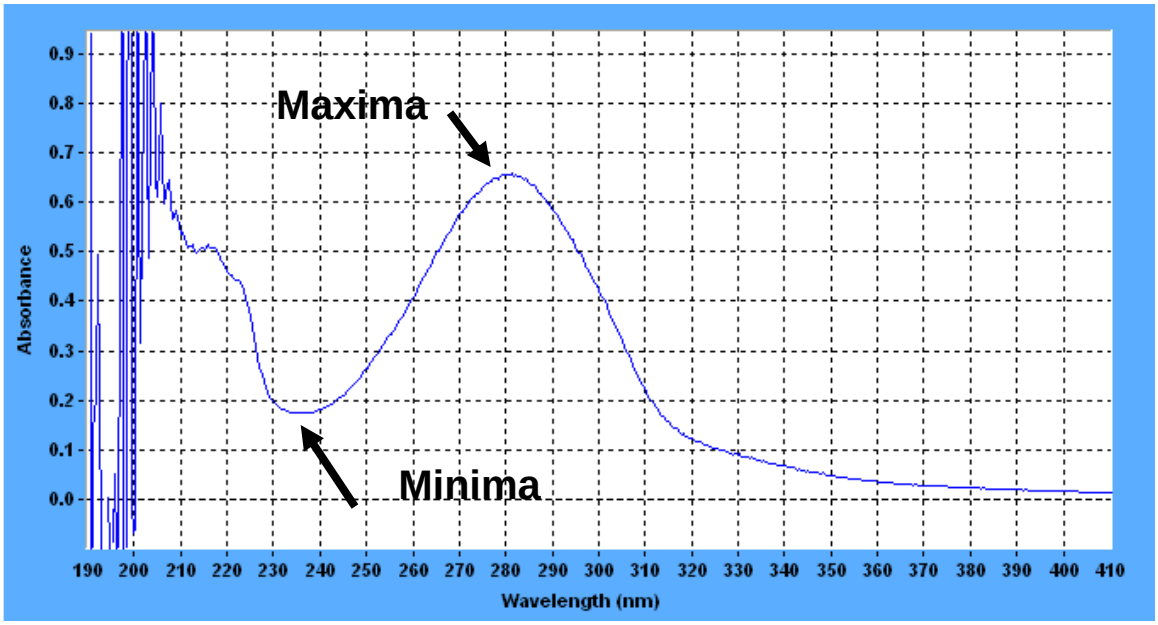


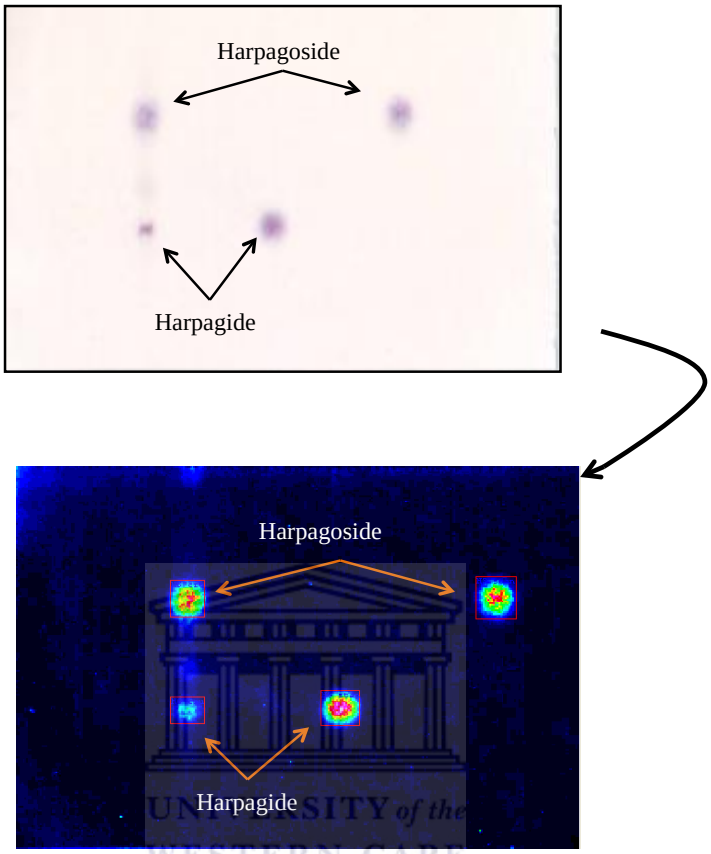
Figure 4.8. UV spectrum of pure harpagoside standard dissolved in PBS.



4.2.2 Quantification of harpagoside and harpagide by densitometry.

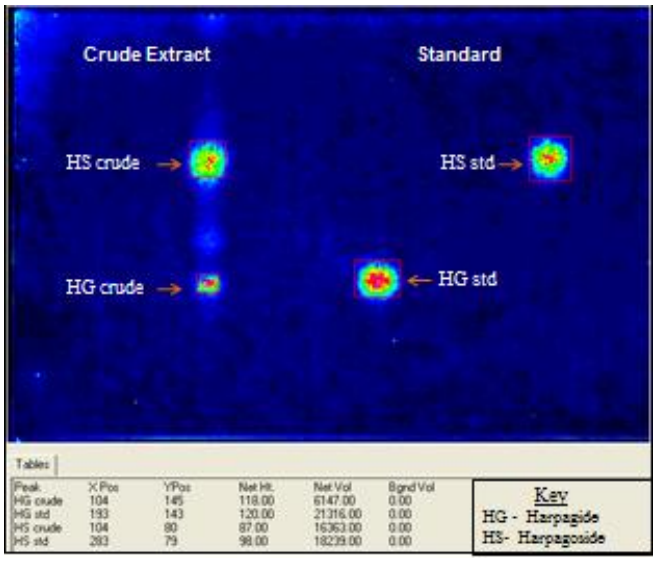
The TLC plates containing the harpagide and harpagoside from *Harpagophytum procumbens* extract were quantitatively densitometrically analyzed for their harpagoside and harpagide contents using the Biosoft® Quantiscan software. After spraying the TLC plate, it was scanned and digitally manipulated with the software (figure 4.9).

Figure 4.9. Sprayed TLC plate digitally converted with Biosoft® Quantiscan software.



The densitometry software allows the spots on the TLC plates to be analyzed on X – position and Y- positions. From figure 4.10 it can be observed that harpagoside in the crude extract appears on the same Y- position (80) as that of the harpagoside standard (79). Similarly, the developed spots of harpagide in the crude extract and the harpagide standard also appear along the same Y- position with values of 145 and 143 units respectively.

Figure 4.10. Digital densitometry of TLC spots of harpagoside and harpagide.



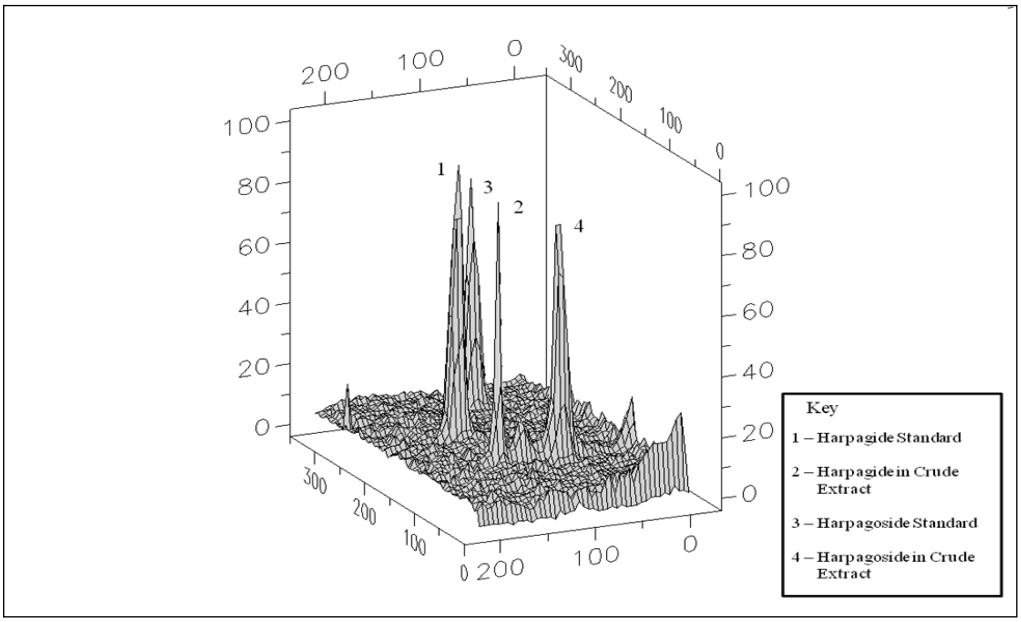
The densitometric analysis revealed a total volume count for harpagoside and harpagide from *Harpagophytum procumbens* extract of 16363 units and 6147 units respectively. In addition, a known quantity (0.07 mg) of harpagoside and harpagide standards where spotted on the same TLC plate resulting in net volumes of 18239 units and 21316 units respectively. The quantities were extrapolated with a standard curve using the values of TLC spotted harpagoside and harpagide standards (table 4.4). The harpagoside and harpagide in the extract represented quantities of 0.06mg and 0.02 mg respectively. The sample standards spotted on the same plate produced a quantity of 0.07 mg for harpagoside and 0.07 mg for harpagide from the standard curve.

Table 4.4. Harpagoside and harpagide standards spotted on TLC plates with corresponding densitometric volumes.

Quantity of pure standard (mg)	Harpagoside volume (units)	Harpagide volume (units)
0.08	21411	24418
0.06	16670	18831
0.04	10888	12552
0.02	5222	6214

Figure 4.11 is a 3 dimensional graphical quantitative and positional representation generated by the Biosoft® software of the developed spots on the TLC plate where the volume of the peaks is proportional to its concentration. It can also be seen that the two harpagide peaks (1 and 2) appear on the same axis and the two harpagoside peaks appear on the same axis (3 and 4).

Figure 4.11. Three dimensional quantitative and positional representation TLC harpagide and harpagoside spots.



The quantitative analyses of the compounds screened on the developed TLC plates indicated that the tested *Harpagophytum procumbens* extract contained 3% harpagoside and 1% harpagide. This result is similar to that of Bruneton (1999) who reported the quantity of iridiode glycosides, which include harpagoside and harpagide, present in the *Harpagophytum procumbens* crude extract to be between 0.5% and 3%. Wichtl (2004) reported the quantity of harpagoside present in the crude extract to be 1.5% and varies with the quality of the crude drug.

The quantity of components yielded may also vary depending on the extract technique implemented. Different extraction techniques may result in diverse fractions of the crude being yielded (Loew *et al.,* 2001). These extraction techniques may vary in the solvent systems being used, temperature as well as crude drug particle size (Gunther *et al.,* 2006).

Gunther *et al.,* (2006) demonstrated through experimental manipulation of the mentioned variables that harpagoside yields of between 20 and 30% were obtainable from the crude *Harpagophytum procumbens* extract. However, the study also revealed that when conventional extraction procedures were implemented, the harpagoside content in the crude extract was as low as 2.5%. The conventional techniques involve particle size reduction, and agitation with a solvent similar to the technique used in our study.

4.2.3 High pressure liquid chromatography (HPLC)

The chromatogram consisting of harpagide, harpagoside and chlorocresol standards are presented in figure 4.12.

Figure 4.12 Chromatogram of *Harpagophytum procumbens* extract. Mobile phase: 30-60% methanol gradient at wavelength 210 *nm.*

The chromatograms of the crude *Harpagophytum procumbens* extract (figure 4.12) and the standards (figure 4.13) were superimposed in order to identify the harpagoside and harpagide in the crude extract (figure 4.14). The retention times of the standards, which corresponded to the peaks on the chromatogram of the crude extract, were identified. This chromatogram indicates retention times of 2.2 minutes for harpagide, 8.7 minutes for harpagoside and 9.3 minutes for the chlorocresol used as internal standards. This method was also utilized for partition co-efficient experiments with the harpagoside and harpagide marker compounds with the various permeation enhancers and gel formulations.

Figure 4.13 Chromatogram of pure harpagoside and harpagide drug standards. Mobile phase: 30-60% methanol gradient at wavelength 210 *nm*.

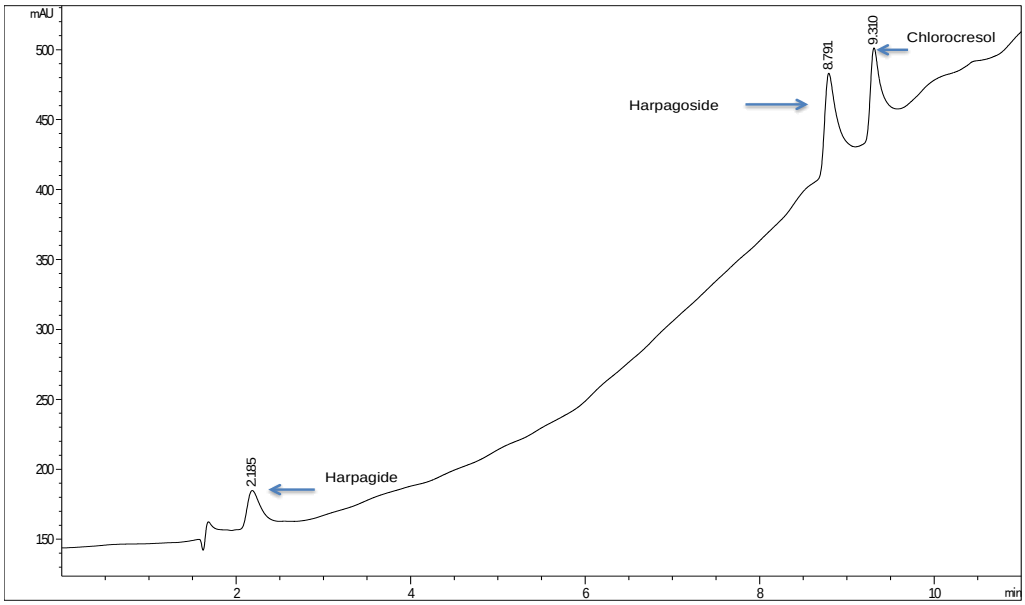
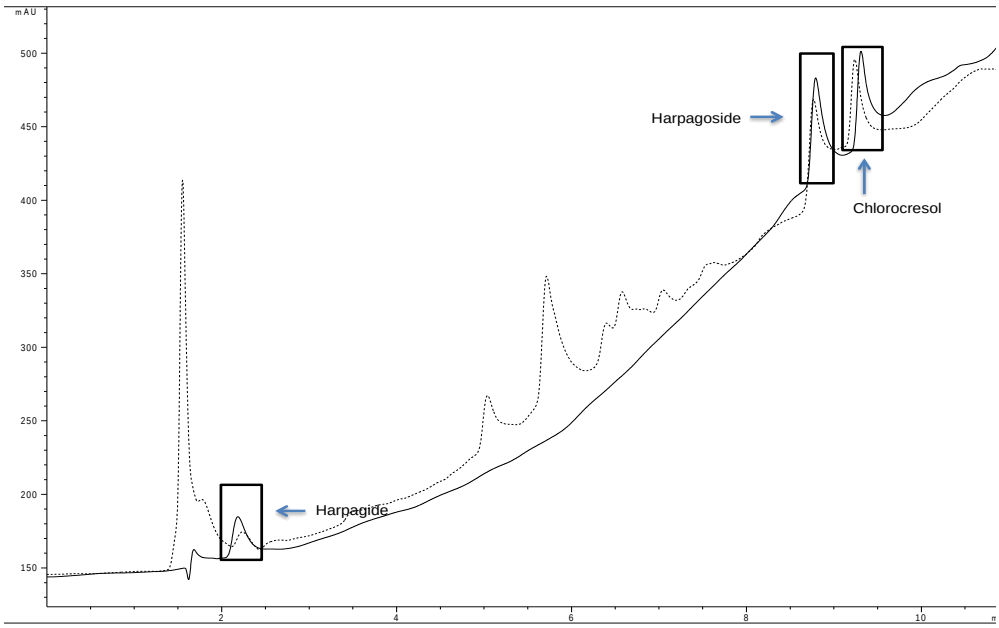


Figure 4.14 Superimposed chromatogram of *Harpagophytum procumbens* extract, pure harpagoside and harpagide standards. Mobile phase: 30-60% methanol gradient at wavelength 210 *nm*.



Due to sensitivity and interfering substances, the HPLC method used was modified. An isocratic HPLC method was used at two different wavelengths. For the harpagide, a 20% methanol/buffer (50 mM K_2HPO_4 + KH_2PO_4) was used at a wavelength at 210 *nm*. For harpagoside, a 60% methanol/buffer (50 mM K_2HPO_4 + KH_2PO_4) mobile phase was used at a wavelength of 278 *nm*. These wavelengths represent the maximum UV absorbance for harpagide and harpagoside respectively.

In these systems, harpagide standard had a retention time of 2.1 minutes (figure 4.15) and 4.5 minutes for harpagoside (figure 4.16).

Figure 4.15 Chromatogram of pure harpagide standard. Mobile phase: 20% methanol/buffer (50mM K_2HPO_4 + KH_2PO_4) at wavelength 210 *nm*.

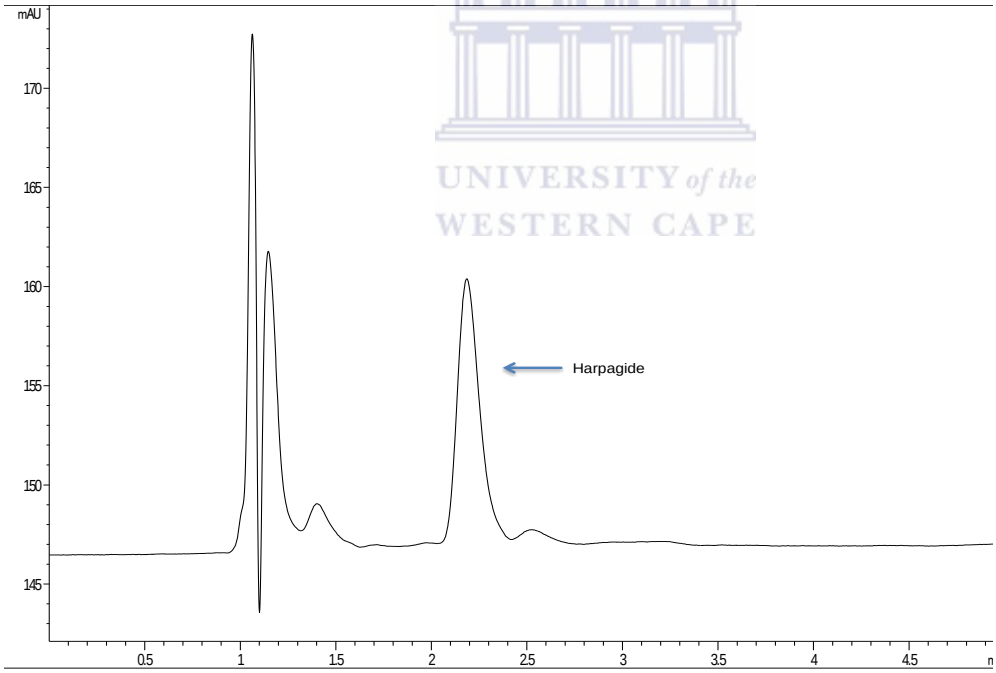
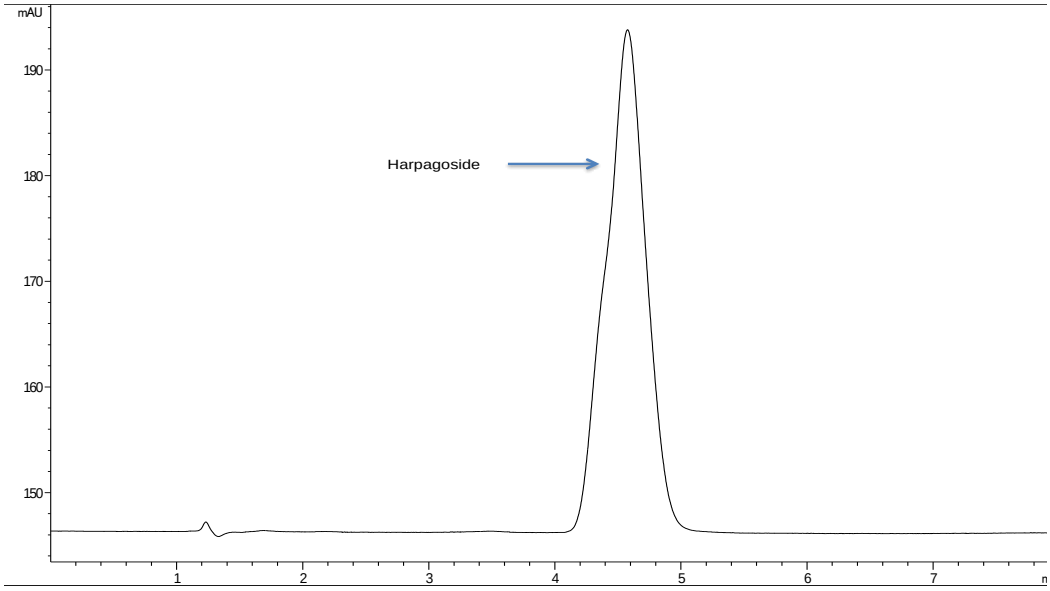


Figure 4.16 Chromatogram of pure harpagoside standard. Mobile phase: 60% methanol/buffer (50 mM K_2HPO_4 + KH_2PO_4) at wavelength 278 *nm*.



With the isocratic mobile phase method, the harpagide peak was identified in the crude sample by superimposing the chromatogram of *Harpagophytum procumbens* with this sample containing spiked quantities of harpagide standard (figure 4.17), This method of identification was used for the identification for harpagoside as well (figure 4.18).

Figure 4.17 Superimposed chromatograms of *Harpagophytum procumbens* extract alone, and spiked with pure harpagide standard. Mobile phase: 20% methanol/buffer (50 mM K_2HPO_4 + KH_2PO_4) at wavelength 210 *nm*.

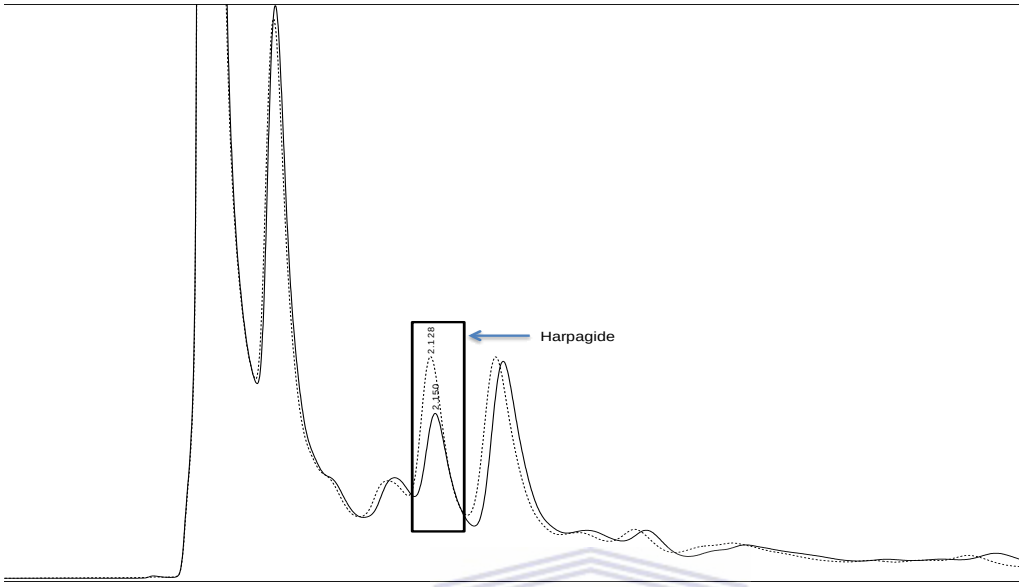
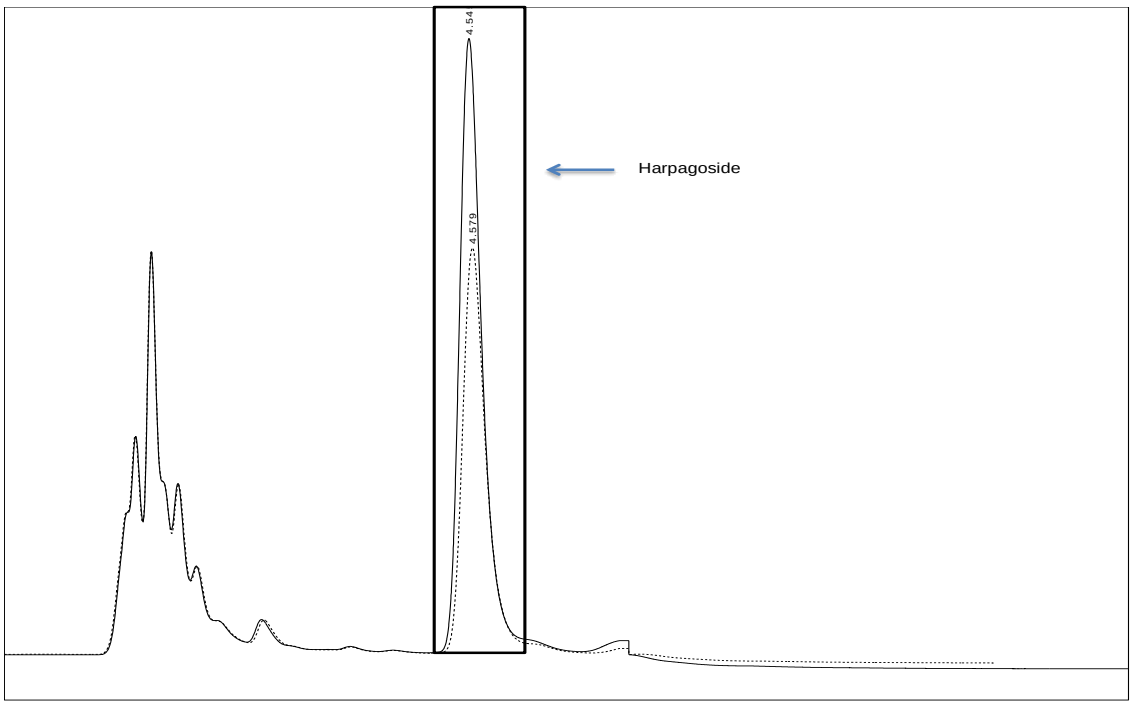


Figure 4.18 Superimposed chromatograms of *Harpagophytum procumbens* extract alone, and spiked with pure harpagoside standard. Mobile phase 60% methanol/buffer (50 mM K_2HPO_4 + KH_2PO_4) at wavelength 278 *nm*.



Confirmative identification of harpagide and harpagoside was performed by comparing the UV spectra of the identified harpagide and harpagoside peaks in the crude *Harpagophytum procumbens* sample with that of pure standards, with the harpagide not displaying a UV spectra (figure 4.19) and harpagoside with a peak maxima to minima at wavelength of 280 nm and 233 nm, with a ratio of 1,187 (figure 4.20). The UV spectra of the identified harpagide and harpagoside peaks in the crude sample corresponded to the respective spectra of the harpagide and harpagoside standards.

Figure 4.19. UV spectrum of the harpagide peak from crude *Harpagophytum procumbens* extract.

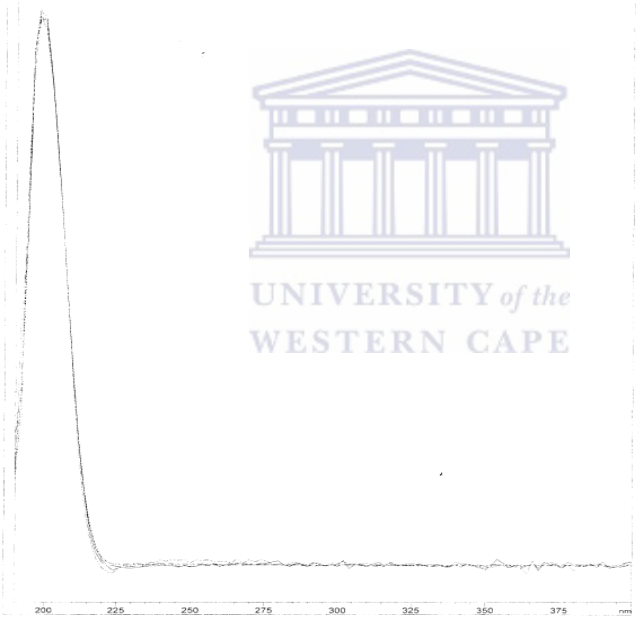
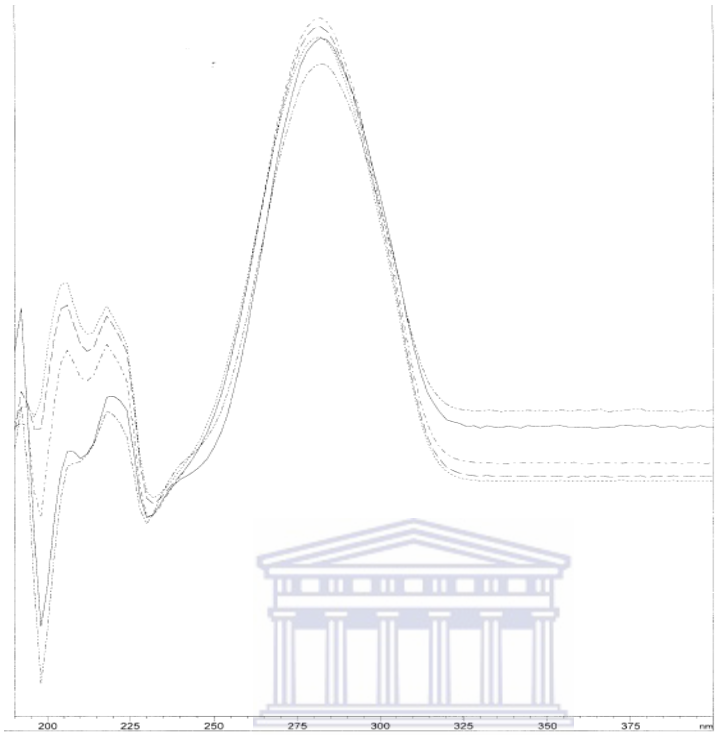


Figure 4.20 UV spectrum of the harpagoside peak from crude *Harpagophytum procumbens* extract.



4.3 Conclusion

Harpagide and harpagoside marker compounds were identified in the crude *Harpagophytum procumbens* extract. Two-dimensional thin layer chromatography of the identified harpagide in the crude extract yielded an identical R_f = 0.19 value and dark red colour when compared to the harpagide standard. One-dimensional chromatography of the harpagoside compound yielded an R_f value of 0.68 with a dark red colour, which was identical to the harpagoside standard. As with the harpagide standard, the harpagide identified in the crude extract also did not display a UV spectrum. The UV spectra of the identified harpagoside on the TLC plate in the crude extract and harpagoside standard were identical with a peak maxima to minima ratio of 1.87. The HPLC analysis of the crude extract identified harpagide and harpagoside by having identical retention times and UV spectra to the respective standards. Harpagide and harpagoside were densitometrically quantified in the crude *Harpagophytum procumbens* extract to be 1% and 3% respectively.

CHAPTER FIVE: Pre- Transdermal Cyclooxygenase-2 enzyme inhibition

Content of this section has been reproduced from the Article titled "*Direct inhibition of Cyclooxygenase-2 enzyme by an extract of Harpagophytum procumbens, harpagoside and harpagide*" by *N Ebrahim and R Uebel, African Journal of Pharmacy and Pharmacology Vol. 5(20), 2209-2212, 29 November, 2011* (See annexure I for full-length article).

The *Harpagophytum procumbens* extract and the two marker compounds (harpagide and harpagoside) were tested for their pharmacological ability to directly inhibit the Cyclooxygenase-2 enzyme, which would be an indication of their anti-inflammatory properties. Further, the COX-2 inhibition experiments were also used to determine whether the crude extract retained its COX-2 inhibition activity after permeation across human skin membrane.

5.1 Methods

5.1.1 Pre-Permeation COX-2 enzyme inhibition activity.

COX-2 activity was determined spectrophotometrically (Cintra® 202, Australia) by measuring UV absorption change of the reaction mixture at a wavelength of 610 *nm* exactly 8 minutes after initiating the reaction against the appropriate blank. COX-2 inhibition experiments were based on the methods reported by Copeland *et al., (1994)* and Corazzi *et al.,* (2005) with minor changes.

5.1.1.1 Purified COX-2 enzyme

Purified COX-2 (ovine) enzyme (Cayman Chemical, USA) was stored at -80°C and maintained on dry ice during experiments. Twenty-five units (4µl) of the enzyme was added to the enzyme reaction buffer solution.

5.1.1.2 Enzyme reaction Buffer

The buffer consisted of 100 mM sodium phosphate pH 6.5, 0.5 µM hematin (Sigma-Aldrich, South Africa), and 1mg/ml gelatin (Sigma-Aldrich, South Africa).

5.1.1.3 Enzymatic reaction

The enzymatic reaction was initiated by adding 100 µM of arachadonic acid (Sigma-Aldrich, South Africa) and 50 µM N,N,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) (Sigma-Aldrich, South Africa) to a final reaction mixture of 1000 µl buffer solution in laboratory temperatures not exceeding 20º C. Arachadonic acid and TMPD were freshly prepared when experiments were performed. For COX-2 inhibitory activity determination, *Harpagophytum procumbens* extract, harpagoside and harpagide were dissolved in dimethsulphoxide (DMSO) (Merck, South Africa) and added to the reaction solution buffer 15 minutes before arachadonic acid and TMPD were added. DuP-697 was dissolved in dimethylformamide (Merck, South Africa) instead of DMSO due to its relatively poor solubility in DMSO. The enzyme activity was measured by determining the rate of TMPD oxidation as indicated by the increase in the absorbance at 610 *nm*.

5.1.1.4 Spectroscopy

Fixed wavelength, confirmative UV-spectroscopy (Cintra 202, Australia) was performed with the *Harpagophytum procumbens* extract harpagoside and harpagide at different

concentrations in phosphate buffered saline (PBS). Fixed wavelength (610 *nm)* UV spectroscopy was performed with various reaction mixtures. Absorbance was measured against phosphate buffered saline (PBS) blank. The fixed wavelength absorbance reading was recorded at 30 second intervals for 10 minutes. No change of initial absorption values indicated 100% inhibition and maximum absorption change in the reaction without added inhibitors indicated zero inhibition. Data was fitted into GraphPad® Prism version 5 software.

The following reaction mixtures were prepared:

- **Standard COX-2 reaction mixture** - Arachadonic acid (AA) 100 µM + N,N,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM +COX-2 enzyme 4 µl (25 units) + buffer solution up to 1000 µl.
- **Sample reaction mixture** -Arachadonic acid (AA) 100 µM + N,N,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM + COX-2 enzyme 4 µl (25 units) + ***Harpagophytum procumbens*** **crude extract or harpagoside or harpagide 50** µl + buffer solution up to 1000 µl.
- **Reference inhibition reaction mixture** - Arachadonic acid (AA) 100 µM + N,N-,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM + COX-2 enzyme 4 µl (25 units) + **DuP-697 50 µl** + buffer solution up to 1000 µl.

5.2 Results and Discussion

5.2.1 COX-2 enzyme inhibition

Figures 5.1 to 5.4 represent the illustrative absorbance versus time graphs for the COX-2 inhibitory activity of various compounds including the crude *Harpagophytum procumbens* extract, harpagoside, harpagide and reference compound, DuP-697. The absorbance is associated with the oxidation of TMPD due to the activity of the COX-2 enzyme. Therefore, increased absorbance readings are associated with higher enzymatic activity and lower absorbance reading would indicate decreased COX-2 enzymatic activity (greater inhibition). The graphs were extrapolated and fitted to time zero using GraphPad® Prism 5 software. The standard reaction contains all the components of the reaction mixture excluding inhibitors. COX-2 absorbance at time 8 minutes was used in the measurement of inhibitory activity as this was determined to be the point of maximum absorbance from the data. The absorbance versus time graph indicates that inhibitory activity for most of the reactions were similar for the first 3 minutes of the reaction, the exceptions being the potent DuP-697 inhibitor and *Harpagophytum procumbens* extract at 2mg concentration.

Figure 5.1 UV absorbance vs. time of *Harpagophytum procumbens* extract at various dilutions.

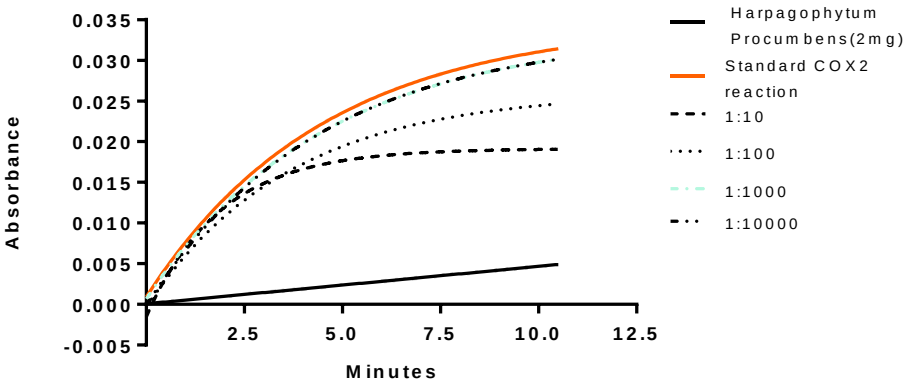


Figure 5.2 UV absorbance vs. time of *Harpagophytum procumbens* and harpagide at various dilutions.

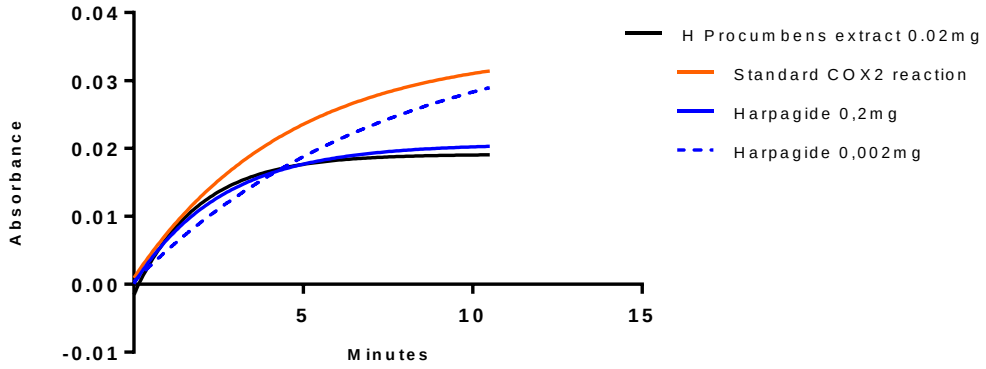


Figure 5.3 UV absorbance vs. time of *Harpagophytum procumbens* and harpagoside at various dilutions.

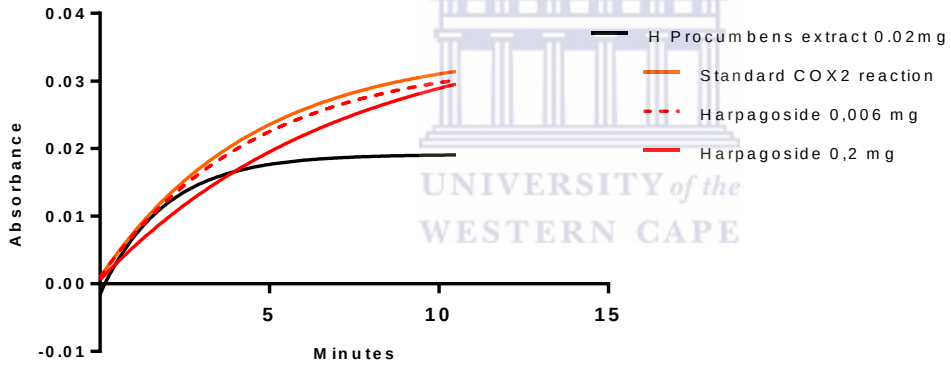
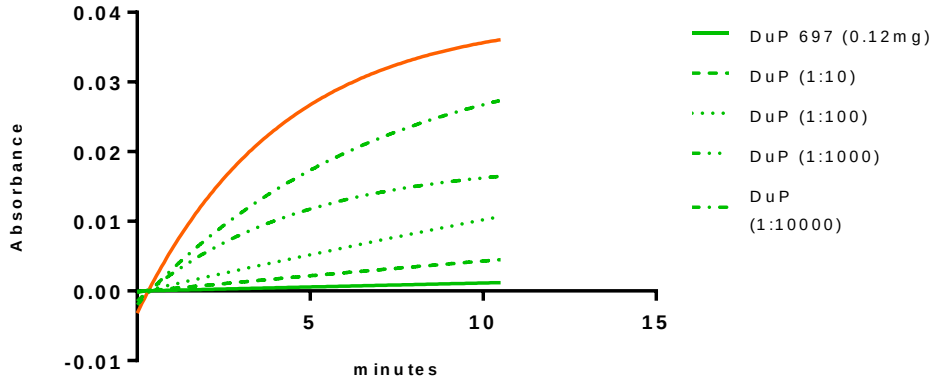


Figure 5.4 UV absorbance vs. time of DuP-697 at various dilutions.



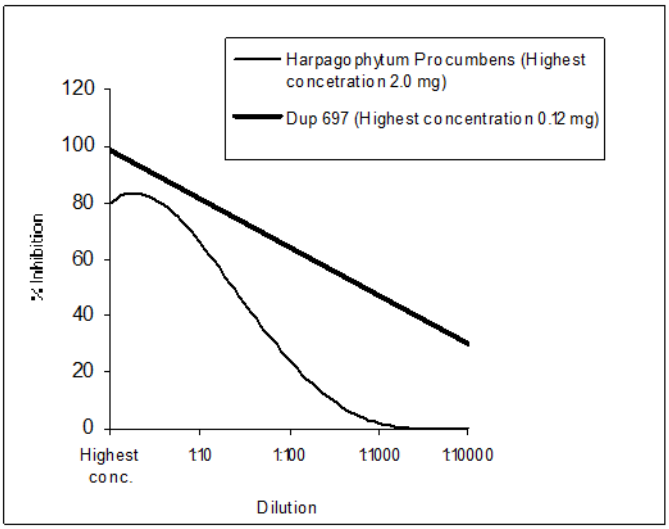
The COX-2 inhibitory activity of *Harpagophytum procumbens* and DuP-697 were tested at various dilutions with the highest concentration being 2.0 mg and 0.12 mg respectively. At these maximum concentrations, 80% inhibition was achieved for the *Harpagophytum procumbens* and 96% for DuP-697 as can be seen from table 5.1. At the maximum dilution tested, 1:10 000 the *Harpagophytum procumbens* did not display any inhibitory activity while the DuP-697 displayed 18% inhibitory effect.

Table 5.1 COX-2 % inhibition of *Harpagophytum procumbens* and DuP-697 at varying dilutions.(Highest concentration-*Harpagophytum procumbens* 2.0mg and DuP-697 0.12mg) (Mean±SD, n=3)

	Highest concentration	**(1:10)**	**(1:100)**	**(1:1000)**	**(1:10 000)**
Harpagophytum procumbens	80% (2.1)	68%(2.1)	24% (1.0)	2 % (0.3)	0%(0)
Dup -697	98% (0.6)	85% (0.5)	66% (0.5)	58.5 (0.6)	18% (0.5)

Figure 5.5 compares the COX-2 inhibition of *Harpagophytum procumbens* and DuP-697 extract at 10 fold dilutions, starting at 2mg (80% inhibition) and 0.12 mg (98% inhibition) respectively. The DuP-697 starting concentrations was 17 times lower than that of the extract but exerted a greater inhibitory effect at lower concentrations (1:1000 dilution) than *Harpagophytum procumbens* extract. The difference in the inhibition decreases to less than 20% at higher extract concentrations (1:10 dilution).

Figure 5.5 COX-2 inhibition curves (pre-permeation)of DuP-697 and *Harpagophytum procumbens* extract at various dilutions.



When comparing the *Harpagophytum procumbens* extract to the DuP-697 inhibitor, the resultant inhibition curve indicated a non-linear COX-2 inhibition at low and high concentrations. This could suggest the presence of possible synergistic or polyvalent activity of compounds in the extract.

Figure 5.6 Direct COX-2 enzyme inhibition (pre-permeation) by *Harpagophytum procumbens* extract, harpagoside and harpagide. ***(n=3; mean ±S.D.)***

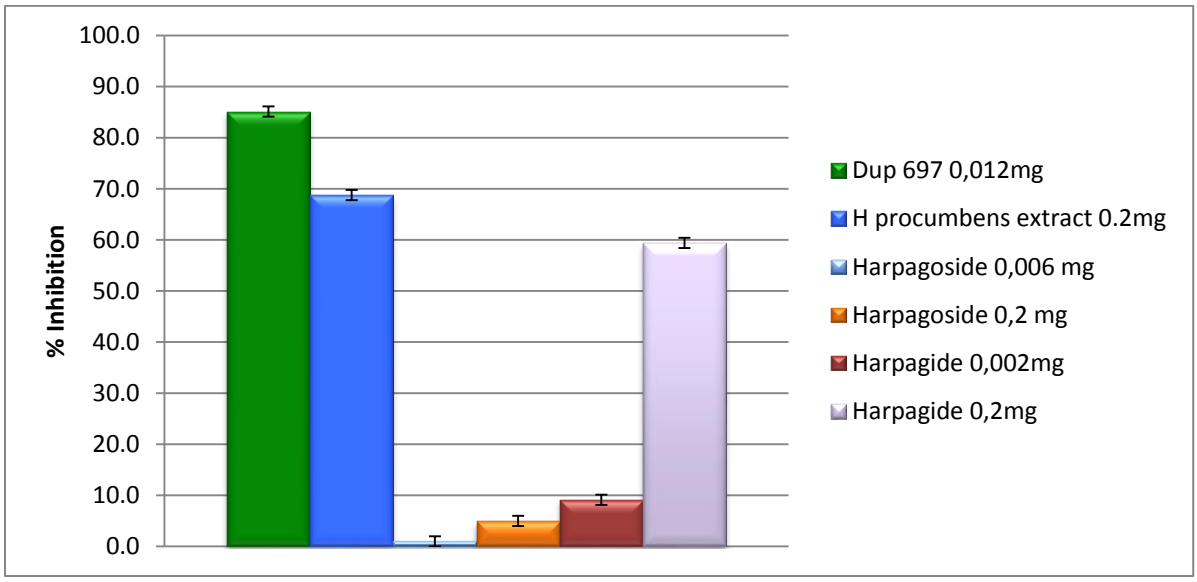


Figure 5.6 illustrates direct COX-2 inhibition by *Harpagophytum procumbens* extract as well as harpagoside and harpagide. At a concentration of 0.2 mg, *Harpagophytum procumbens* extract demonstrated direct enzyme inhibition of 68.3% (the highest concentration at the linear part of the crude extract's inhibition), harpagoside 5% and harpagide 60%. When enzyme inhibition of harpagoside and harpagide were tested at 0.006 mg and 0.002mg respectively, this is the quantity that these compounds are represented in 0.2 mg of the crude *Harpagophytum procumbens* extract, only 1% and 9% direct inhibition were obtained respectively. The One Way ANOVA test indicated the data was statistically significant. The Tukey's multiple comparison indicated that the difference in the % COX-2 inhibition for all samples tested in Figure 4.6 was statistically significant *($P \leq 0.05$)* when compared to the reference compound DuP-697 (1:10 dilution. 0.012mg).

5.2.2 IC_{50} values

The calculated IC_{50} values derived from inhibition studies are listed in table 5.2. The reference compound DuP-697 resulted in the lowest IC_{50} value (0.000134 mg/ml). Harpagide achieved higher COX-2 inhibition activity (IC_{50} of 0.1186 mg/ml) than harpagoside as direct inhibitor but less than *Harpagophytum procumbens* extract and DuP-697.

Table 5.2. IC_{50} values of DuP-697, *Harpagophytum procumbens* extract, harpagide and harpagoside as COX-2 inhibitors.

Compound	**IC_{50} mg/ml**
DuP-697	0.000134
Harpagophytum procumbens extract	0.1046
Harpagide	0.1186
Harpagoside	104.1

The COX-2 inhibition experiments demonstrate direct inhibition (direct inhibition of the enzyme activity by the inhibitor) of the cyclooxygenase-2 enzyme with a crude extract of *Harpagophytum procumbens*, harpagide and to a lesser extent harpagoside.

Previous studies report mainly on the inhibitory activity of *Harpagophytum procumbens* and its active compounds through indirect mechanisms of activity. This includes mechanisms of reduced enzyme expression or measuring enzymatic output through various biochemical pathways and not direct inhibition as in this study. Examples of indirect inhibition are studies measuring COX-2 inhibition through TPA-induced COX-2 expression where the crude *Harpagophytum procumbens* extract inhibited the *in vivo* activation of transcription factors such as activator protein-1 and cyclic AMP (Kundu *et al.,* 2005) in mice skin. Another example of measuring indirect COX-2 inhibition is shown in the study Jang *et al.,* (2003) that measured inhibition by observing lipopolysaccharide-stimulated cyclooxygenase expression by measuring prostaglandin synthesis in mouse fibroblast cells.

When comparing the results of this study with other in vitro or in vivo studies which focus on indirect inhibition were, however, able to compare the extent to which inhibition of the COX-2 by *Harpagophytum procumbens* and its active components has occurred even though the mechanisms of achieving this inhibition is different.

The extent of inhibition activity of the crude extract in this study is similar to the results obtained by Jang *et al.,* (2003) and Kundu *et al.,* (2005). Jang *et al.,* (2003) reported COX-2 inhibition of up to 70% percent through inhibition of expression of prostaglandin-2 in the presence of *Harpagophytum procumbens*. The Kundu *et al.,* (2005) study revealed a 46% percent COX-2 inhibitory effect by the crude extract *Harpagophytum procumbens* when compared to their standard reaction not containing the crude extract. Benito *et al.,* (2000) also reported that harpagoside was able to produce 30% inhibition of the release of the prostaglandin-2 which is a product of the cyclooxygenase enzymes, the same study indicated that harpagide was unable to produce prostaglandin-2 inhibition.

Abdelouahab and Heard (2008) reported that *Harpagophytum procumbens* and harpagoside produced COX-2 expression of about 50% in the porcine ear epidermis. Further, their study also indicated that instead of producing inhibitory COX-2 inhibitory effects as found in our study, harpagide increased COX-2 expression, which the authors suggested contributed to an inflammatory response.

The Abdelouahab and Heard (2008) study indicated that with the exception of harpagide that the other individual compounds from the extract i.e. harpagoside, 8-coumaroylhapagide and verbascoside individually produced greater inhibition than the crude extract of *Harpagophytum procumbens*. Our results as seen from figure 4.6 indicate that the crude extract produced greater inhibition than the individual components when comparing them at the same concentrations.

When enzyme inhibition of harpagoside and harpagide were tested at 0.006 mg and 0.002mg respectively which is the quantity that these compounds are represented in 0.2 mg of the extract, only 1% and 9% direct inhibition were demonstrated respectively. Similarly, Fiebich *et al.,* (2001) reported no COX-2 inhibitory activity by harpagoside and harpagide in preventing the release of TNF-α synthase (a cytokine which is present during inflammatory conditions) at concentrations of up to 0.01mg.

When the inhibitory effects of *Harpagophytum procumbens* extract, harpagoside and harpagide were observed clinically and in animal studies, differences also occurred with respect to whether the crude extract or individual components conferred greater inhibitory activity.

Clinical studies revealed favourable results for the relief of various inflammatory conditions, which include osteoarthritis and back pain with *Harpagophytum procumbens* and harpagoside (Gagnier *et al.,* 2004; Chrubasik *et al.,* 2002; *Chantre et al.,* 2000) when compared to other compounds such as diacerein and refocoxib.

Animal studies involving COX-2 inhibition by *Harpagophytum procumbens* extract indicated significant reduction in pain and inflammation induced by experimental test procedures (Anderson *et al.,*, 2004; Lanhers *et al.,*, 1992). These animal studies tend to indicate that compounds such as harpagoside do demonstrate anti-inflammatory properties (McGregor *et al.,*, 2005; Lanhers *et al.,*, 1992).

The greater COX-2 direct inhibition exerted by the *Harpagophytum procumbens* extract compared to the combined inhibition of the harpagide and harpagoside may be as a result of other compounds in the extract. Gyurkovska *et al.,* (2011) tested the inhibition (indirect) of COX-2 expression by crude extracts and components of *Harpagophytum procumbens*. It yielded strong inhibitory effects and suggested that the high activity is as a result of synergistic activity between the various components of the crude extract. It should be considered that crude extracts including *Harpagophytum procumbens* may contain a range of active compounds which all contribute to their efficacy which may not necessarily indicate synergy rather polyvalency (Williamson, 2001).

Various studies reported differences in the activity of the crude extract with its constituents such as harpagoside and harpagide. However, what is generally observed is that the activity of the crude extract displayed better COX-2 inhibitory activity when compared to individual components.

5.3 Conclusion

Harpagophytum procumbens extract produced direct inhibition of COX-2 enzyme, but less than the reference inhibitor DuP-697. COX-2 inhibition by *Harpagophytum procumbens* extract was greater than that of harpagoside and harpagide at the same concentrations. Harpagide had up to 12 times more inhibition activity as direct COX-2 enzyme inhibitor than harpagoside. The calculated combined inhibition of harpagoside and harpagide at the concentrations as they appear in the extract, is 10% compared to 68% by the *Harpagophytum procumbens* extract. However, the combination of these two components, with other compounds, which form part of the extract, has an even greater direct inhibition of COX-2 enzymes. This difference in COX-2 inhibition may be attributed to interplay expressed by other components in the extract. The IC_{50} values indicate that the *Harpagophytum procumbens* crude extract had the lowest IC_{50} concentration when compared to the harpagide and harpagoside.

CHAPTER SIX: Physiochemical properties

The physicochemical properties of a drug substance are important determinants forthier permeation ability across the skin due to the barrier properties of the stratum corneum. These properties must to be understood when considering the mechanisms of permeation across the stratum corneum. The physiochemical properties of harpagide and harpagoside were determined and how these properties were affected by the presence of permeation enhancers and gel formulations. This provided an indication of permeation enhancer and gel formulations to use for permeation studies.

6.1 Methods

6.1.1 Physiochemical properties of harpagoside and harpagide

6.1.1.1 Determination of solubility of purified harpagoside and harpagide

The aqueous solubility of harpagide and harpagoside in the crude extract *Harpagophytum procumbens* was determined by preparing solutions with increasing concentrations in phosphate buffer solution (PBS) at pH 7. The mixtures were stirred with a magnetic stirrer in a water-jacketed container at a 32°C for 24 hours. After 24 hours, solutions were filtered and analyzed by TLC for solubility determination. Maximum solubility was determined at the concentration where further addition of crude extract did not result in the increase in intensity of TLC spots with densitometric analysis.

6.1.1.2 Experimental partition coefficient

Partition coefficient experiments were performed with the marker compounds (harpagoside and harpagide) on its own; in the presence of permeation enhancers and gel formulation combinations.

The following method was used for determining the partition co-efficient. Test samples were added to pre-saturated PBS buffer, followed by mixing with pre-saturated *n*-octanol. This mixture was stoppered and agitated for 75 min followed by centrifuging at 4000 rpm for 45 min resulting in the formation of two layers. Samples from the PBS layer were withdrawn from the mixture and analyzed by HPLC. It was filtered (0.45 µm) prior to being analyzed. The *n*-octanol-PBS partition coefficients (log D) were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase to the concentrations in the PBS. The experiments were performed in triplicate.

6.1.1.2.1 Preparation of pre-saturated *n*-octanol and PBS buffer

Saturated *n*-octanol and PBS (pH 7) solutions were prepared by mixing equal volumes of these solvents together and shaking (Infors HT, Switzerland) for 24 hours. This was followed by separating the two-formed saturated layers from each other with a separating flask.

6.1.1.2.2 *n*-octanol and PBS partitioning of the crude extract

Partition co-efficient values for *Harpagophytum procumbens* crude extract were determined using the method described in 6.1.1.2.

6.1.1.3 Gel Formulation

The following gels were formulated:

6.1.1.3.1 Pluronic® F-127 gel

Distilled water was cooled to a temperature of 4°C in a refrigerator prior to adding to 2.5 g Pluronic® powder (BASF, Germany). The Pluronic® was sprinkled into distilled water (10 ml) with constant agitation on a magnetic stirrer until it completely dissolved in a beaker. The

beaker was sealed with Parafilm® and refrigerated for 24 hours to aid dissolution and removal of air bubbles.

6.1.1.3.2 Carbopol Ultrez 21®

Three hundred milligrams of Carbopol Ultrez 21® (Lubrizol, USA) was sprinkled onto the surface of 10 ml distilled water in a beaker using a sieve to create an even fine sprinkle. This was gently stirred with a magnetic stirrer until it was completely dissolved. The beaker was sealed with Parafilm® and refrigerated for 24 hours to aid dissolution and removal of air bubbles.

6.1.1.3.3 Hydroxypropyl cellulose

Dispersed 250 milligram of hydroxypropyl cellulose in 3 ml ethanol on a magnetic stirrer at low speed in a beaker. Propylene glycol (2.8 ml) was added to the beaker followed by 3 ml distilled water. Stirring continued at low speed until gelling occurred. The beaker was sealed with Parafilm® and refrigerated for 24 hours to aid dissolution and removal of air bubbles.

6.1.1.4 Partition co-efficient determination with various permeation enhancers and gel formulations.

6.1.1.4.1 *n*-octanol and PBS partitioning of harpagoside and harpagide in the crude extract with permeation enhancers.

The enhancers SDS, DMSO, Cineole and Azone® were combined with the crude extract in separate containers. Each contained 7% permeation enhancer. The partition co-efficient values were determined using the method described in 6.1.1.2.

6.1.1.4.2 *n*-octanol and PBS partitioning of harpagoside and harpagide in the crude extract in gel formulations.

The crude extract was added at a concentration of 300 mg/ml in each gel formulation (Carbopol Ultrez 21®, hydroxypropyl cellulose and Pluronic® F-127) and triturated until homogenous in beakers. Partition co-efficient values were determined using the method described in 6.1.1.2.

6.1.1.4.3 *n*-octanol and PBS buffer partitioning of harpagoside and harpagide in the crude extract with gel formulations containing permeation enhancers.

Each gel formulation containing crude drug was distributed into four beakers. The permeation enhancers namely, SDS, DMSO, Cineole and Azone® were slowly incorporated into the three formulated gels individually. The formulations contained 7% permeation enhancers. The literature indicates that, generally the concentration of the enhancers selected ranges between 5%-10%, it was therefore decided to use a 7% concentration in this study. Each beaker contained a different gel-enhancer combination. Partition co-efficient values were determined using the method described in 6.1.1.2.

6.2 Results and Discussion

6.2.1 Aqueous Solubility

Determining the solubility of individual components in crude extracts may be difficult as it contains many compounds with various properties and solubilities. In the case of *Harpagophytum procumbens,* it contains compounds such as iridiode glycosides, sugars, triterpenes, sterols, fats and waxes (Wichtl, 2004) that have varying degrees of aqueous solubility. No specific data is available for the solubility values of harpagoside and harpagide in various solvents including PBS.

Table 6.1 Aqueous solubility of harpagoside and harpagide
(Mean±SD, n=2).

Aqueous Solubility
Harpagoside 30.3 mg/ml (0.8485)
Harpagide 58.6 mg/ml (1.131)

Table 6.1 represents the solubility of harpagoside and harpagide in PBS 7. These values may give an indication of how solubility will affect permeation across the stratum corneum. Harpagide has a higher solubility with a value of (58.6 mg/ml) when compared to harpagoside (30.3 mg/ml). Compounds with longer carbon chains tend to have decreased aqueous solubility as reported by Yano *et al.,* 1986 investigating the skin permeability of various salicylates. The lower aqueous solubility of the harpagoside may also be associated with the carbon side chain attached to its O-ring structures, which may impart more lipophilic properties. This O-ring is absent in the harpagide. Gerber *et al.,* (2006) suggests that compounds with aqueous solubility less than 1mg/ml may be problematic to permeate across the stratum corneum. Swart *et al.,* 2005 reported when investigating the solubility of glycosides in an aqueous solvent (pH 7), that an increased transdermal permeation was

observed when the aqueous solubility of the glycosides increased. Owing to the fact that our purified harpagoside and harpagide yielded aqueous solubility values more than 1mg/ml would suggest that the aqueous solubility of these compounds would not be the main restricting factor affecting transdermal permeation.

6.2.2 Partition Co-efficient

The experimental octanol-PBS (pH 7) partition coefficient (Log D) values were determined. Higher Log D values indicate more lipophilic compounds. The Log D values of the harpagoside and harpagide in *Harpagophytum procumbens* extract were determined, as well as with gel formulations and permeation enhancers (SDS, DMSO, 1.8 Cineole and Azone®). The Log D value for the harpagoside present in the extract was calculated to be 0.278 and -0.125 for harpagide. (table 6.2).

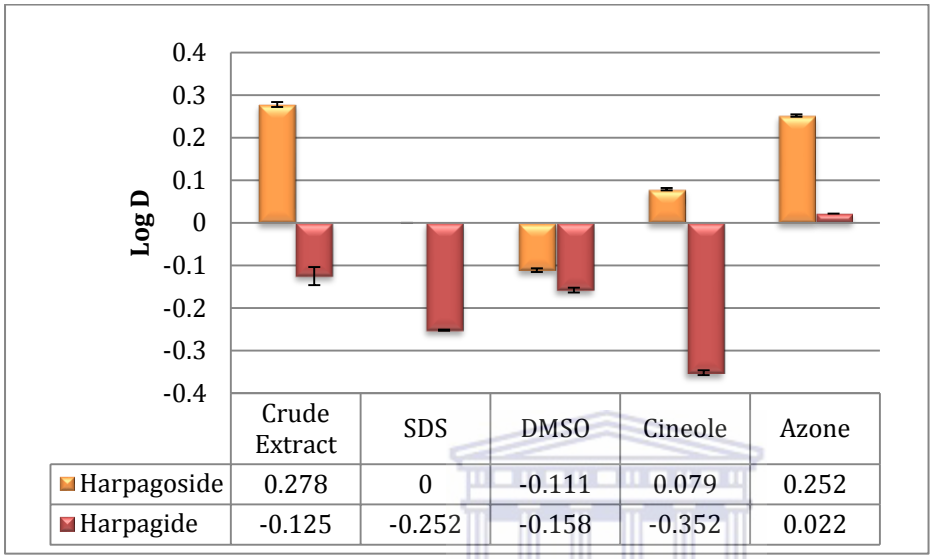
Table 6.2 Log D values of harpagoside and harpagide from crude extract (Mean±SD, n=3).

Log D
Harpagoside 0.278 (0.005)
Harpagide - 0.125 (0.002)

The resultant higher Log D value for harpagoside compared to harpagide is what was expected, as the harpagide is more polar than the harpagoside as its chemical structure consists of a five-member ring with hydroxyl groups and a glycoside attached. The harpagoside has the same structure but has an unsaturated five-carbon chain with a benzene ring, which is more non-polar, and therefore, would likely increase lipophilic properties.

All the permeation enhancers tested decreased the Log D of harpagoside where Azone® produced the least reduction in log D (figure 6.1). DMSO resulted in the harpagoside shifting from a lipophilic value to a hydrophilic value of -0.111.

Figure 6.1. Log D values for harpagoside and harpagide with various permeation enhancers (n=2).



	Crude Extract	SDS	DMSO	Cineole	Azone
Harpagoside	0.278	0	-0.111	0.079	0.252
Harpagide	-0.125	-0.252	-0.158	-0.352	0.022

The harpagide presented a Log D value of -0.125. When enhancers were added to the crude extract, the Log D decreased except for Azone®.

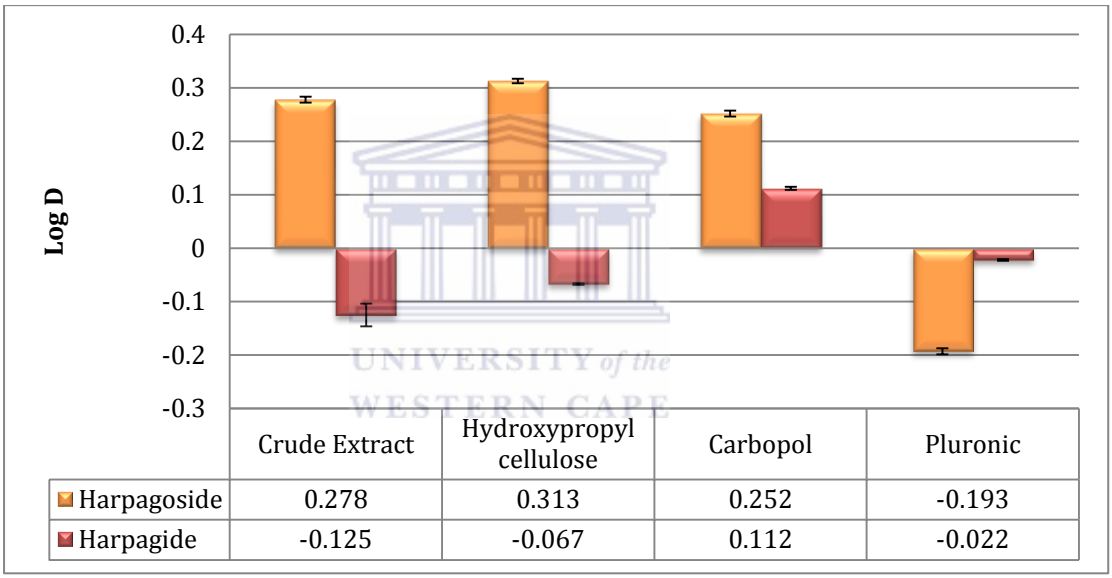
The terpene,1.8 Cineole caused the biggest reduction in log D (-0.352) for harpagide. The partition co-efficient of harpagoside, which has lipophilic properties, also displayed decreased Log D values in the presence of cineole even though cineole is lipophilic in nature (range of 2.6-2.8) (Copolovici and Niinemets, 2005). It is suggested that the activity of terpenes are dependent on the physiochemical properties of the penetrant and that the introduction of cineole may result in a decrease in the permeation of a penetrant (Sinha and Kaur, 2000; Gao and Singh, 1997).

The Two Way analysis of variance (ANOVA) test indicated the partition co-efficient permeation enhancer data was statistically significant. The Tukey's multiple comparison

post-test indicated that the changes in the log D values for harpagoside and harpagide in the presence of each of the permeation enhancers was statistically significant *(P* ≤ 0.05).

Figure 6.2 represents the effect of various gel formulations on the partition co-efficient of harpagoside and harpagide. Hydroxypropyl cellulose gel formulation caused the highest increase in the log D value of harpagoside (0.313) which is higher than the log D value of the harpagoside component on its own 0.278. The Pluronic® F-127 hydrogel formulation decreased the log D value of harpagoside (- 0.193).

Figure 6.2. Log D values for hapagoside and harpagide with gel formulations (n=2).



	Crude Extract	Hydroxypropyl cellulose	Carbopol	Pluronic
Harpagoside	0.278	0.313	0.252	-0.193
Harpagide	-0.125	-0.067	0.112	-0.022

All the gel formulations increased the log D values of harpagide; with the Carbopol Ultrez 21® producing the largest increase to the lipophilic region.

The extent to which the harpagoside partitioned with the gels may be related to the excipients used in the different formulations. The hydroxypropyl cellulose gel consists of ethanol, water and polypropylene glycol, which has good gelling properties and allows for the incorporation of permeation enhancers (Janathraprapapa and Stagni, 2007). This gel formulation consisted of the lowest water concentration when compared to the other two formulations. It resulted in a higher Log D value for harpagoside. The presence of ethanol

may also have contributed as it has non-polar properties imparting more lipophilic tendencies.

The Carbopol Ultrez 21® consists of hydrophobically modified cross-linked polyacrylate polymers (Lubrizol, USA) which may have resulted in partitioning of harpagoside and harpagide in the lipophilic region even though it had a high water content in its formulation.

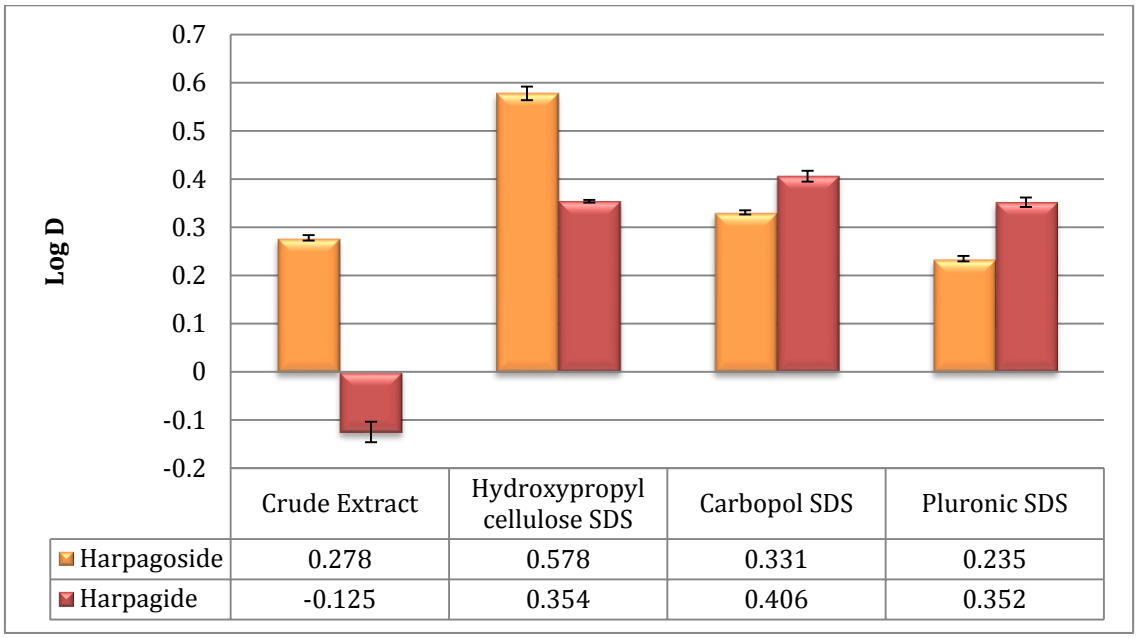
The permeation enhancer that produced the least favourable Log D results was excluded from this study. This was cineole as it reduced harpagide Log D value further from the recommended 1-2 Log D value for transdermal permeation (Gerber *et al.,* 2006).

Various combinations of the remaining permeation enhancers and gel formulations were tested in order to determine the effect on the partition coefficient values of harpagoside and harpagide.

Figure 6.3 represents the log D values of harpagoside and harpagide with SDS when incorporated into the 3 different gel formulations. Its combination with hydroxypropyl cellulose reveals a log D value of 0.578 for harpagoside, which is almost double the value when compared to the log D value of the harpagoside only (without the presence of an enhancer or gel formulation). This SDS hydroxypropyl cellulose value is also higher than the log D value of harpagoside when combined with hydroxypropyl cellulose Gel Only combination (0.313) (figure 6.2).

The combination of the SDS Pluronic® Gel formulation increased the Log D of harpagoside (0.235) compared to Pluronic® Gel only (-0.191). However, this value was still less than the log D value of harpagoside when no enhancer or gel was incorporated.

Figure 6.3 Log D values of harpagoside and harpagide with gel formulations containing SDS (n=2).



	Crude Extract	Hydroxypropyl cellulose SDS	Carbopol SDS	Pluronic SDS
Harpagoside	0.278	0.578	0.331	0.235
Harpagide	-0.125	0.354	0.406	0.352

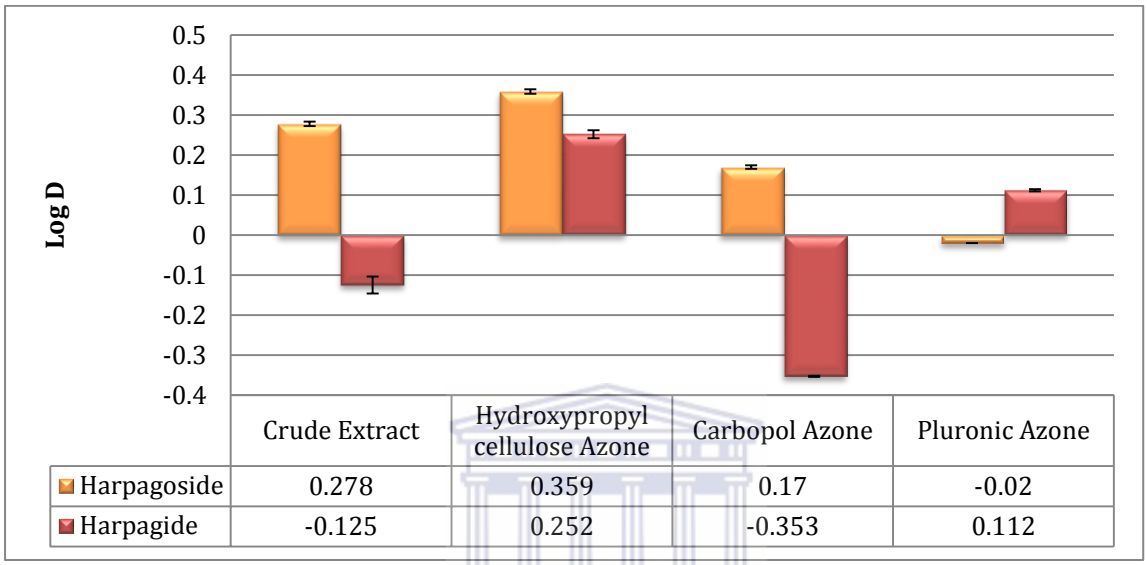
All 3 gel formulations containing SDS resulted in an increase in Log D of harpagide. Hydroxypropyl cellulose and Carbopol Ultrez 21® formulations with SDS increased the log D values of harpagoside and harpagide more than the values of these compounds when tested as an extract only (without permeation enhancer or gel). All changes in Log D values for harpagoside and harpagide with SDS and the various gel formulations were deemed statistically significant *(P* ≤ 0.05).

Figure 6.4 represents the resultant log D values of harpagoside and harpagide when the permeation enhancer Azone® was incorporated in the 3 gel formulations. The Azone® hydroxypropyl cellulose combination yielded higher Log D values than when harpagoside and harpagide were combined with Azone® (figure 6.1) and hydroxypropyl cellulose gel (figure 6.2) alone. The combination of Azone® with the hydroxypropyl cellulose gel increased Log D of harpagoside and harpagide with values of 0.359 and 0.252 respectively, both statistically significant *(P* ≤ 0.05).

Jantharaprapapa and Stagni (2007) suggested that a higher solubility of a compound in a vehicle (gel) lowers the chemical potential of the permeation enhancer, which results in a

decrease in Log D. This could explain why the harpagide log D value is lower when Azone® was incorporated into the Carbopol Ultrez 21® formulation, suggesting good solubility between harpagide and aqueous based Carbopol Ultrez 21®.

Figure 6.4 Log D values of harpagoside and harpagide with gel formulations containing Azone® (n=2). .

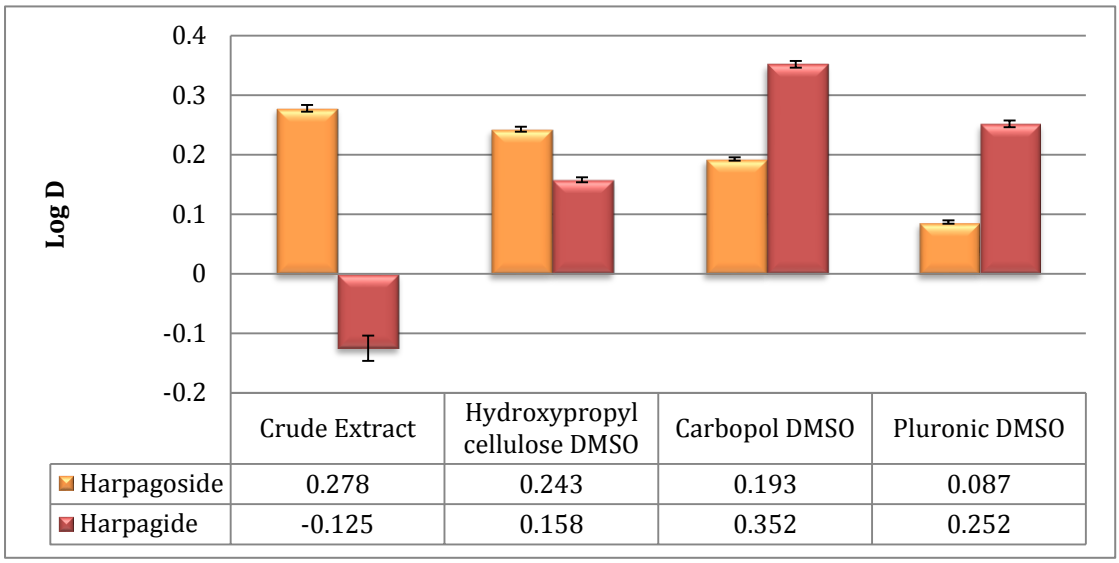


	Crude Extract	Hydroxypropyl cellulose Azone	Carbopol Azone	Pluronic Azone
Harpagoside	0.278	0.359	0.17	-0.02
Harpagide	-0.125	0.252	-0.353	0.112

The Pluronic® and Carbopol Ultrez 21® gels when formulated with Azone® resulted in log D values lower than when harpagoside and harpagide were tested without enhancers or gels .The Log D value for harpagide with the Azone® Carbopol Ultrez 21® gel resulted in the biggest decrease when compared to other gel combinations.

All gel combinations with DMSO resulted in decreased lipophilicity of harpagoside (figure 6.5). When DMSO was incorporated into the gel formulations harpagide had the largest in log D (0.352) with the Carbopol Ultrez 21® gel, followed by the Pluronic® gel, both statistically significant *(P* ≤ 0.05) compared to the crude extract only value.

Figure 6.5 Log D values of harpagoside and harpagide with gel formulations containing DMSO (n=2).



	Crude Extract	Hydroxypropyl cellulose DMSO	Carbopol DMSO	Pluronic DMSO
Harpagoside	0.278	0.243	0.193	0.087
Harpagide	-0.125	0.158	0.352	0.252

All DMSO gel formulations resulted in log D values of harpagoside and harpagide being higher compared to the DMSO crude extract only combination tested in figure 6.1.

For harpagoside, the hydroxypropyl cellulose formulated with SDS yielded the highest log D value of 0.578. This was followed by the hydroxypropyl cellulose and Azone® gel with a log D value of 0.359.

The highest log D value (0.406) for harpagide was obtained with the combination of the Carbopol Ultrez 21® containing SDS. This was followed by a value of 0.35 which was obtained by 3 different gel – enhancer combinations namely, hydroxypropyl cellulose with SDS; Pluronic® with SDS and Carbopol Ultrez 21® with DMSO.

The hydroxypropyl cellulose gel with Azone® and hydroxypropyl SDS formulations yielded the highest Log D values for harpagoside as well as increasing the log D value of harpagide when compared to the crude extract log D value on its own (i.e. no enhancer or gel formulation). Ghafourian *et al.,* 2004 suggests that increased log D values of certain enhancer-gel formulations may be as a result of synergy between these excipients.

The Carbopol Ultrez 21® - SDS formulation was not selected for permeation experiments even though it yielded the highest harpagide log D value. The reason for its omission is that the corresponding harpagoside log D value was less than the crude extract on its own (i.e. no enhancer or gel formulation). DMSO was the second permeation enhancer excluded as it reduced the harpagoside and harpagide into the hydrophilic range and further, from the log D 1-2 range, Pluronic® was the gel formulation excluded, as it caused the largest reduction of log D for harpagoside.

The properties of penetrants includes the extent of its ionization and its ability to form ion complexes in the presence of other compounds which impact on its partitioning to either the aqueous or lipophilic phase in a system (Inagi *et al.,* 1980). It could be considered that the permeation enhancers may play a part in the ion complex formation of penetrants to affect its partitioning ability. Considering that the skin is comprised of lipophilic and hydrophilic regions, the compound permeating across it should have properties demonstrating some affinity for both regions (Sloan *et al.,* 2006; Kalia *et al.,* 2001). It is therefore, suggested that log D values required for good compound permeability are in the range of 1-2 coupled with an aqueous solubility of more than 1mg/ml (Gerber *et al.,* 2006; Naik *et al.,* 2000). The aim is to formulate a gel preparation with an enhancer to get as close as possible to these values. However, combinations of higher or lower solubility and log D values may ultimately affect the flux values obtained by compounds across the skin as demonstrated by Gerber *et al.,* 2006. This together with the action of the selected gel formulation and enhancers on the skin may play a significant role in increasing the flux values of harpagoside and harpagide.

In order to perform the permeation studies with synthetic membranes using Franz diffusional cells, one gel formulation (hydroxypropyl cellulose) with 2 enhancer (Azone®, SDS) combinations were selected. Combinations were selected based on increasing lipophilicity achieved by the gels and enhancer combinations.

6.3 Conclusion

Harpagoside displayed a higher Log D (0.278) value, when compared to harpagide (-0.125). Harpagoside (30.3 mg/ml) and harpagide (58.6 mg/ml) displayed aqueous solubility values above the 1mg/ml.

Hydroxypropyl cellulose gel formulation caused the highest increase in the log D value of harpagoside (0.313) which is higher than the log D value of the harpagoside component on its own 0.278. All the gel formulations increased the log D values of harpagide with Carbopol Ultrez 21® producing the largest increase.

All 3 gel types containing SDS resulted in an increase in the Log D of harpagide. The combination of Azone® with the hydroxypropyl cellulose gel increased the Log D values of harpagoside and harpagide to 0.359 and 0.252 respectively. All gel combinations with DMSO resulted in decreased Log D of harpagoside.

Hydroxypropyl cellulose containing Azone® and hydroxypropyl cellulose containing SDS were the gel formulation combinations selected for permeation studies. These formulations yielded log D values between 0-1 for harpagoside and harpagide.

CHAPTER SEVEN Permeation studies

7.1 Synthetic membranes

Permeation studies were performed with the selected gel-enhancer formulations (hydroxypropyl cellulose gel containing 300 mg/ml crude extract and 7% permeation enhancers, either Azone® or SDS) on synthetic membranes namely Sil-Tec ® (hydrophobic properties) and Tuffyrn® (hydrophilic properties). This was performed in order to determine the permeation profile of harpagoside and harpagide in a gel formulation (with and without enhancers). Plotting the average cumulative amount per area versus time represented an indication of the release characteristics and flux of the two marker compounds from the various gel formulations. These experiments were performed prior to transdermal permeation studies using human skin samples.

7.1.1 Methods

7.1.1.1 Synthetic membrane preparation

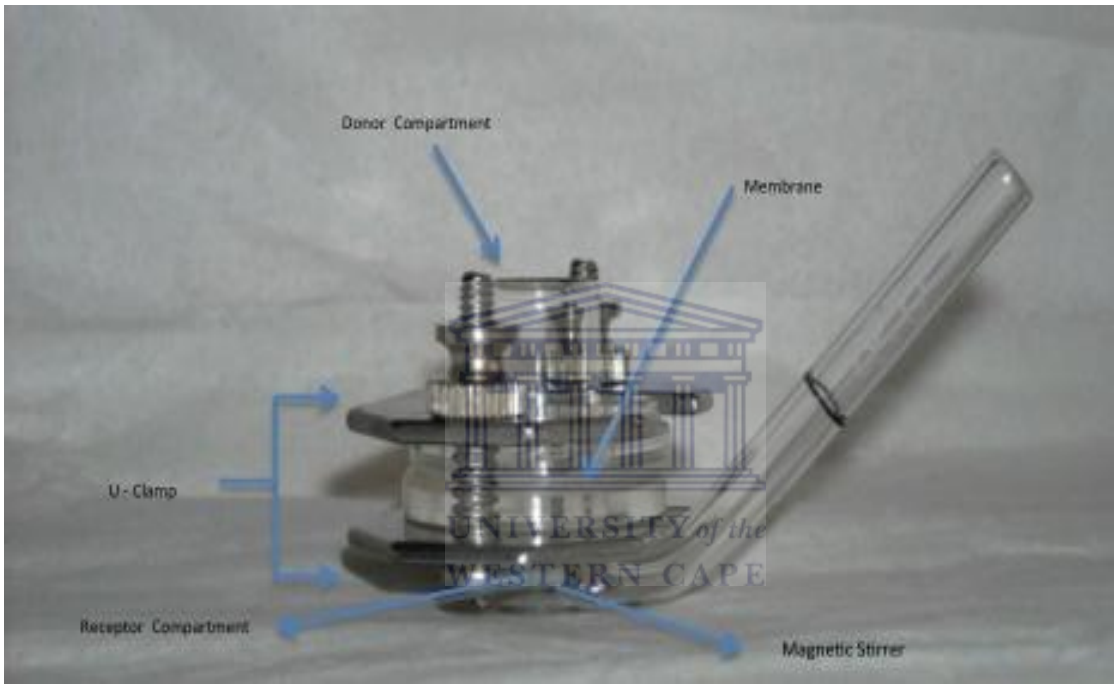
The membranes selected for permeation studies for the synthetic membrane experiments were Sil-Tec® (hydrophobic properties) and Tuffyrn® (hydrophilic properties). Prior to placement into Franz diffusion cells, the membranes were rinsed by soaking it in a PBS buffer solution for 10 minutes, placed on Whatman® filter paper and allowed to air dry.

7.1.1.2 Franz diffusion cell

Vertical Franz diffusion cells with 2.0 ml receptor compartments and 1.0751cm^2 effective diffusion area were used for the permeation studies (figure 7.1). A stirrer bar was placed in the receptor solution cavity of the bottom section of the Franz diffusional cell. The membrane

was carefully mounted on the bottom section of the Franz cell. The two sections of the Franz cells were attached. Vacuum grease was applied to the circumference in order to prevent leakage of the cells. A spring loaded U clamp was used to tighten the upper and lower parts of the Franz cell with the membrane separating the donor and receptor compartments of the Franz cell.

Figure 7.1 Franz diffusional cell with U – clamp



The receptor compartments were filled with 2 ml isotonic PBS (pH 7.4). This was gently introduced with tubing attached to a syringe to prevent air bubbles from being introduced into the receptor compartment that usually settles at the membrane causing a reduction in surface area. The donor compartment was filled with 1 ml of the test sample and covered with Parafilm® to prevent evaporation of components within the sample for the duration of the experiment. An excess amount of sample was present in the donor compartments at all times during the experiments. The Franz cells were placed in a water bath onto specifically moulded holders, which allow for only the receptor compartment to be exposed to the water (37°C) circulating in the water bath. The base of the water bath was fitted with a multi stage

magnetic stirrer. The entire volume of the receptor was withdrawn from each Franz cell and replaced with fresh PBS buffer preheated at 37°C with the aid of tubing attached to a syringe. Each Franz cell had its own set of tubing and syringes. Receptor compartment withdrawal and replacement occurred after 2, 4, 6, 8, 10 and 12 hours. The experiments were conducted over a 12 hour period.

7.1.1.3 Donor formulations

The donor compartment of the Franz diffusional cells was filled with the different gel formulations. The top of the donor compartment was closed with Parafilm® to prevent possible loss during experimentation.

7.1.1.4 Solid Phase Extraction

Samples taken from the receptor compartment had to be concentrated prior to analysis. For this purpose, solid phase extraction of the solutes was performed. The solid phase extraction parameters with respect to flow rate, solvent system and column bonding phase type was determined using the Phenomenex® Strata® preparation method development software version 1. A Strata®-X 30mg/1ml 8B-S100-TAK solid phase, polymeric RP sorbent extraction column was selected. Solid phase extraction flow rates of 3-6ml/min were used. The solid phase extraction cartridge was conditioned with 1ml methanol for 10-20 seconds followed by washing with 1ml distilled water for 10-20 seconds. The sample (the contents of the receptor compartment of each Franz cell at each time interval) was loaded to the cartridge with a flow rate for 1-2ml/min and washed with 1ml water with a flow rate between 1-2ml/min.

The cartridge was dried under vacuum for 1 minute and then eluted with 1ml methanol/acetonitrile (1:1) solution. The elution solvent was allowed to soak into the sorbent

for 60 seconds and eluted using a flow rate of between to 1-2ml/min. New cartridges were used for each sample.

The elution solvent was removed and transferred to 2 ml Greiner® vials and placed in a digital dry bath, set at a temperature of 37º C under a gentle stream of nitrogen through a manifold. The contents were reconstituted with methanol.

7.1.1.5 Determination of Flux

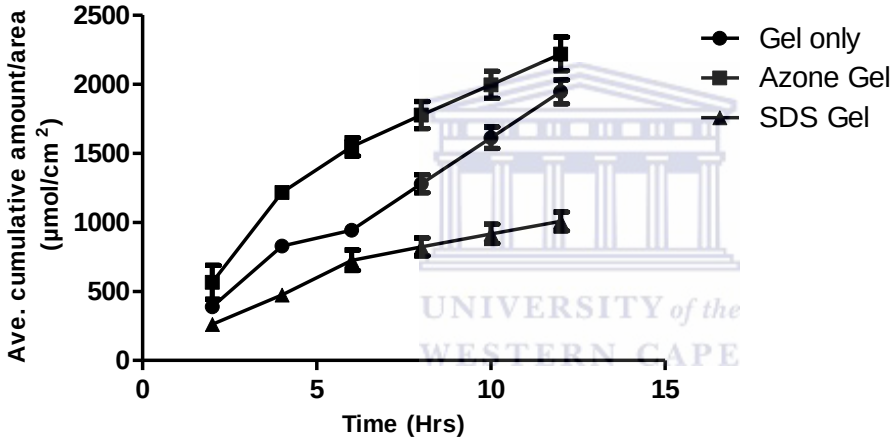
The samples were assayed by HPLC for quantification. The mean flux of harpagoside and harpagide was calculated by plotting the average cumulative amount of marker compound (in receptor compartment) per area versus time. The slope of the linear portion of the curve represents the average (mean) flux value. Data was fitted into GraphPad Prism® version 5 software for steady state curve and flux value extrapolation.

7.1.2 Results and Discussion

7.1.2.1 Flux Values

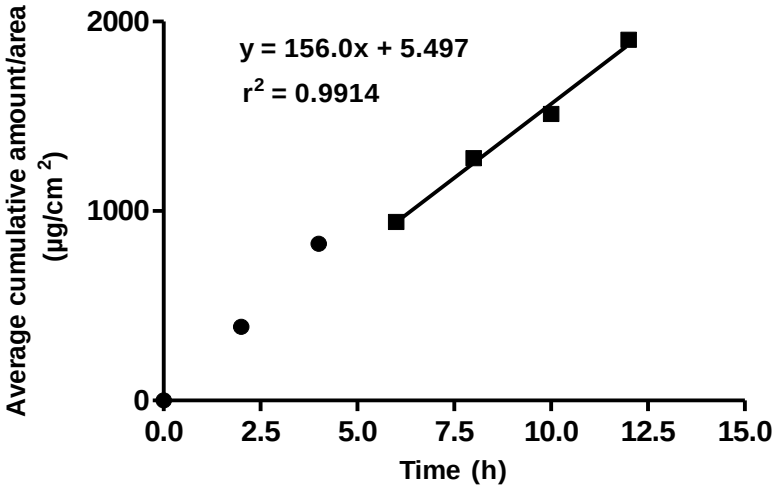
Figure 7.2 represents the cumulative quantities of harpagide that permeated through the hydrophobic synthetic Sil-Tec® membrane over a 12 hour period with 3 gel formulations, 2 of which contained the permeation enhancers.

Figure 7.2 Cumulative amount per unit area of harpagide in various formulations per unit area that penetrated through Sil-Tec® vs. time.



The average value of the slope of the linear segment of the graph above was used to determine the flux value (figure 7.3). Flux values for all permeation studies with Sil-Tec® and Tuffyrn® membranes were determined in this manner (annexure II and III).

Figure 7.3 The average cumulative amount of harpagide that penetrated Sil-Tec® with the Gel only formulation as a function of time (n=2).



The solubility test of the harpagoside and harpagide demonstrated that PBS as receptor fluid is not a rate-limiting step in this permeation process as the maximum solubility concentration was not reached in the receptor solution of the Franz cells. Solubility limitations did not influence the values obtained for the various formulations.

7.1.2.2 Synthetic hydrophobic membrane (Sil-Tec®) and hydrophilic membrane (Tuffyrn®).

The flux values of harpagoside and harpagide in the 3 gel formulations where compared after permeating across the hydrophobic synthetic Sil-Tec® membrane. The gel formulation consisting of the permeation enhancer Azone® increased the flux value of harpagoside to 374.3 µg/cm^2 (Table 7.1) compared the gel formulation without any enhancers (27.1 µg/cm^2) which was statistically significant ($P \leq 0.05$).

Table 7.1 Flux values and enhancement ratio (ER) of harpagoside with various gel formulations permeating through the hydrophobic Sil-Tec® membrane(Mean±SD, n=2).

Hydrophobic membrane (Sil-Tec®)– Harpagoside	Flux (µg.cm^{-2}.h^{-1})	Enhancement ratio (ER)
Gel only	27.1 (1.3)	
Gel + Azone®	374.3 (44.5)	13.9
Gel + SDS	3.9 (0)	0.1

The Azone formulation resulted in an enhancement ratio of 13.9. Conversely, the SDS formulation decreased the flux value of harpagoside to 3.9 µg.cm^{-2}.h^{-1}. When comparing the flux values of harpagide across the Sil-Tec® membrane (table 7.2), the flux values obtained for the Azone® gel (150 (µg.cm^{-2}.h^{-1}) was similar to that of the Gel only formulation (156 (µg.cm^{-2}.h^{-1}).

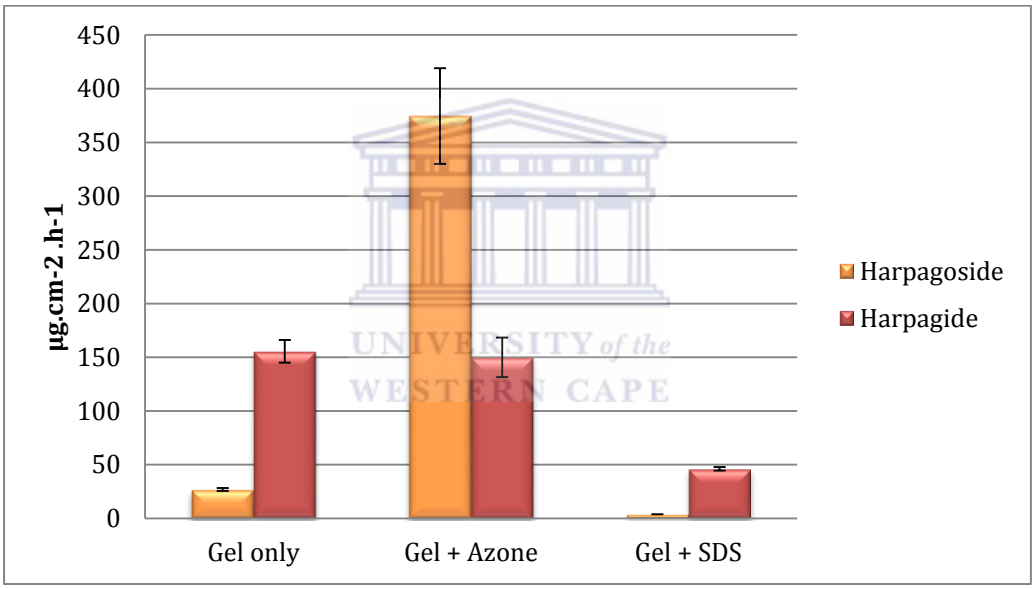
Table 7.2 Flux values and enhancement ratio (ER) of harpagide and with various gel formulations permeating through the hydrophobic Sil-Tec® membrane (Mean±SD, n=2).

Hydrophobic membrane (Sil-Tec®) – Harpagide	Flux (µg.cm^{-2}.h^{-1})	Enhancement ratio (ER)
Gel only	156 (10.6)	
Gel + Azone®	150 (18.3)	1
Gel + SDS	46.2 (1.6)	0.3

The SDS Gel decreased the flux of harpagide resulting in a value of 46.2 µg.cm^{-2}.h^{-1}. This reduction is similar to the SDS gel reducing the flux value of harpagoside, when compared to the Gel only formulation (figure 7.4).

Harpagide flux values were higher than harpagoside for the Gel only formulation as well as the SDS gel formulation. The only increase in flux achieved with this Sil-Tec® was with the Azone® Gel formulation which increased the flux value of harpagoside.

Figure 7.4 Flux values of harpagide and harpagoside with various gel formulations permeating through Sil-Tec® membrane (n=2).



The flux value for harpagoside permeating across the synthetic hydrophilic Tuffyrn® membrane was 537 µg.cm^{-2}.h^{-1} for the Gel only formulation as seen in table 7.3. When the flux values of this formulation was compared to formulations containing Azone® and SDS the values decreased resulting in enhancement ratios of less than 1. Azone® gel demonstrated the greatest decrease in the reduction of flux (90.1 (µg.cm^{-2}.h^{-1}) when compared to the Gel only sample.

Table 7.3 Flux values and enhancement ratio (ER) of harpagoside with various gel formulations permeating through the hydrophilic Tuffyrn® membrane (Mean±SD, n=2).

Hydrophilic membrane (Tuffyrn®) – Harpagoside	Flux (µg.cm^{-2}.h^{-1})	Enhancement Ratio (ER)
Gel only	537.1 (33.2)	
Gel + Azone®	90.1 (1.3)	0.2
Gel + SDS	217.4 (4.2)	0.4

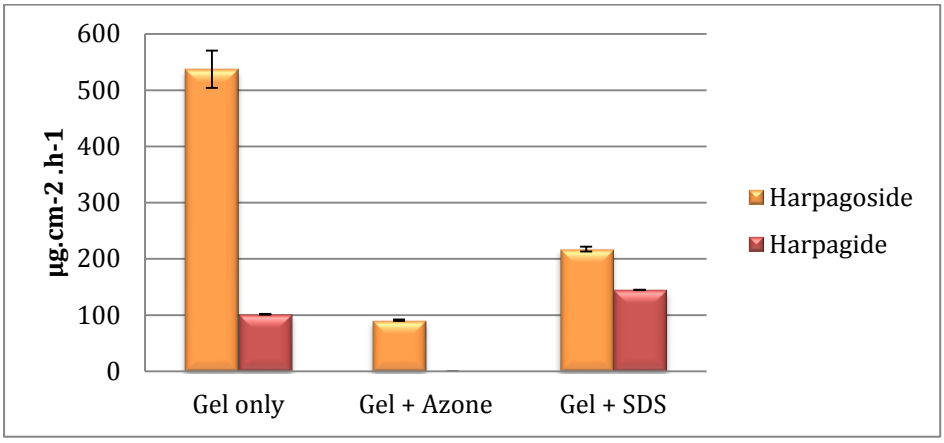
The SDS gel formulation increased the flux value of harpagide to 144.8 µg.cm^{-2}.h^{-1} across the Tuffyrn® membrane (table 7.4.) Incorporation of Azone® into the formulation resulted in harpagide content not being detected in the receptor compartment of the Franz cell (This experiment was repeated for verification and yielded the same result), indicating that the flux value of the Azone® formulation was less than that of the Gel only formulation.

Table 7.4 Flux values and enhancement ratio (ER) of harpagide and with various gel formulations permeating through the hydrophilic Tuffyrn® membrane. (Mean±SD, n=2)

Hydrophilic membrane (Tuffyrn®) – Harpagide	Flux (µg.cm^{-2}.h^{-1})	Enhancement Ratio (ER)
Gel only	101.1 (0.7)	
Gel + Azone®	-	-
Gel + SDS	144.8 (0.21)	1.4

The flux value of harpagide in SDS Gel with this Tuffyrn® membrane was the only flux value more than its respective Gel only formulation (Figure 7.5).

Figure 7.5 Flux values of harpagide and harpagoside with various gel formulations permeating through Tuffyrn® membrane (n=2).



When observing flux values of harpagoside for the Gel only formulations the results indicate that the hydrophilic membrane produced higher flux values compared to the lipophilic membrane. This result is similar to Clement *et al.* (2000) who reported that caffeine with a log D value of -0.1 displayed higher flux values through a hydrophilic (633 µg.cm^{-2}.h^{-1}) polysulphane membrane compared to the lipophilic silicone membrane (9.5 (µg.cm^{-2}.h^{-1}). A factor contributing to the lower permeation in the lipophilic membrane is that the formulation used in the Clement *et al.,* (2000) study was an alcohol based gel with hydrophilic properties and therefore, possibly had less affinity for the lipophilic membrane as suggested by the authors. Further, our Gel formulation is also a hydro-alcoholic base, making it to have more hydrophilic properties possibly contributing to higher permeation in the hydrophilic membrane as observed.

The permeation enhancers also displayed an effect on the flux of the marker compounds across both synthetic membranes as the flux values differed when compared to the Gel only formulation. This could be due to the interaction of the enhancer on the active compound or

gel formulation as well as possible interaction with the membranes. Azone® has surfactant properties, enhancing wetting of the gel on the surface of the synthetic membranes which leads to increased flux values as suggested by Ferreira *et al.,* (1994). They suggested that an emulsion formulation containing a surfactant had better permeation compared to an aqueous solution due to better wetting, resulting in better contact. This suggests that increases in flux rates for both membrane types with Azone® could be expected and not only for the hydrophobic membrane. However, the effect of the permeation enhancers in the gel formulation did not produce a large increase in the enhancement ratio across both membranes except for harpagoside with Azone® on the Sil-Tec® membrane.

When SDS, which has good surfactant properties, was incorporated into the formulation a general decrease in flux was observed. Despite permeation enhancers providing wetting properties to the membranes, other factors resulted in decreased flux rates. The efficacy of compounds such as SDS in the reduction of surface tension for better wetting can be affected by formulation excipients including the penetrant (Boulenc *et al.,* 1995). The excipients present in the formulation may affect the physical integrity of the membrane or the active compound may bind to the membrane (Thakker and Chern 2003). This could explain the general reduction in permeation in the presence of SDS in the formulation.

Permeation enhancers may also affect the electronic charge potential of the marker compounds which may promote or hinder permeation as compounds may prefer to permeate a membrane as ion pairs making it more or less polar. This was reported by Tantishaiyakul *et al.,* (2004), where benzoate permeated through a silicone membrane preferentially as an ion pair rather than a single ion. Therefore, compounds related to good wetting properties may not necessarily increase the rate of permeation as other factors may override the effect of surfactant properties of the permeation enhancers.

Due to the hydrophobic nature of the Sil-Tec® synthetic membrane, it would be expected that hydrophobic compounds would have higher flux rates than hydrophilic compounds. Our

partition coefficient results indicate that harpagoside is more hydrophobic than harpagide. Yet the flux value of harpagide with the Gel only formulation which permeated across the hydrophobic Sil-Tec® membrane was higher than harpagoside. Conversely, the hydrophilic Tuffyrn® membrane yielded a higher flux value for harpagoside than harpagide with the same Gel only formulation. Although lipophilicity is an important factor to consider in determining the permeation potential of compounds across membranes, other factors also contribute to this process. These include molecular weight and size as well as chemical composition and orientation. Gel formulation excipients including permeation enhancers influence these factors resulting in differing permeation rates between compounds as can be seen by the action of the Azone® and SDS.

7.1.3 Conclusion

The permeation enhancer used in the formulation and the nature of the synthetic membranes affected the harpagoside and harpagide permeation profile. For harpagide, all flux values decreased when permeation enhancers were added except for the SDS Gel with the Tuffryn® membrane. For harpagoside, Azone® Gel on the Sil-Tec® membrane showed higher flux values.

The role of the permeation enhancer on the flux value may be due to it affecting the release from the gel and influencing the availability of harpagoside and harpagide at the surface of the synthetic membrane or the membrane could be the rate-limiting step. Harpagide and harpagoside responded differently with respect to flux values obtained in the presence of permeation enhancers. The flux value of harpagide in the Gel only formulation that permeated across the hydrophobic Sil-Tec® membrane was higher than that of harpagoside and the hydrophilic Tuffyrn® membrane yielded a higher flux value for harpagoside than harpagide with the same Gel only formulation. Generally, no direct distinction can be made that would indicate that one of the membranes had higher rates of permeation over the other. The results do suggest that the marker compounds are being released from the formulations albeit to varying degrees.

Following the determination that harpagoside and harpagide have diffused from the gel dosage form and permeated across the membrane with the various formulated gels, transdermal permeation experimental studies were performed with human skin. The formulations included the Gel only formulation as well as gel formulations consisting of Azone® and SDS.

7.2 Transdermal permeation

7.2.1 Methods

Transdermal permeation studies were performed with the selected gel-enhancer formulations (hydroxypropyl cellulose gel containing 300 mg/ml crude extract and 7% permeation enhancers, either Azone® or SDS) as with synthetic membranes. The same apparatus and methods were used for transdermal permeation studies as used for the synthetic membranes. This included the Franz diffusional cells with PBS pH 7.4 receptor solutions with a multi stage magnetic stirrer in a water bath at 37°C. The samples were withdrawn and replaced at 2 hour intervals for 12 hours. This was followed by solid phase extraction of the sample in the receptor solution. For transdermal permeation studies, human epidermal skin was used.

7.2.1.1 Human skin membrane preparation

Female human abdominal skin was used for the transdermal permeation studies. Skin samples were obtained from abdominoplasty surgical procedures performed by plastic surgeons. Samples were maintained under cold chain conditions during transportation from the hospital to the University. Ethics approval was obtained from the Ethics Committee of University of the Western Cape (ethics approval number 09/7/40). Patient medical records were not accessed and patient confidentiality was maintained. The specimens received contained full thickness skin, which included the all skin layers including the fatty hypodermis with a thickness of about 7 cm. A sharp knife, scalpel and forceps were used to carefully separate the fatty hypodermis resulting in only the full thickness skin with the epidermis as seen in figure 7.6.

Following the removal of the fatty hypodermis, the stratum corneum was removed by first immersing the full thickness skin in 60°C HPLC water for 1 minute. A glass plate used during

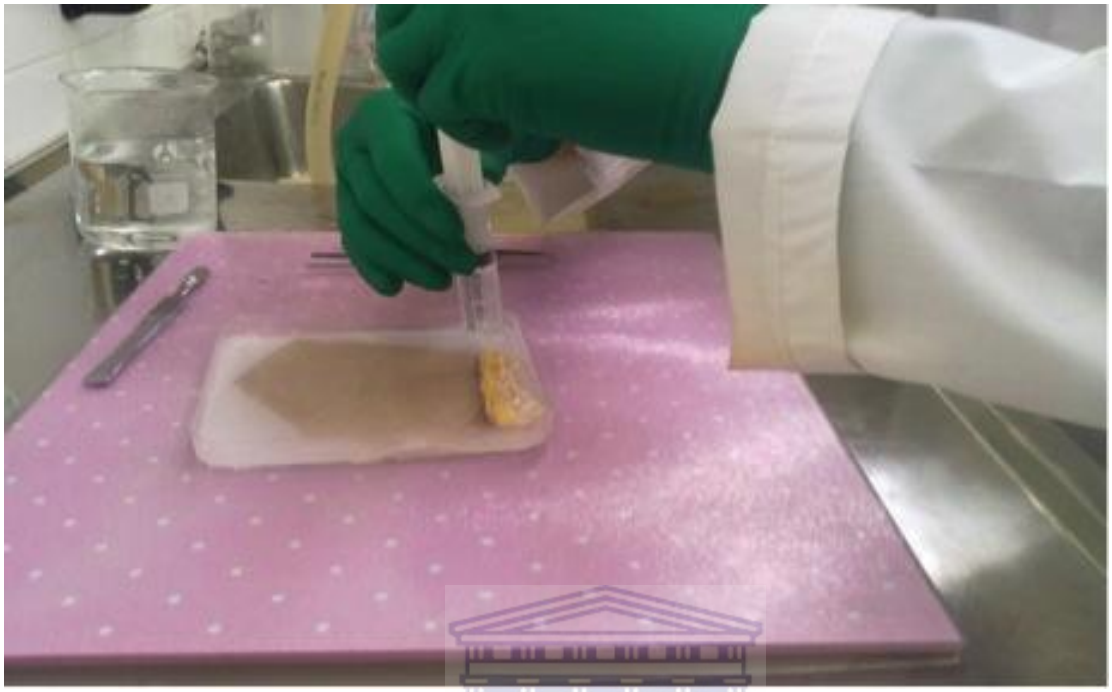
the removal of the stratum corneum was maintained at a temperature of 37°C with circulating water. The epidermis was gently teased away from the skin with forceps and scalpel. Special care was taken that the integrity of the thin delicate stratum corneum was not compromised.

Figure 7.6 Full thickness human abdominal skin after hypodermis removal.

It was placed in a shallow container with HPLC grade water and carefully placed on Whatman® filter paper. The water was drained from the container with a syringe resulting in the stratum corneum distributing uniformly on the filter paper (figure 7.7)

The stratum corneum was sealed in foil, stored in a designated freezer at -20ºC. All biological waste material was disposed in a bio hazardous container and removed weekly by designated contractors. Prior to use, the stratum corneum was thawed and examined visually with the aid of a colony plate reader due to its magnification and lighting abilities, for any defects, before it was mounted on the Franz diffusion cells.

Figure 7.7 Stratum corneum after the removal of the dermal layers drained on Whatman® filter paper.



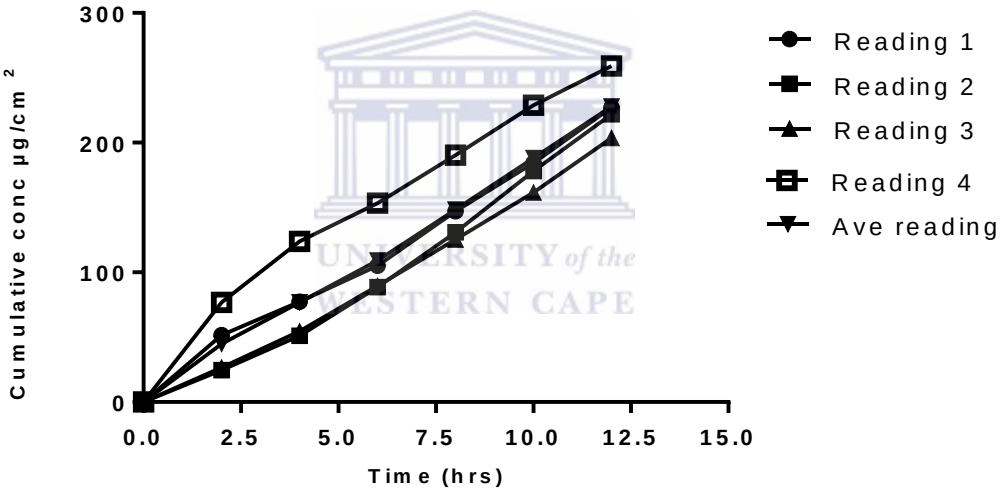
The same Franz diffusional method and donor solutions used with the synthetic membrane were applied to the transdermal permeation study. The stratum corneum was carefully mounted to the receptor compartment of the Franz cell with the external side of the stratum corneum facing upward. Silicone grease was applied on the edges of the donor and receptor compartment of the Franz cells. These compartments were clamped together with a U-clamp to hold the cells in place. Samples were inserted into the donor compartment of the Franz cells.

7.2.2 Results and discussion

7.2.2.1 Flux values

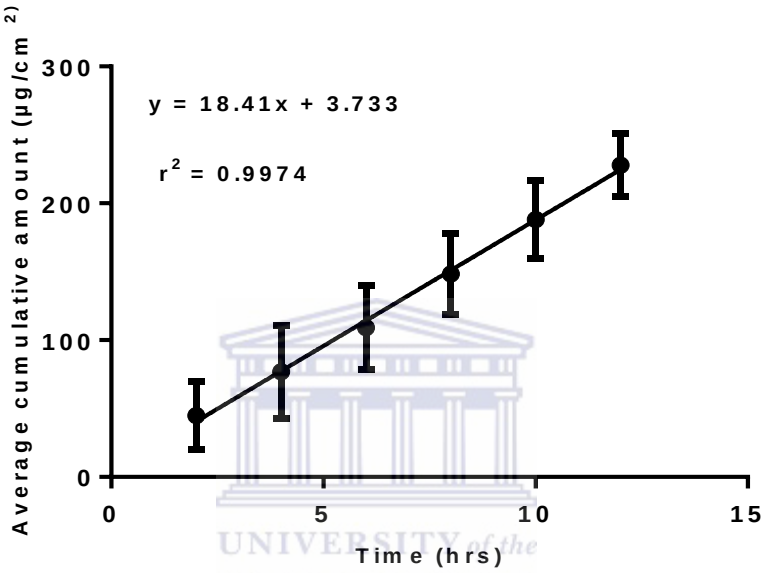
The mean flux of harpagoside and harpagide permeating through the human skin was calculated by plotting the average cumulative amount per area versus time. The slope of the linear portion of the curve represents the average (mean) flux value. Figure 7.8 represents the cumulative 2 hourly readings of the Franz cells for harpagoside with the Gel Only formulation.

Figure 7.8 The cumulative amount of harpagoside per area in each Franz cell permeating the skin as a function of time.



The average cumulative concentration curve reading was used to determine the flux value as seen in figure 7.9 .The average flux value for harpagoside and harpagide with the various gel formulations were calculated in the same manner (plotting the average cumulative amount per area versus time- annexure IV)

Figure 7.9 The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate average flux for harpagoside (n=4).



The changes in flux values across the human stratum corneum upon addition of permeation enhancers Azone® and SDS in our study are listed for harpagoside in table 7.5. The gel formulation consisting of the Azone® yielded an enhancement ratio of 14 when compared to the Gel only formulation (statistically significant $P \leq 0.05$). The SDS Gel formulation (14.16 $\mu g.cm^{-2}.h^{-1}$) yielded the lowest value, even less than the Gel only formulation (18.4 $\mu g.cm^{-2}.h^{-1}$).

Table 7.5 Average flux and enhancement ratio for harpagoside with various gel formulations. (Mean±SD, n=4).

Human epidermis– Harpagoside	Flux (μg.cm^{-2}.h^{-1})	Enhancement ratio (ER)
Gel only	18.4 (1.9)	
Gel + Azone®	256.8 (54.6)	14
Gel + SDS	14.1 (1.9)	0.8

The gel formulation consisting of Azone® also yielded the highest flux value with an enhancement ratio of 8 when compared to the Gel only formulation for harpagide (table 7.6). Diez-Sales *et al.* (1996) who investigated the effect of enhancers on skin permeation reported that Azone produced increased transdermal permeation of various compounds ranging from hydrophilic to lipophilic across human skin. Niazy (1996) also demonstrated that Azone® increased skin permeation of dihyroergotamine across the human skin 55 times compared to a formulation not containing Azone®. Similarly, Degim *et al.,* (1999) reported that Azone® resulted in a 3-fold increase of naproxen through human skin after pretreatment of the skin with Azone®. The gel formulation consisting of Azone® yielded the best permeation results for harpagoside and harpagide compounds.

Table 7.6 Average flux and enhancement ratio for harpagide with various gel formulations. (Mean±SD, n=4).

Human epidermis– Harpagide	Flux (μg.cm^{-2}.h^{-1})	Enhancement ratio (ER)
Gel only	5.05 (0.6)	
Gel + Azone®	38.7 (1.2)	8
Gel + SDS	36.4 (8.5)	7

The SDS gel formulation yielded a result with an enhancement ratio of 7 for the harpagide compound compared to the gel only formulation. The difference between these 2 formulations was not statistically significant. The SDS Gel formulation resulted in a decrease in the flux value compared to the Gel only formulation and an enhancement ratio of less than 1 for harpagoside. The mechanism of permeation enhancement by SDS could be attributed to the insertions and disruption of the bilayer of the stratum corneum (Ribuad *et al.,* 1994). Borra-Blasco *et al.* 1997 reported when assessing the permeation of four different compounds with varying lipophicities that the effect of SDS decreases as the lipophilicity of the compounds increases. Similarly, Copovi (2006) indicated that SDS had no effect on compounds with a log P value of more than 3 and the most hydrophilic compound (log P -0.95) tested 5-flourouracil, displayed the largest enhancement ratio. The difference in the enhancement ratio between harpagoside and harpagide in the presence of SDS may also be due to SDS affecting the polarity of the stratum corneum because hydrophobic interaction of the SDS alkyl chain with the skin structure would leave the end sulphate group of the SDS compound exposed, this would create more sites in the membrane which could allow polar molecules to partition across the stratum corneum more easily (Borra-Blasco *et al.,* 1997).

The solvents and co-solvents in the gel formulation selected consist of ethanol, propylene glycol and water (1:1:1), as used by Janathraprapapa and Stagni (2007) who demonstrated that this formulation allowed for the addition of various permeation enhancers and contributed to the permeation of compounds across a synthetic membrane. The ethanol and propylene glycol in this formulation may also contribute towards an increase in the flux value of the compounds. However, a maximum concentration should be considered as the various formulations tested in the Janathraprapapa and Stagni (2007) study indicated that high concentrations of ethanol and propylene glycol in the gel formulation decreased the permeation profile values and an ideal seems to be equal quantities of the co-solvents i.e. ethanol, propylene glycol and water. The increase in the concentration of the co-solvent propylene glycol beyond 30% may result in a decrease in flux as demonstrated by Thomas

and Panchagula (2003) when testing various concentrations of propylene glycol with AZT permeation across rat epidermis.

Ethanol is an effective solvent for polar and non polar groups of structures and it is feasible that small quantities are capable of leaching significant quantities of non-covalently bound amphophilic stratum corneum lipids which would affect the skin barrier and act as permeation enhancer (Heard *et al.,* 2006). At lower concentrations, ethanol (30%) may increase the lipid fluidity of the stratum corneum at polar interfaces (Suhonen *et al.,* 1999), which could result in increased flux values of compounds in formulations. Water as a co-solvent may as a result of its polar nature interact with the polar head groups of lipids in stratum corneum, upsetting the packing nature of the polar arrangement (Suhonen *et al.,* 1999), therefore promoting permeation. Hydrophilic tendencies of ethanol and propylene glycol may also increase the hydrophilic properties of the gel formulation favouring the harpagide more than harpagoside.

7.2.2.2 Solubility and Molecular weight vs. flux

When comparing the Gel only flux values of the two marker compounds with their relative solubilities, the harpagoside with the lower aqueous solubility of the 2 marker compounds displayed the highest permeation across the human skin for the Gel only and the Azone® gel preparation (table 7.7). It also displayed the largest ER with Azone® Gel when compared to harpagide.

Harpagoside which has a higher molecular weight compared to harpagide displayed the higher flux reading than with the Gel only formulation. Higher molecular weight compounds are usually associated with the lower flux values (Malan SF *et al.,* 2002). The volume that the compounds occupy in their structural orientation should also be considered. Compounds with a molecular weight less than 500 Dalton are considered good candidates for transdermal permeation (Bos and Meinardi 2000).

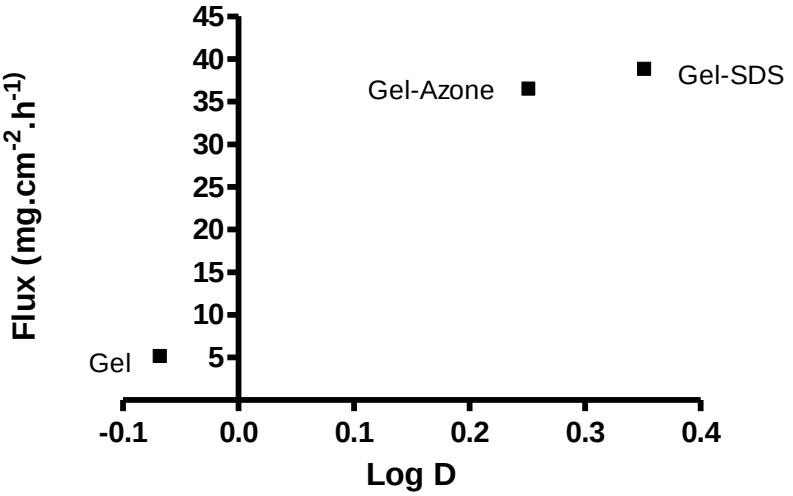
Table 7.7. Physiological properties of harpagoside and harpagide compared to the flux values of the various gel formulations.

Compound	Molecular Weight (mg)	Solubility (mg/m)l	Flux – Gel ($\mu g.cm^{-2}.h^{-1}$)	Flux- Azone® ($\mu g.cm^{-2}.h^{-1}$)	Flux-SDS ($\mu g.cm^{-2}.h^{-1}$)
Harpagoside	494.5	30.3 mg/ml	18.41	256.8	14.16
Harpagide	364.3	58.6 mg/ml	5.05	38.7	36.4

7.2.2.3 Partition Co-efficient vs. flux

Figure 7.10 demonstrates that formulations, which produced higher partition co-efficient values for harpagide, resulted in higher flux values, where the gel formulation consisting of SDS had the highest log D and flux.

Figure 7.10 The relationship between flux and partition co-efficient of different gel formulations for harpagide.



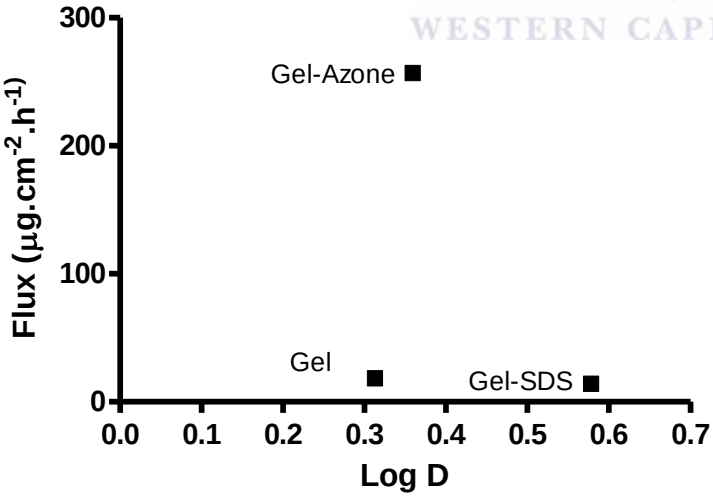
For harpagoside, increased flux values were obtained upon addition of the Azone® and SDS compared to the Gel only formulation (figure 7.11). The Azone® formulation showed the

largest increase in the flux value even though it had a lower log D value (0.359) when compared to the SDS gel formulation (0.578)

The SDS gel formulation did not increase the flux value of harpagoside with an increase in the log D value when compared to the Gel only formulation. Its flux value was less than that of the Gel only formulation even though the log D value increased.

The suggested partition co-efficient value for compounds to be used for transdermal permeation should be between 1-2 (Gerber *et al.,* 2006). This range offers a better chance of permeation across the various skin layers such as the more hydrophobic stratum corneum and the hydrophilic lipid bilayer (Bos and Meinardi 2000).

Figure 7.11 The relationship between flux and partition co-efficient of different gel formulations for harpagoside.



When the relationship between the physiochemical properties of the Gel only formulation with that of the enhancer formulations was observed, it is seen that the Azone® had a

greater impact on the increase in the flux value on the compound with lower aqueous solubility i.e. harpagoside, with an ER = 14 compared to the more aqueous soluble harpagide, compound (ER = 8). Harpagoside also had a higher log D value than harpagide.

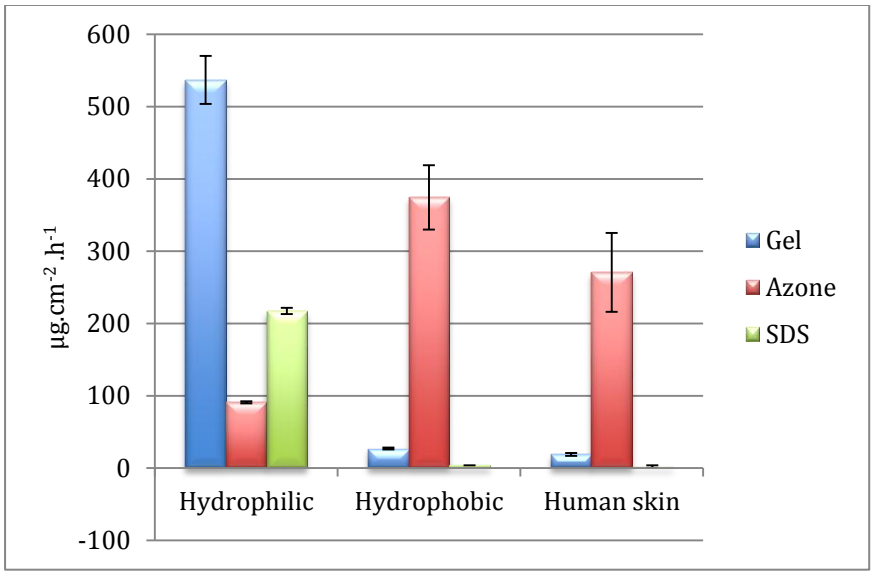
The addition of SDS to the gel formulation resulted in a decrease in flux for harpagoside and an increase for harpagide.

When assessing the physiochemical properties of harpagoside and harpagide no clear pattern emerges that would indicate how these properties affect the flux values. The permeation enhancers, however, do seem to have an impact by generally causing an increase in the flux rate of the marker compounds. This increased flux may be as a result of the permeation enhancers influencing the physiochemical properties of harpagoside and harpagide to varying degrees as well as direct interaction with the human stratum corneum rendering it more permeable.

7.2.2.4 Human epidermis vs. Synthetic membranes

When comparing the flux values for harpagoside with the human epidermis and synthetic membranes, it is observed that the synthetic membranes yielded the highest flux values for all three formulations (figure 7.12). The synthetic hydrophilic Tuffryn® membrane yielded the highest values for the Gel only and SDS gel formulations and the hydrophobic Sil-Tec® membrane for Azone® gel formulation. The Sil-Tec® membrane and human epidermis both demonstrated a higher flux value for Azone® gel and a lower SDS gel flux value compared to the Tuffryn® membrane.

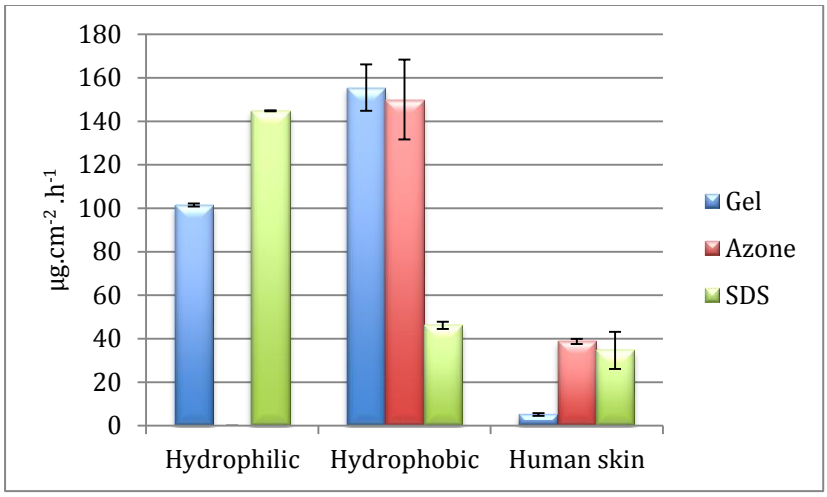
Figure 7.12 Flux values of harpagoside in various gel formulations permeating through various membranes.



When comparing the flux rates across the stratum corneum and synthetic membrane for harpagide as seen in figure 7.13, it was observed that the synthetic membranes have the highest permeation rates in all three formulations. Flux across the stratum corneum was higher when Azone® and SDS were added to the gel formulation compared to the Gel only preparations. This was not the case with the Sil-Tec® and Tuffyrn membranes where the former had a decrease in flux upon addition of both enhancers and the latter had a decrease in flux with the Azone® formulation. The Gel only flux values of the synthetic membranes were at least 5 times higher than that of the human epidermis.

When comparing the flux of harpagoside and harpagide across the human stratum corneum and synthetic membranes, no real similarities could be drawn even though the human and hydrophobic membrane displayed some similarity with harpagoside.

Figure 7.13 Flux values of harpagide in various gel formulations permeating through various membranes.



The study by Jantharaprapapa and Stagni (2007) investigating the permeability of meloxicam across synthetic and human membranes revealed no relationship between the various formulations tested with these membranes. What is noted in their study is that the flux values for the synthetic membrane were higher than that of the human skin. Similarly, our study generally indicated that the flux values of harpagoside and harpagide were higher in the synthetic membranes. The Jantharaprapapa and Stagni (2007) study also indicated that enhancement ratios obtained with permeation enhancers were higher through human skin compared to the synthetic membrane. This result was also reflected in this study were the presence of Azone® and SDS as enhancers displayed higher enhancement ratios with human skin compared to the synthetic membranes. Fang *et al.,* (1999) indicated when testing the permeation characteristics of the anti-inflammatory diclofenac sodium, a synthetic membrane yielded higher flux values obtained when compared to human skin and rat skin. The authors suggested that the large difference in flux values between synthetic and human membrane may be due to the higher permeability of the diclofenac through cellulose in the hydrophilic membrane related to it being less dense in structure compared to the skin. When comparing the flux values of the hydrophilic membrane and the human skin with the 3 formulations, the differences were statistically significant with the harpagoside. The

harpagide also yielded statistical significance except with the Azone® gel formulation. There may be other factors contributing to the permeation differences, such as the way the physio-chemical properties of the marker compounds interacting more actively with the human skin compared to the synthetic membranes. What is also worth considering is that permeation across the human skin involves a complex structure such as lipid bilayers whereas permeation across a synthetic membrane is a passive diffusional barrier (Barbato *et al.,* 1998). This would further suggest that physiochemical properties of the marker compounds would be more important to consider in active human skin rather than passive synthetic membranes as can be seen by the lower overall flux values in the human skin compared to that in the synthetic membrane.

In our study, synthetic membranes were not good indicators of how these compounds would permeate human skin but it provided information regarding the permeation profiles of the various gel formulations across hydrophilic and hydrophobic membranes. This is useful considering the large quantity and variety of compounds such as waxes and sterols present in crude plant extracts including *Harpagophytum procumbens,* which could ultimately affect the permeation process.

When comparing flux values of hydrophilic and hydrophobic membranes with the 3 Gel formulations, the flux differences were statistically significant for harpagoside and harpagide except for the Azone® Gel formulation for harpagide. This suggests that there are no similarities between the 2 synthetic membranes besides generally having higher flux values than the human skin. Synthetic membrane permeation systems were developed to predict drug permeation across the skin, however, these membrane models merely act as passive diffusional barriers (Barbato *et al.,* 1998) and does not take into account the complexity of the human epidermis. The permeation profiles of harpagoside and harpagide are dependent on the characteristics of the membrane as well as the physiochemical properties of the formulation and excipients, which includes permeation enhancers. This observation is similar

to Wang *et al.* (2006) who observed that the formulation components as well as membranes contributed to the permeation of penetrants.

7.3 Conclusion

Transdermal permeation

The gel formulation with Azone® as enhancer yielded an enhancement ratio of 14 when compared to the Gel only formulation for harpagoside. The SDS Gel formulation did not yield an increase in flux for harpagoside.

When flux values of harpagide which is more hydrophilic than harpagoside was observed it can be seen that the gel formulation consisting of Azone® yielded the highest value with an enhancement ratio of 8 when compared to the Gel only formulation. The SDS Gel formulation yielded an enhancement ratio of 7 with harpagide.

The formulations producing higher partition co-efficient values for harpagide, namely Azone® and SDS formulations resulted in higher flux values compared to the Gel only formulation. The same was not observed with harpagoside.

Human epidermis vs. Synthetic membranes

There were generally no similarities between the synthetic membranes besides having higher flux values than human skin. The effect of permeation enhancers in the gel formulation did not produce a large increase in the enhancement ratio across the synthetic membranes except in the case of harpagoside with Azone® for Sil-Tec® membrane. Enhancement ratios achieved with human skin was higher than synthetic membranes. Synthetic membranes were not a good indicator of how these compounds would permeate human skin but it provided useful information regarding the permeation profiles of the various gel formulations across hydrophilic and hydrophobic membranes. However, Sil-Tec® membrane could serve as an indicator of possible permeation pattern of human stratum corneum for harpagoside.

The use of synthetic membranes for preliminary permeation studies with plant compounds is important as it provides vital information with respect to the release and availability of active compounds from a gel matrix to the surface of a membrane rendering it available for permeation across a membrane. This approach therefore, has limited applicability in determining the complex processes involved in percutaneous release. It should rather be applied as a tool for determining active compound release from topical dosage forms. This will be of significance with plant material with its variety of known and unknown compounds, which could interact with formulation excipients, which may affect the release of active compounds from these formulations.

In the following chapter, post transdermal COX-2 inhibition permeation studies was performed to establish whether the crude extract of *Harpagophytum procumbens* extract has maintained its direct COX -2 inhibition activity after permeating through the stratum corneum. The Azone® gel formulation was selected as transdermal vehicle to transport active ingredients of the crude extract through the human stratum corneum because it increased the flux values of harpagoside and harpagide across the stratum corneum.

CHAPTER EIGHT Post- Transdermal Cyclooxygenase-2 enzyme inhibition.

It was established that the *Harpagophytum procumbens* extract possessed COX-2 inhibitory properties through direct enzyme inhibition. In order to determine whether the crude extract maintained its activity after incorporation into a gel formulation followed by *in vitro* transdermal permeation, post permeation COX-2 inhibition experiments were performed. This was done after the extract that was formulated into an Azone® gel was exposed to human skin in permeation studies.

8.1 Methods

The interval that yielded the largest quantity of crude extract over the 12 hour permeation period in the Franz cell receptor compartment was identified and used to perform the post permeation COX-2 inhibition experiments. This was selected to maximize the possibility to obtain COX-2 inhibition. The sample Azone® gel contained 300 mg/ml crude extract in the donor compartment of the Franz cells. An Azone® gel only formulation excluding the crude extract was run in parallel to this sample.

The sample gel and the Azone® gel only preparation were exposed to the identical experimental procedure, which included sample preparation, transdermal permeation and solid phase extraction.

8.1.1 Post-permeation direct COX-2 enzyme inhibition.

For post-permeation COX-2 inhibition studies, the same methodology used in pre-permeation experiments were implemented (section 5.1.1). These experiments, however, did have different reaction mixtures and included solid phase extraction techniques.

8.1.1.1 Solid Phase Extraction

In order to concentrate diluted crude extract collected after transdermal permeation studies in the receptor compartment of the Franz cell, solid phase extraction was performed for sample enrichment. These enriched samples were added to the COX- reaction mixture to determine enzyme inhibition. Solid phase extraction was performed on the sample gel and the Azone® only gel. The same solid phase extraction methods and parameters were used as described in section 7.1.1.4.

8.1.1.2 Reaction mixtures

The following reaction mixtures were used for the post-permeated COX-2 inhibition experiments.

COX-2 reaction mixture (standard reaction) - Arachadonic acid (AA) 100 µM + N,N-,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM + COX-2 enzyme 4 µl (25 units) + buffer solution up to 1000 µl.

DuP-697 reaction mixture – This is the reference inhibition reaction which consists of Arachadonic acid (AA) 100 µM + N,N,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM +COX-2 enzyme 4 µl (25 units) + **DuP-697 50 µl** + buffer solution up to 1000 µl.

Sample Gel reaction mixture (Post-permeation) - Arachadonic acid (AA) 100 µM + N,N-,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM + COX-2 enzyme 4 µl (25 units) + **Azone® gel formulation content containing *Harpagophytum procumbens* crude extract 50µl after solid phase** + buffer solution up to 1000 µl.

Gel formulation reaction mixture (Post-permeation) – Arachadonic acid (AA) 100 µM + N,N,N',N'– tetramethyl-*p*-phenylenediamine (TMPD) 50 µM + COX-2 enzyme 4 µl (25 units) + **Azone® gel formulation content after solid phase extraction** + buffer solution up to 1000 µl. This is the same as the sample gel formulation reaction mixture except it does not

contain the crude extract. This was to determine whether the excipients of the formulation and solid phase extraction techniques influenced the COX-2 inhibition results.

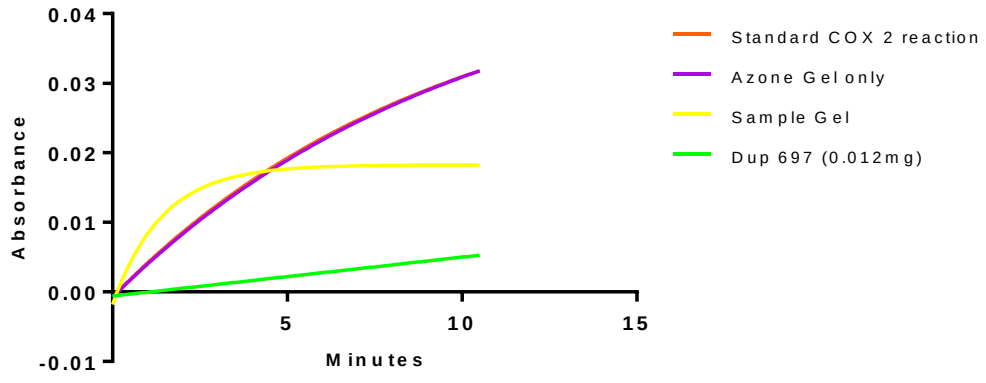
8.2 Results and discussion

The 10 hour Azone® Gel sample yielded the highest quantity of extract when compared to the other time intervals in the Franz permeation experiments. This yielded a quantity of 1.9 mg from the receptor compartment of the Franz cell after solid phase extraction. This consisted of the crude extract and possible trace components of the gel formulation.

Figures 8.1 represents the absorbance versus time graphs for the COX-2 inhibitory activity of the *Harpagophytum procumbens* extract containing the transdermally permeated 10 hour Sample Gel (sample Azone® Gel containing *Harpagophytum procumbens*), permeated Azone® Gel only, the reference inhibitor compound (DuP-697) and the standard COX-2 reaction.

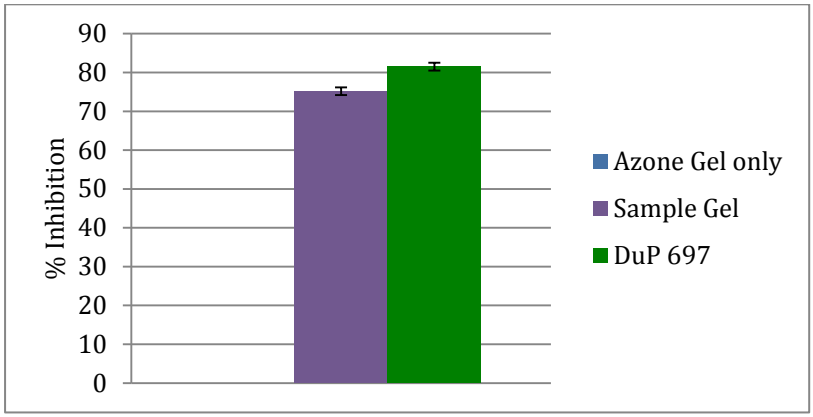
The standard COX-2 reaction and the permeated Azone Gel only preparation yielded identical inhibition curves. This suggests that the Azone Gel formulation excipients did not contribute to COX-2 enzyme inhibition. As expected the DuP-697 curve demonstrated the greatest COX-2 enzyme inhibition.

Figure 8.1 UV absorbance vs. time of post transdermal permeated gel samples.



The DuP-697 (0.012 mg) COX-2 inhibitory activity yielded COX-2 inhibition of 83% (figure 8.2). The post permeation COX-2 inhibitory effect of the sample containing crude *Harpagophytum procumbens* yielded an inhibitory effect of 77%.

Figure 8.2 Direct COX-2 enzyme inhibition (post-permeation) by Azone® gel only, Sample gel and DuP-697. ***(n=3; mean ±S.D.)***



This result is similar to the COX-2 inhibition achieved with the 2mg crude extract (80%), in the pre permeation COX-2 inhibition experiments. The Azone® Gel only formulation did not display any COX-2 enzyme inhibitory activity (0%), which suggests that the excipients in this formulation did not contribute to COX-2 inhibitory activity, and that the inhibitory activity observed with the *Harpagophytum procumbens* Azone® Gel was as a result of the activity of the crude extract.

A study by Ouitas and Heard (2009) evaluated the ability of *Harpagophytum Procumbens* to retain its ability to indirectly inhibit COX-2 inhibition properties after permeation across full thickness excised pigskin. The results of the their study demonstrates indirect (COX-2 expression) COX2 inhibitory effects of *Harpagophytum procumbens* extract after the permeation process but about 25% combined decrease in activity compared to the reference extract which did not permeate the pig skin.

The skin consists of enzymes that may possibly contribute to the breakdown of various compounds in the crude extract resulting in decreased inhibitory effects. The permeation process could also have resulted in not all components of the crude extract permeating the skin, resulting in lower levels of some components of the extract in the Franz receptor

compartment. These factors are suggested by Ouitas and Heard (2009) as being contributors to the weaker inhibitory effects of the permeated samples in their study.

In our study, there was only a 3% reduction in COX2 inhibition between the pre and post tested samples. The composition and ratio of the permeated crude drug may have changed between the pre and post COX2 samples, however, both samples produced COX-2 inhibition. This may be due to the possibility that the ratio and composition of the crude extract remained unchanged after permeation across the stratum corneum or, the composition of the crude extract changed possibly with a lower concentration of the crude extract but with higher proportions of permeable compounds such as harpagide, which imparts COX-2 inhibition. The possibility of the excipients of the gel formulation contributing inhibitory effects can be excluded as figure 7.2 illustrates that the gel sample did not impart any COX-2 inhibition.

The 77% inhibitory activity achieved by the post permeated crude sample could be considered to be mainly attributed to the activity of harpagide rather than harpagoside. This suggestion is substantiated in figure 5.6 where the 0.2 mg harpagide compound resulted in 60% of the COX-2 enzyme where the same concentration of harpagoside only yielded 5% activity. The rest of the inhibitory activity could be attributed to the other components that permeated through the stratum corneum in the 1.9 mg yield.

The quantity of *Harpagophytum procumbens* extract, which permeated at its maximal quantity at time interval 10 hours, was more than the quantity required to obtain the IC_{50} level, which was 0.1046 mg/ml (table 5.2).

8.3 Conclusion

The post-permeated sample gel displayed 77% COX-2 inhibitory properties compared to 80% obtained in the pre-permeated COX-2 inhibition experiments (Chapter 5). The Azone® gel only formulation (without crude extract) did not display any COX-2 enzyme inhibitory activity. The experiments proved that *Harpagophytum procumbens* extract formulated into an Azone® gel, maintains its direct COX-2 inhibition properties after it penetrated human skin samples.

Future studies could combine the direct and indirect mechanisms of COX-2 inhibition in order to determine the total effect on COX-2 activity by the *Harpagophytum procumbens* extract.

CHAPTER NINE Summary and final conclusions.

The following objectives were set:

- Develop a standardized extraction method to yield a consistent crude *Harpagophytum procumbens* extract.
- Perform quality, purity and identity tests by means of Thin layer Chromatography and High Pressure Liquid Chromatography on the *Harpagophytum procumbens* crude extract.
- Develop a method and experimentally measure the direct COX–2 enzyme inhibitory activity of the crude extract of *Harpagophytum procumbens*, harpagoside and harpagide glycosides.
- Experimentally determine the aqueous solubility, partition co-efficient of harpagoside and harpagide components.
- To formulate the crude extract into a transdermal topical gel preparation.
- Experimentally perform partition coefficient studies with selected permeation enhancers and gel formulation combinations of the *Harpagophytum procumbens* crude extract containing harpagoside and harpagide.
- Experimentally determine the permeation (flux) of harpagoside and harpagide compounds from the crude extract in gel formulations across synthetic membranes.
- Experimentally determine the permeation (flux) of harpagoside and harpagide compounds from the crude extract in gel formulations across the human skin (transdermal).
- Determine the relationship between the flux values of the synthetic membrane and human skin.

- Determine the extent to which the gel formulation retained the ability of the crude extract to maintain direct COX 2 enzyme inhibition after transdermal permeation.

Goals and objectives were achieved by a systematic approach.

Extraction and marker identification

The *Harpagophytum procumbens* extract was effectively extracted from the roots of this medicinal plant. The harpagoside and harpagide were identified and quantified in the extract using various analytical techniques.

Pre- COX-2 direct inhibition studies

The direct COX-2 inhibition properties of harpagoside and harpagide compounds as well the crude extract was determined. It was established that harpagide had a greater inhibition on the enzyme than harpagoside. The crude extract however had an even larger inhibition activity on the enzyme positively indicating polyvalence or synergism of compounds in the extract. The IC_{50} values indicated that the *Harpagophytum procumbens* crude extract had the lowest IC_{50} concentration when compared to harpagide and harpagoside.

Partition co-efficient

The partition coefficient results indicate that harpagoside displayed a higher Log D than harpagide. The addition of permeation enhancers resulted in changes in the partitioning of the 2 marker compounds.

Azone® resulted in the lowest reduction in log D for harpagoside and produced the largest increase in log D for harpagide compared to other permeation enhancers tested.

Hydroxypropyl cellulose gel produced the highest increase in the log D value for harpagoside. All the gel formulations increased the log D values for harpagide with Carbopol Ultrez 21® producing the largest increase.

The hydroxypropyl cellulose gel formulated with SDS and Azone® yielded log D values between 0-1 for harpagoside and harpagide. The hydroxypropyl gel was used for all permeation experiments.

Synthetic membrane permeation

The flux value of harpagide with the Gel only formulation across the hydrophobic Sil-Tec® membrane was higher than harpagoside. Conversely the hydrophilic Tuffyrn® membrane yielded a higher flux value for harpagoside than harpagide with the same Gel only formulation. Incorporation of the crude extract in the Azone® hydroxypropyl cellulose formulation resulted in an enhancement ratio (ER) of about 14 for harpagoside with the Sil-Tec® membrane compared to the gel only preparation. The hydroxypropyl cellulose gel with permeation enhancers (Azone® and SDS®) did not result in an increase in permeation of harpagide for this membrane. The formulation of Azone® and SDS in the gel resulted in decreased flux for harpagoside on the Tuffyrn® membrane. A minimal increase was obtained for harpagide in the SDS gel with an ER of 1.4. The permeation results across the synthetic membranes suggested that the marker compounds were released from the formulations albeit to varying degrees.

Transdermal permeation experiments

The gel formulations used in the transdermal permeation experiments were hydroxypropyl cellulose with Azone® and SDS. The formulation with Azone® resulted in increased flux to produce an ER =14 for harpagoside with no enhancement with SDS obtained. For harpagide similar enhancement ratios were obtained with Azone® and SDS, 8 and 7 respectively. Overall, formulations containing Azone® obtained the highest permeation across the human skin. Enhancement ratios achieved with the human skin was generally higher than that of

the synthetic membranes. The permeation profiles of harpagoside and harpagide were dependent on the characteristics of the membrane as well as the physiochemical properties of the permeation enhancers.

Post permeation COX-2 inhibition experiments

The Azone® gel formulation was selected for the post transdermal permeation COX-2 inhibition studies because it resulted in the highest flux values for harpagoside and harpagide. The crude extract retained its COX-2 inhibitory activity after permeation across the human skin with 77% inhibition achieved. The inhibitory effects of the pre and post transdermal permeated samples were similar.

The *Harpagophytum procumbens* crude extract was successfully formulated into a topical gel consisting of hydroxypropyl cellulose, water, propylene glycol and ethanol with Azone® as permeation enhancer. This formulation enhanced the transdermal flux of the harpagide and harpagoside and probably other extract components. Further, the preparation maintained the ability of the crude extract to effectively permeate the skin without losing its COX-2 enzyme inhibition activity. This topical gel preparation has the potential to be utilized as an effective natural anti-inflammatory transdermal dosage form. In order for the current formulation to be as a suitable pharmaceutical preparation with clinical potential, further pharmaceutical, physical and chemical analysis must be performed.

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ANNEXURES

Annexure I:

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Full Length Research Paper

Direct inhibition of cyclooxygenase-2 enzyme by an extract of *Harpagophytum procumbens*, harpagoside and harpagide

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A methanolic extract of Harpagophytum procumbens as well as harpagoside and harpagide were tested as direct inhibitors of cyclooxygenase-2 enzyme (COX-2). The *H. procumbens* extract demonstrated direct inhibition (68%) of COX-2 enzyme. The concentration of harpagoside and harpagide equivalent to that found in the extract (3 and 1% respectively) contributed 1.5 and 13% to this inhibition. Results indicated direct COX-2 enzyme inhibition by the *H. procumbens* extract due to possible synergistic activity of active components in the extract, which include harpagide and harpagoside.

Key words: Harpagophytum procumbens, harpagoside, harpagide, cylcooxygenase-2, devils claw, synergism.

INTRODUCTION

Devil's claw (*Harpagophytum procumbens*) is a plant geographically located in many regions throughout Southern Africa. It's typical habitat is open savannahs and arid regions. The plant root has been used for the treatment of osteoarthritic conditions and has analgesic properties (Van Wyk et al., 1997). Devil's claw contains iridiode glycosides, sugars, triterpenes, sterols and fats (Wichtl, 2004). The iridiode glycosides (harpagoside and harpagide) are believed to have medicinal properties (Van Wyk et al., 1997).

H. procumbens extract and its various active components have anti-inflammatory properties expressed by different mechanisms of activity (Fiebich et al., 2001; Loew et al., 2001; Benito et al., 2000). The clinical efficacy of *H. procumbens* for inflammatory conditions has been reported (Chrubasik et al., 2002). These reveal favourable results for the relief of osteoarthritic conditions with H. procumbens and harpagoside as compared to anti-arthritic drugs, refecoxib and diacerein (Gagnier et al., 2004; Chantre et al., 2000). Questions have been raised about the clinical assessment criteria used in some studies (McGregor et al., 2005).

Studies involving COX-2 inhibition by *H. procumbens* extract in animals indicate significant reduction in pain and inflammation induced by experimental test procedures. These include Freund's adjuvant induced arthritis and carrageenan-induced rat paw oedema (Anderson et al., 2004; Lanhers et al., 1992). However, animal studies tend to indicate that isolated components, such as harpagoside do not demonstrate anti-inflammatory properties (McGregor et al., 2005; Lanhers et al., 1992).

Two forms of cyclooxygenase are known, namely, cyclooxygenase-1 (COX-1) and cylclooxygenase-2 (COX-2). COX-1 is a constitutive enzyme and COX-2 is induced in inflammatory cells by inflammatory stimuli (Rang et al., 2003). Anti-inflammatory properties of *H. procumbens* extract and its active components have been attributed to the inhibition of activities related to the pathways of COX-1 (Jang et al., 2003) and COX-2 (Abdelouahab and Heard, 2008), the latter being more frequently investigated.

Cyclooxygenase enzyme inhibition studies may be categorized by (a) measuring direct inhibition of enzyme activity by the inhibitor; (b) measuring the inhibition of enzyme activity through reduced enzyme expression; or (c) measuring other biochemical activities due to reduced enzyme output. The last two are categorized as indirect

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measurements of inhibition as demonstrated by McGregor et al. (2005) and Kundu et al. (2005) with the *H. procumbens* extract, respectively.

In vitro studies investigating constituents of *H. procumbens* extract, such as harpagoside and harpagide reveal opposing results in its ability to inhibit COX-2 activity (Abdelouahab and Heard, 2008; Fiebich et al., 2001). This may be related to differences in the method of inhibition assessment and plant extract composition (McGregor et al., 2005; Loew et al., 2001). The *H. procumbens* extract with all its constituents seem to be the most effective in the inhibition of COX-2 as compared to its individual constituents, which may be related to synergy (Abdelouahab and Heard, 2008).

Synergistic interactions of compounds may play an important role in improved therapeutic activity of medicinal plants. In addition, crude extracts may also offer better stability of various active compounds and may include the presence of unknown constituents (Williamson, 2001).

The present study demonstrates direct inhibition of COX-2 enzyme (in contrast to others which focus on indirect inhibition) by an extract of *H. procumbens* as well as pure harpagoside and harpagide. DuP-697 [5-bromo-2-(4-flourophenyl)-3-(4-(methylsulfonyl)-thiophene)], a member of the diaryl heterocyclic group of selective COX-2 inhibitors, which includes MK-966 recoxib and celocoxib, was used as the reference inhibitor. Copeland et al. (1994) demonstrated significant direct COX-2 enzyme inhibition by DuP-697.

Harpagoside was identified in the H. procumbens extract by thin layer chromatography (TLC) and after purification by UV-spectroscopy. Harpagide was identified in the extract by TLC after spraying with a visualization reagent. Weak UV absorption characteristics of harpa gide rendered confirmative UV spectroscopy unfeasible. Quantification of both compounds was done by quantitative TLC.

METHODOLOGY

Preparation of *H. procumbens* extract

Dried *H. procumbens* (A. White Chemist, South Africa) was powdered using a hammer mill (Cullati, Germany). Methanol (Sigma-Aldrich, South Africa) was added to a final powder concentration of 100 mg/ml. The samples were vortexed for 5 min followed by shaking at 400 rpm (Infors HT, Switzerland) for 5 min at room temperature. Thereafter, it was centrifuged at 8000 rpm for 5 min (Hëttich, Germany) and the methanol phase was carefully removed from the pellet. This supernatant was concentrated by the removal of the methanol at 35℃ under a gentle stream of nitrogen resulting in a resinous H. procumbens extract which was stored (-20℃).

Identification of the extract components

Thin layer chromatography analysis was performed on methanol reconstituted solutions of the resinous crude extract (400 mg/ml) with a mobile phase of butanol (Merck, South Africa), acetic acid (Merck, South Africa) and water (12:3:5). Five microlitres of the sample was applied to the TLC plates (silica gel 60, F_{254}, Merck, South Africa) and chromatographed over a plate length of 8 cm with pure harpagoside and harpagide (PhytoPlan, Germany) in methanol as reference chemical standards. The TLC plates were air dried, observed under UV light (254 nm), compounds marked, and they were sprayed with a solution containing 1% (w/v) vanillin (Sigma-Aldrich, South Africa) in ethanolic sulfuric acid, by means of an aerosol spraying device. It was then heated (5 min, 80℃) in an oven yielding identifiable visible spots. The chromatographed compounds on the plate were quantified using a scanner connected to a laptop computer with specialized Quantiscan software version 3 (Biosoft, England) with the ability to quantify and differentiate between chromatographed compounds.

In order to perform confirmative UV-spectroscopy (Cintra 202, Australia) on the chromatographed harpagoside, further purification was necessary. This was achieved by following preparative TLC methods under the same conditions. Chromatographed harpagoside bands on the TLC plates were removed from the plates, extracted with methanol and the UV spectrum of the TLC purified harpagoside was determined by UV spectroscopy in phosphate buffered saline solution.

Determination of cyclooxygenase- 2 inhibition

COX-2 inhibition experiments were based on the methods reported by Copeland et al. (1994) and Corazzi et al. (2005) with minor changes. Twenty-five units of purified COX-2 (ovine) enzyme (Cayman Chemical, USA) were added to 700 µl of enzyme reaction buffer. The buffer consisted of 100 mM sodium phosphate pH 6.5, 0.5 µM hematin (Sigma-Aldrich, South Africa) and 1 mg/ml gelatin (Sigma-Aldrich, South Africa). The enzymatic reaction was initiated by adding 100 µM of arachadonic acid (Sigma-Aldrich, South Africa) and 50 µM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (Sigma-Aldrich, South Africa) to a final reaction mixture of 1 ml.

Various concentrations of COX-2 inhibitors, namely; H. procumbens extract, harpagoside and harpagide were dissolved in dimethsulphoxide (DMSO) (Merck, South Africa) and added to the reaction solution buffer 15 min before arachadonic acid and TMPD were added. DuP-697 was dissolved in dimethylformamide (Merck, South Africa) instead of DMSO. All determinations were performed in triplicate.

COX-2 activity was determined by measuring UV absorption change of the reaction mixture at wavelength 610 nm exactly 8 min after initiating the reaction against the appropriate blank. No change of initial absorption values indicated 100% inhibition and maximum absorption change in the reaction without added inhibitors indicated zero inhibition. Data was fitted into GraphPad Prism version 5 software for the calculation of mean, standard deviation (SD) and IC_{50} values.

RESULTS AND DISCUSSION

TLC of harpagoside present in the *H. procumbens* extract showed an R_f value of 0.68 when viewed under the UV light, which corresponded to the harpagoside standard. UV-spectrometry of the purified compound was identical to that of the harpagoside standard with the ratio of peak maxima to minima at wavelength 280 and 233 nm of 1.87. TLC of harpagide present in the extract could not be visualized on the plates under UV light. It was however

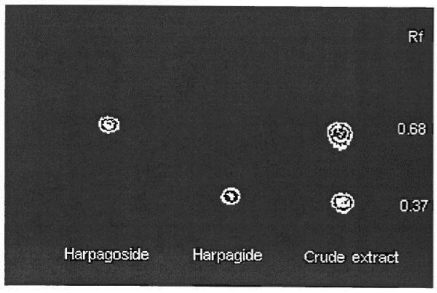

Figure 1. Quantitative thin layer chromatography of *H. procumbens* extract with harpagoside and harpagide reference standards. Harpagoside R_f 0.68; Harpagide R_f 0.37; Mobile Phase: butanol, acetic acid and water (12:3:5). Harpagide spots appeared reddish upon spraying with 1% vanillin in ethanolic sulfuric acid spray reagent.

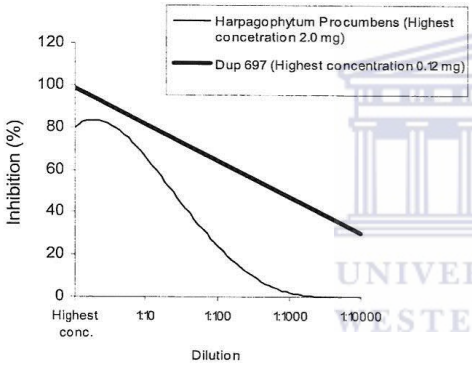

Figure 2. DuP-697 and *H. procumbens* extract COX-2 inhibition curves at various dilutions.

identified after spraying the plate with the 1% vanillin in ethanolic sulfuric acid spray reagent. It yielded a reddish spot, which correlated in colour and R_f value (0.37) with the harpagide standard (Figure 1). The TLC plates containing the harpagoside and harpagide were quantitatively analyzed using the Biosoft® Quantiscan software as described by Nikolova et al., (2004). Bruneton (1999) reported the quantity of iridiode glycosides, which include harpagoside and harpagide, present in the crude extract of H. procumbens to be between 0.5 and 3%. Quantitative analyses of the compounds screened on the developed TLC plates showed that the tested H. procumbens extract contained 3% harpagoside and 1%

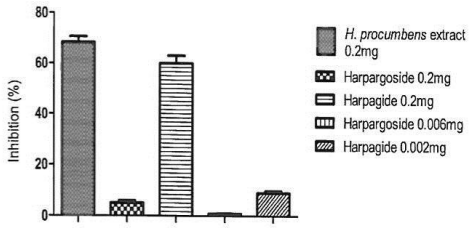

Figure 3. Direct inhibition (%) of COX-2 (25 units) activity by *H. procumbens* extract, harpagoside and harpagide. (n = 3; mean ± S.D.).

harpagide.

Figure 2 illustrates the comparison of COX-2 inhibition between DuP-697 and Harpagophytum procumbens extract at 10 fold dilutions, starting at 0.12 mg (98% inhibition) and 2 mg (80% inhibition) respectively. In addition to the fact that the DuP-697 concentrations were 17 times lower than that of the extract, it exerted a greater inhibitory effect (40%) at lower concentrations (1:1000 dilution) compared to the extract. This difference in inhibition decreases to less than 20% at higher extract concentrations (1:10 dilution). Compared to the DuP-697, the Harpagophytum procumbens extract inhibition curve indicated a non-linear COX-2 inhibition at high and low concentrations, which may be explained by possible synergistic activity of compounds in the extract.

Under these experimental conditions, higher concentrations of Harpagophytum procumbens extract could not be tested because of solubility limitations of the extract in the reaction buffer. Figure 3 illustrates direct COX-2 inhibition by H. procumbens extract as well as harpagoside and harpagide at 0.2 mg. This was the highest concentration at the linear part of the extract's inhibition curve (Figure 2). At a concentration of 0.2 mg, H. procumbens extract showed direct enzyme inhibition of 68.3%, harpagoside 5% and harpagide 60% (Figure 3). The inhibition activity of the crude extract is similar to the results obtained by Jang et al. (2003) and Kundu et al. (2005). Their studies evaluated indirect inhibitory activity through COX-2 expression. Abdelouahab and Heard (2008) reported that harpagoside had higher inhibitory activity than harpagide and that the latter increased COX-expression.

When enzyme inhibition of harpagoside and harpagide were tested at 0.006 and 0.002 mg, respectively, which is the quantity that these compounds are represented in 0.2 mg of the extract, only 1 and 9% direct inhibition were demonstrated, respectively. Fiebich et al. (2001) demonstrated a similar trend where no COX-2 inhibitory effects (TNFα synthase inhibition) by harpagoside and harpagide were observed at concentrations of up to 0.01 mg.

The contrasting COX-2 inhibitory activity reported may be

Table 1. IC_{50} values of DuP-697, *H. procumbens* extract, harpagide and harpagoside as COX-2 inhibitors.

Compound	IC_{50} (mg/ml)
DuP-697	0.000134
H. Procumbens extract	0.1046
Harpagide	0.1186
Harpagoside	104.1

as a result of the total extract containing different fractions of components resulting in synergistic, complimentary or antagonistic effects (Loew et al., 2001).

The calculated IC_{50} values derived from inhibition studies of the inhibitors used are listed in Table 1. It confirms higher inhibition activity of harpagide than harpagoside as direct inhibitor but less than H. procumbens extract and DuP-697. Combined COX-2 inhibition of harpagoside and harpagide at the quantities present in 0.2 mg extract was 10% as compared to 68.3% in the H. procumbens extract. This greater COX-2 direct inhibition exerted by the H. procumbens extract may be as a result of other compounds in the extract possibly with synergistic effects as compared to the combined inhibition of the harpagide and harpagoside. This effect was demonstrated in Fiebich et al (2001) study where the inhibition is attributed to H. procumbens extract and not to the individual components, such as the harpagoside. Abdelouahab and Heard (2008) also suggested that the inhibition of COX-2 expression could be attributed to interplay of the active compounds present in the H. procumbens extract. This phenomenon was described by Williamson (2001) with plant extracts *Ginkgo biloba* and *Cannabis sativa.*

Conclusion

H. procumbens extract produced direct inhibition of COX-2 enzyme, but less than DuP-697. COX-2 inhibition by H. procumbens extract is greater than that of harpagoside and harpagide at the same concentrations. Harpagide has up to 12 times more inhibition activity as direct COX-2 enzyme inhibitor than harpagoside. The calculated combined inhibition of harpagoside and harpagide at the concentrations as they appear in the extract is 10% as compared to 68% by the *H. procumbens* extract. However, the combination of these two components, with other compounds, which form part of the extract, has an even greater direct inhibition of COX-2 enzymes.This difference in COX-2 inhibition may be attributed to interplay expressed by other components in the extract.

Future studies could combine the direct and indirect mechanisms of COX-2 inhibition in order to determine the total effect on COX-2 activity by the *H. procumbens* extract which will be a combination of direct and indirect enzyme activity inhibition.

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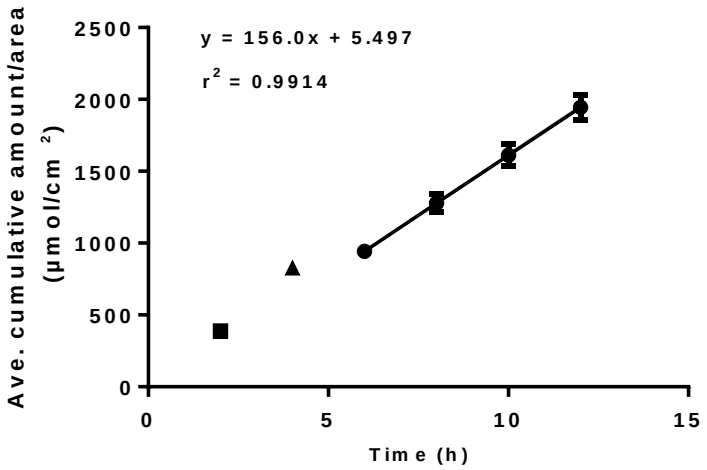
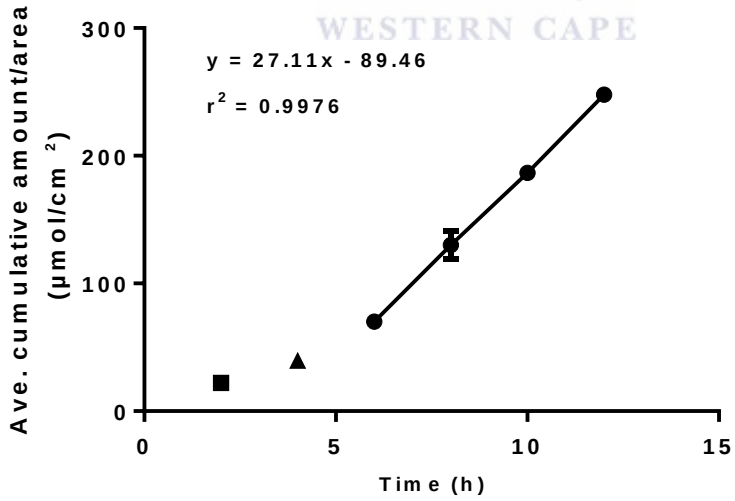
Annexure II: Hydrophobic Membrane

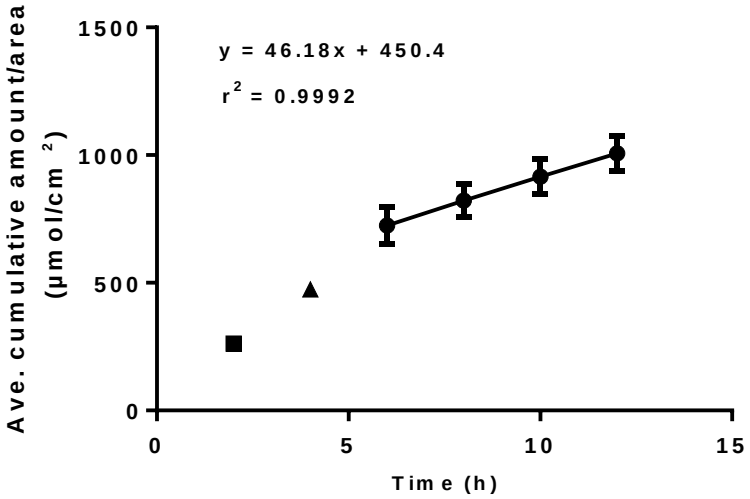
II a. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the gel only preparation.



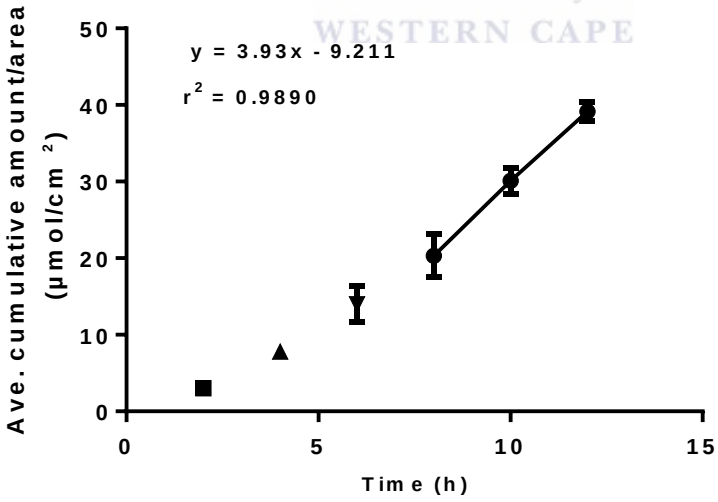
II b. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the gel only preparation with the gel only preparation.



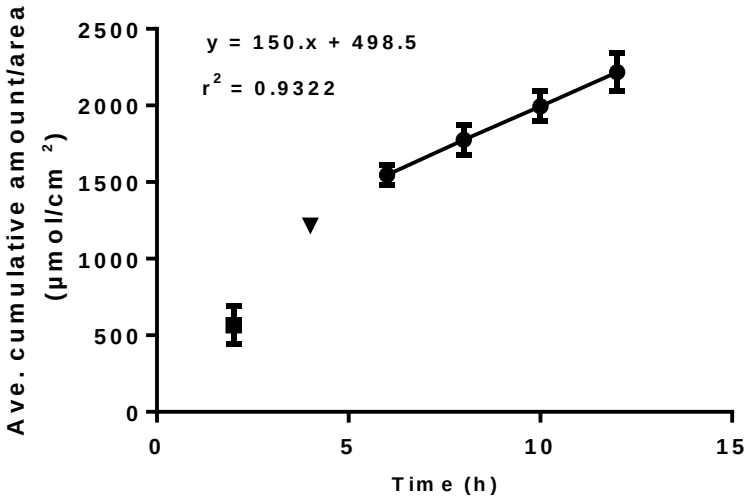
II c. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the SDS- gel only preparation



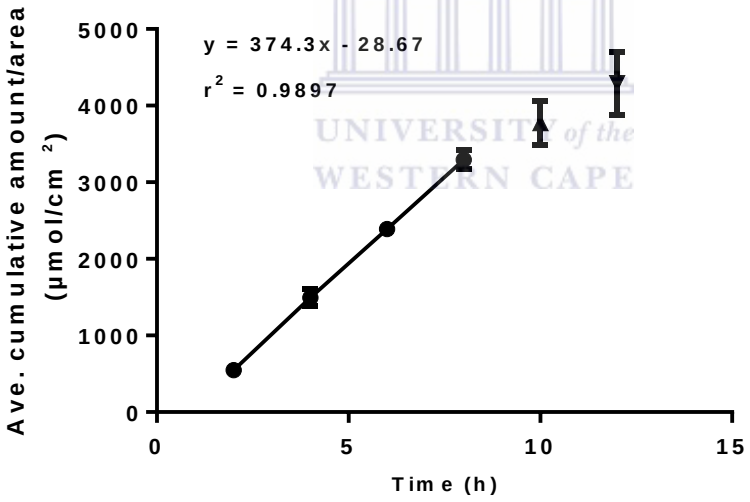
II d. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the SDS- gel preparation.



II e. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the Azone gel only preparation.

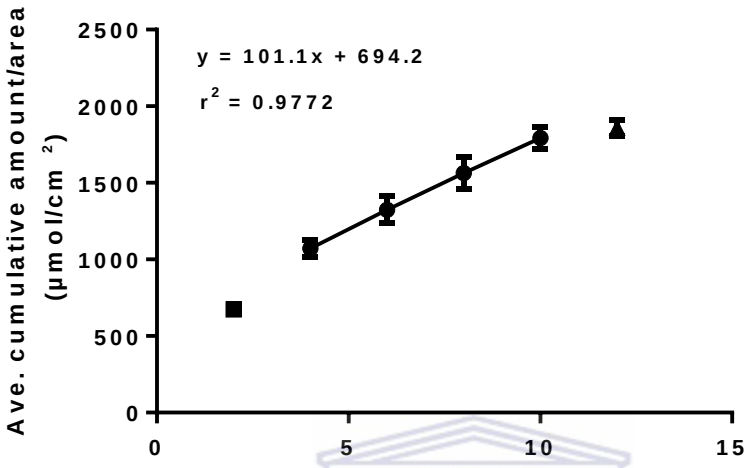


II f. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the Azone gel preparation.

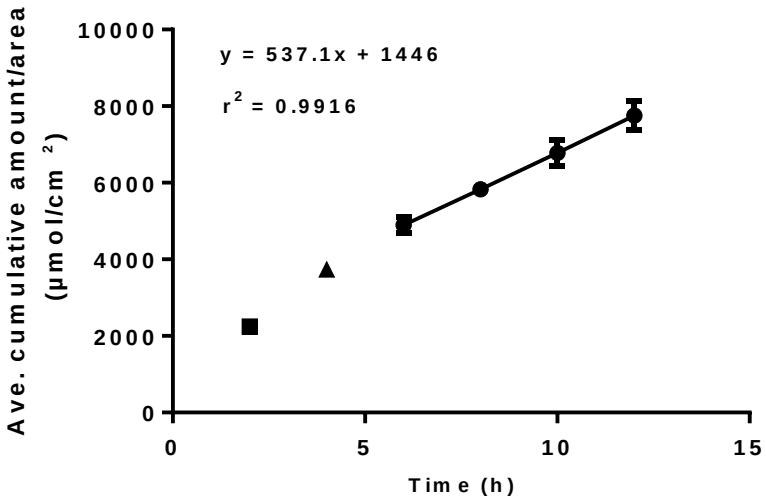


Annexure III: Hydrophilic membrane

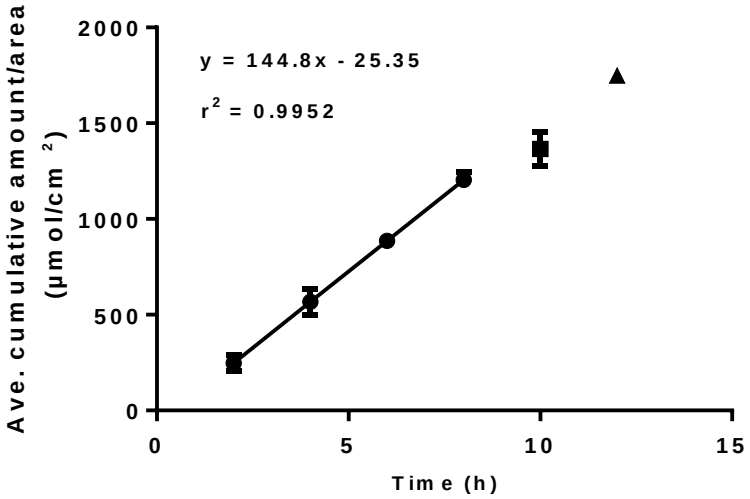
III a. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the gel only preparation.



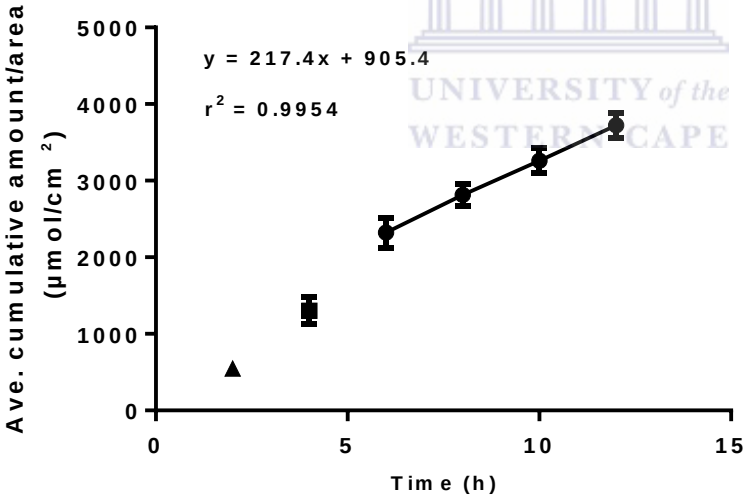
III b. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the gel only preparation.



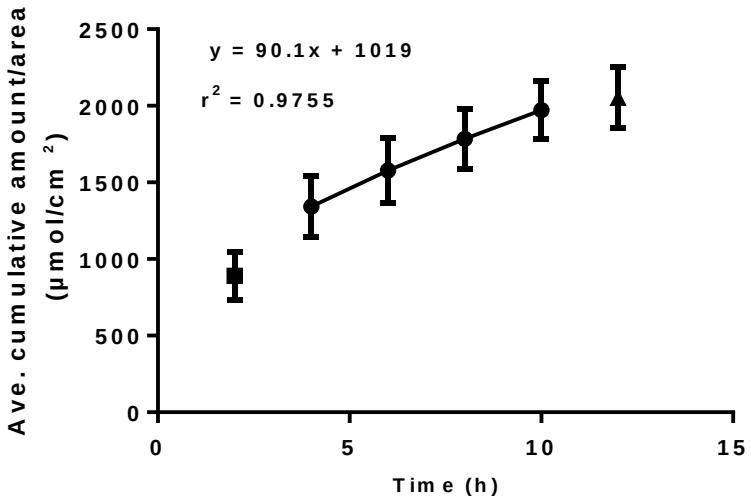
III c. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the SDS gel preparation.



III d. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the SDS gel preparation**.**

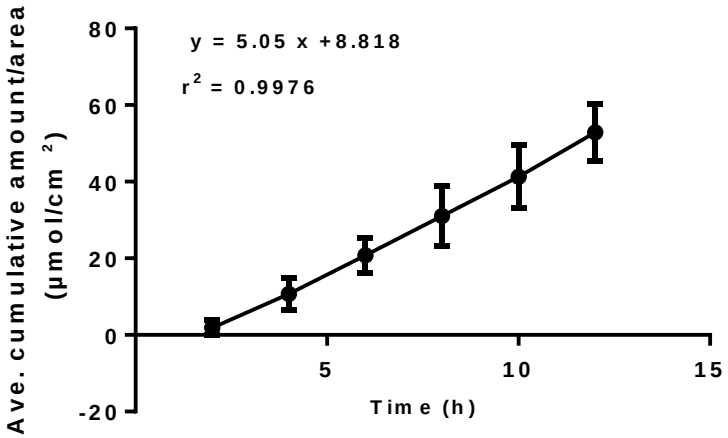


III e. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the gel only preparation Hydrophilic Azone Harpagoside

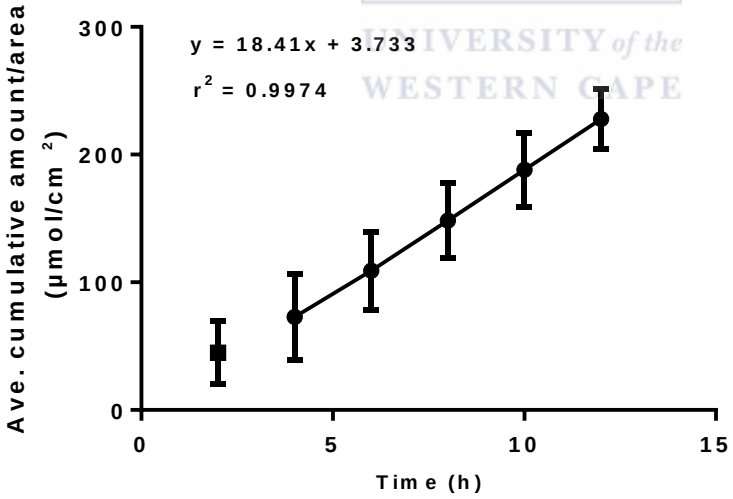


Annexure IV: Human Skin

IV a. The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the gel only preparation.



IV b. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the gel only preparation**.**



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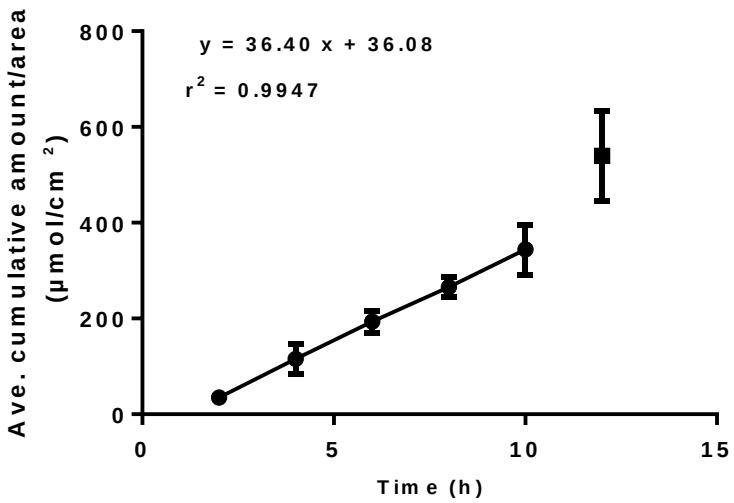
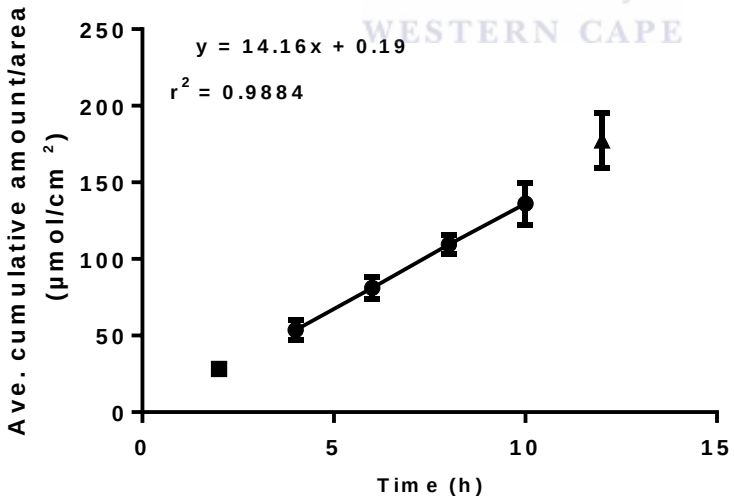
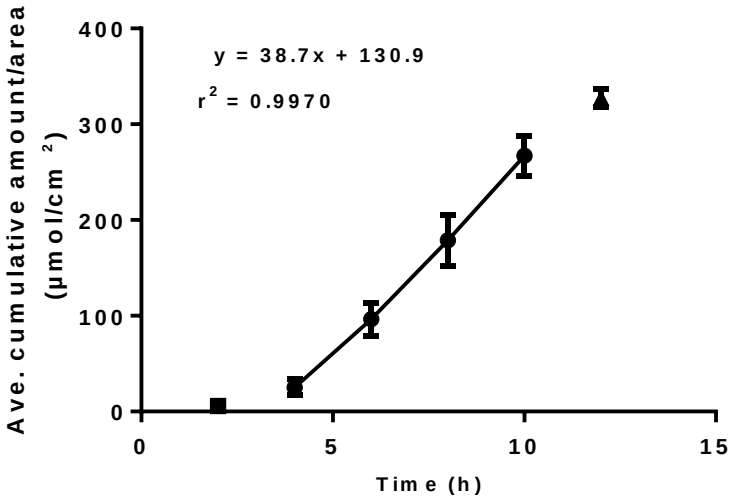


IV d. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the SDS gel preparation.



IV e The average cumulative amount of harpagide, which permeated the skin as a function of time to illustrate the average flux for the harpagide with the Azone gel preparation.



IV f. The average cumulative amount of harpagoside, which permeated the skin as a function of time to illustrate the average flux for the harpagoside with the Azone gel preparation.