Appendix A

Collection, preparation and ^{1}H-NMR fingerprinting of aloe leaf materials

A.1 Introduction

There are more than 360 species of the genus aloe known worldwide (Lee, 2006:1), of which 160 are indigenous to South Africa (Steenkamp & Stewart, 2007:411). Some aloe species have a long history as traditional folk remedies and are generally used, even to this day, to treat conditions such as constipation, arthritis, blood pressure problems, burns, wounds, frostbite, diabetes, eczema, psoriasis and skin cancer (Morton, 1961:311; Reynolds & Dweck, 1999:3, Loots *et al.*, 2007:6891). It can also be used as an excipient in modified release dosage forms (Jani *et al.*, 2007:90) and was found to enhance intestinal drug absorption (Chen *et al.*, 2009:587). Many commercial products such as cosmetics, lotions, sun screens, shampoos, etc. contain whole leaf extracts and pulp (or gel) of *A. vera* due to its soothing, astringent and healing properties (Morton, 1961:311; Choi *et al.*, 2001:535).

Several mechanisms of action have been suggested for *A. vera* and there is much controversy over the active ingredient(s) (Eshun & He, 2004:93). Polysaccharides, as well as miscellaneous bioactive constituents, have been identified from the leaves and roots of the *A. vera* plant (Jia *et al.*, 2008:181). Polysaccharides can exhibit pharmacological as well as physiological activities, and so it can be hypothesised that the mucilaginous gel of the aloe (which consists primarily of polysaccharides) holds the secret to the medicinal properties of this family of plants (Eshun & He, 2004:94).

For example, it has been shown that the moisturising properties of *A. vera* extracts may be due to its polysaccharide-rich composition (Dal'Belo *et al.*, 2006:241), which may be aided by traces of magnesium lactate (Meadows, 1980:51). However, it is considered that the phytoconstituents in the aloe plant act in a concerted way rather than each acting alone (Jia *et al.*, 2008:188).

The main polysaccharide of *A. vera* gel, acetylated mannan (aloverose or acemannan), which consists of a polydispersed β-1,4-linked mannan substituted with O-acetyl groups (Kim, 2006:57), is a proprietary substance covered by many patents (Reynolds & Dweck, 1999:3). Commercially available Carrisyn™ (Acemannan) by Carrington Laboratories, Texas, is one amongst a range of products available (McDaniel & McAnalley, 1987; Reynolds & Dweck, 1999:26).

Regrettably, very little scientific data is available to support the grounds for aloe's therapeutic and medicinal properties (Eshun & He, 2004:91). As the use of aloe is based almost exclusively on research obtained for *A. vera* (Figure A.1 (a)), it is essential for scientists to investigate the pharmaceutical applications of other aloe species (Loots *et al.*, 2007:6891), such as the two species indigenous to South Africa, *A. ferox* (Figure A.1 (b)) and *A. marlothii* (Figure A.1 (c)).

(a) (b) (c)

Figure A.1: The aloe species investigated during this study included (a) *A vera* (Dekumbis, 2013:1); (b) *A. ferox* (Van Wyk, 2002:41) and (c) *A. marlothii*

A.2 Methods

A.2.1 Plant material collection and preparation

Numerous commercially available aloe products contain inadequate amounts of the mucilaginous polysaccharides due to inappropriate processing procedures (Eshun & He, 2004:91). The physiological and pharmaceutical properties of the aloe polysaccharides may be affected due to some types of processing, such as heating, grinding and dehydration (Ramachandra & Rao, 2008:502, 503), therefore it has become vital to harvest, process and distribute the aloe leaves in such a way as to ensure that the essential bioactive components are preserved (Eshun & He, 2004:91; Ramachandra & Rao, 2008:502, 503).

Aloe vera dehydrated gel powder (Daltonmax 700®) and whole leaf material was donated by Improve USA, Inc. (Texas, United States of America); *A. marlothii* leaves were harvested sustainably from natural populations near Koster in the North-West Province of South Africa; *A. ferox* leaves were harvested in the same way near Albertina in the Western Cape Province of South Africa. Care was taken to prevent oxidation and microbial contamination of the inner gel by slowly pealing the leaves from the stem (as can be seen in Figure A.2 (a)).

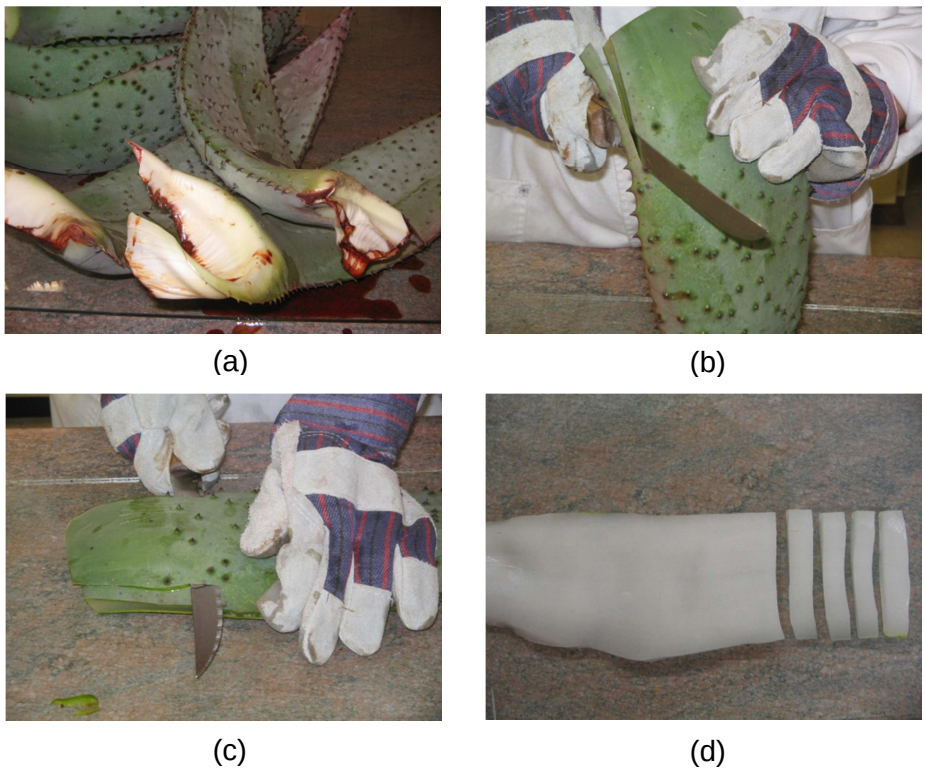


Figure A.2: Processing of *A. marlothii* leaves to demonstrate the method used for aloe leaf processing (a) after harvesting involved the (b) removal of the sharp rinds at the margins of the leaves and (c) the skin layer of the top and bottom flat sides of the leaves to obtain the (d) gel material

It is best to process the leaves immediately after harvesting as the biological activity already starts to lessen six hours after harvesting (at ambient temperatures). It is recommended that the processing of the leaves should be completed within 36 h of its harvesting. This degradative decomposition of the gel matrix can be attributed to natural enzymatic reaction and presence of oxygen which causes the growth of bacteria within the gel (Eshun & He, 2004:93; Ramachandra & Rao, 2008: 504, 508).

The traditional hand-filleting method for processing the *A. marlothii* and *A. ferox* leaves was used, as it was developed to prevent contamination of the gel with the yellow sap (latex/aloin) (Ramachandra & Rao, 2008:505). The sap and the outer leaf of the aloe plant contain apoptosis-inducing anthraquinones, such as aloe emodin and aloin, which are the primary compounds responsible for toxic effects (Eshun and He, 2004:94; Chen *et al.*, 2012:2).

The lower white part of the leaf base, which was attached to the stem of the plant, the tapering point at the leaf top and the sharp spines present at the margins of the leaf were removed with a sharp knife (Figure A.2 (b)). The rind was removed by inserting the knife into the mucilage layer directly underneath the rind (Figure A.2 (c)) from both the top and bottom flat sides of the leaves

(Ramachandra & Rao, 2008:505). The pulp fillet or gel (Figure A.2 (d)) was then rinsed with water to wash off any of the yellow sap that may have come in contact with the pulp fillet.

The gel was liquidised in a kitchen blender either alone or together with parts of the green rind to obtain the gel and whole leaf materials, respectively. Figure A.3 (a) shows the liquidation of the whole leaf material of *A. marlothii* (for demonstration). A freeze dryer (VirTis, United Kingdom, Figure A.3 (b)) was used to lyophilise the gel to produce *A. marlothii* and *A. ferox* gel and whole leaf powder (Jambwa *et al.*, 2011:433; Lebitsa *et al.*, 2012: 298).

(a) (b)

Figure A.3: Aloe gel and whole leaf materials were liquidised in (a) a kitchen blender and (b) subsequently lyophilised with a freeze dryer (VirTis, United Kingdom)

The powders obtained represented the gel and whole leaf materials used during the penetration enhancement studies (Chapter 4 and Appendix D). For the clinical studies (Chapter 3 and Appendix B), a further step was undertaken with the gel materials in order to isolate the polysaccharidic fraction of the gel as described in Section A.2.2.

A.2.2 Precipitation of ethanol insoluble residues

A. ferox 200:1 gel powder containing methanol insoluble residues, or polysaccharidic fraction, was donated by Organic Aloe (Albertina, South Africa). To summarise, the *A. ferox* was prepared by grinding the whole leaf in a hammer mill where after the juice was pressed out, separated and filtered to obtain a clean liquid, the juice was then pumped into a tank and methanol was added; as soon as precipitation of the polysaccharides occurred, it was separated from the liquid by a filtration process; the gel material obtained was then dried with a Niro Spray Dryer in order to obtain a dry powder for commercial use.

The ethanol insoluble residues, or polysaccharidic components, were separated from *A. vera* and *A. marlothii* gel according to the method previously described (Gu *et al.*, 2010:116;

Campestrini *et al.*, 2013:511). Briefly, the powdered aloe leaf gel materials were each mixed with distilled water (RephiLe Direct-Pure UP ultrapure and RO water system, China) and left to mix overnight. It was subsequently filtered through eight layers of cheese cloth in order to remove any insoluble residues. The pH of the filtrate was adjusted to 3.2 with 6 mol/L HCl. Absolute ethanol (Rochelle Chemicals, South Africa) was added to obtain a gel solution: ethanol ratio of 1:4 (v:v). The mixture was then stirred for 30 min and left for at least four hours for the precipitated residue to separate from the fluid. After the water/ethanol liquid was decanted, the solid material was further separated centrifugally (4000 r/min, 10 min). The ethanol insoluble materials from the leaf gel of each aloe species were then snap frozen with liquid nitrogen, lyophilised and subsequently ground with a mortar and pestle.

A.2.3 Proton nuclear magnetic resonance fingerprinting of aloe gel materials

Approximately 30 mg of the gel and whole leaf materials of *A. vera*, *A. marlothii* and *A. ferox* were separately weighed and dissolved in 1.5 ml deuterium oxide (D_2O; Merck, South Africa), whereas approximately 30 mg (*A. vera*), 3 mg (*A. ferox*) and 1 mg (*A. marlothii*) of the precipitated, dried polysaccharidic gel fractions were dissolved in 1.5 ml deuterium oxide. These solutions were filtered through cotton wool and a small quantity of 3-(trimethylsilyl) propionic acid-D4 sodium salt (Merck, South Africa) was added.

The differences in the solution concentrations used for the ^{1}H-NMR fingerprinting can be ascribed to the fact that not all the solutions were clear and non-viscous (normally required for ^{1}H-NMR spectroscopic analysis) after filtration. An Avance III 600 Hz NMR spectrometer (Bruker, Germany) was used to record the ^{1}H-NMR spectra of the solutions. The resultant ^{1}H-NMR spectra were used to identify certain marker molecules and to fingerprint the aloe materials.

A.3 Results

A.3.1 Percentage yield of ethanol insoluble residue

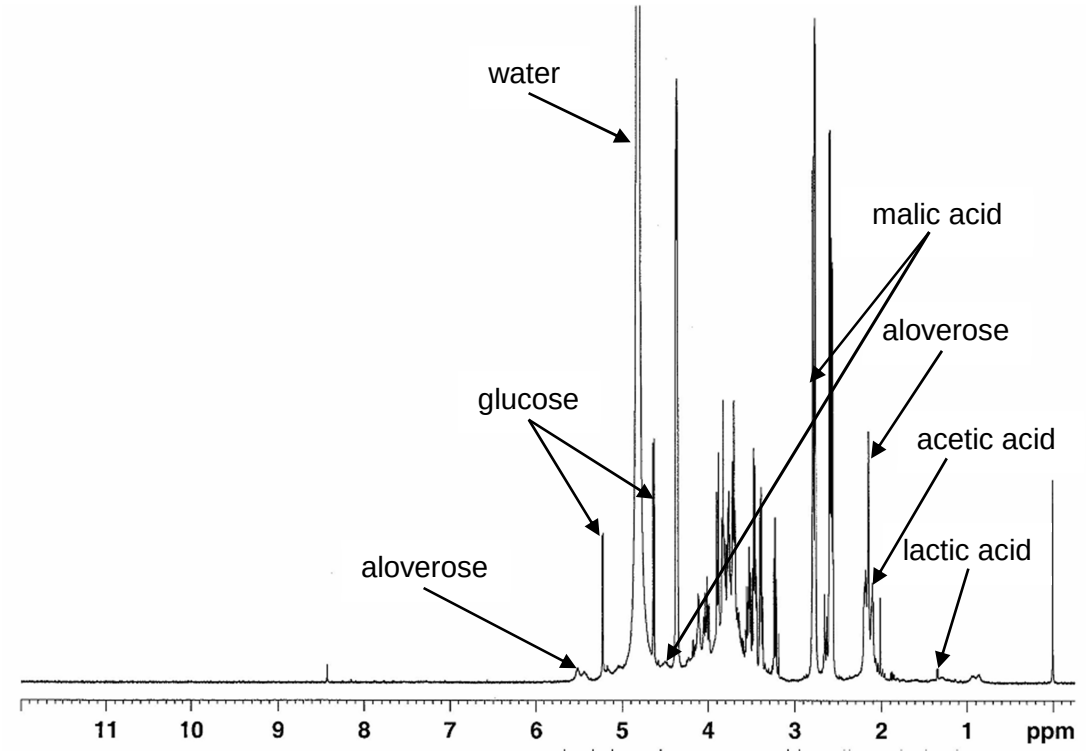
After lyophilisation of the precipitated ethanol insoluble gel materials, the average percentage yield for *A. vera* was 13.81% and for *A. marlothii,* 4.41% of the total pulp material. The percentage yield for the *A. ferox* 200:1 gel powder was not determined as the polysaccharidic fraction was donated by Organic Aloe (Albertina, South Africa).

A.3.2 Nuclear magnetic resonance fingerprinting

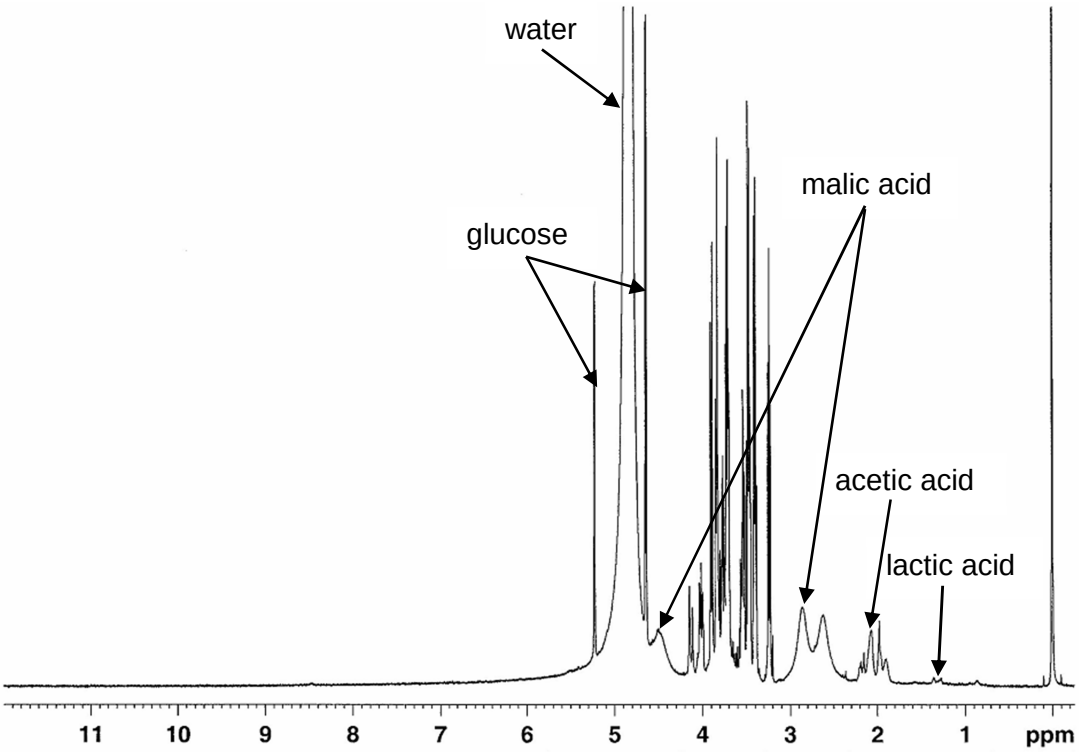
A.3.2.1 Aloe gel materials

The ^{1}H-NMR spectra of *A. vera*, *A. marlothii* and *A. ferox* gel materials are given in Spectrums A.1, A.2 and A.3, respectively.

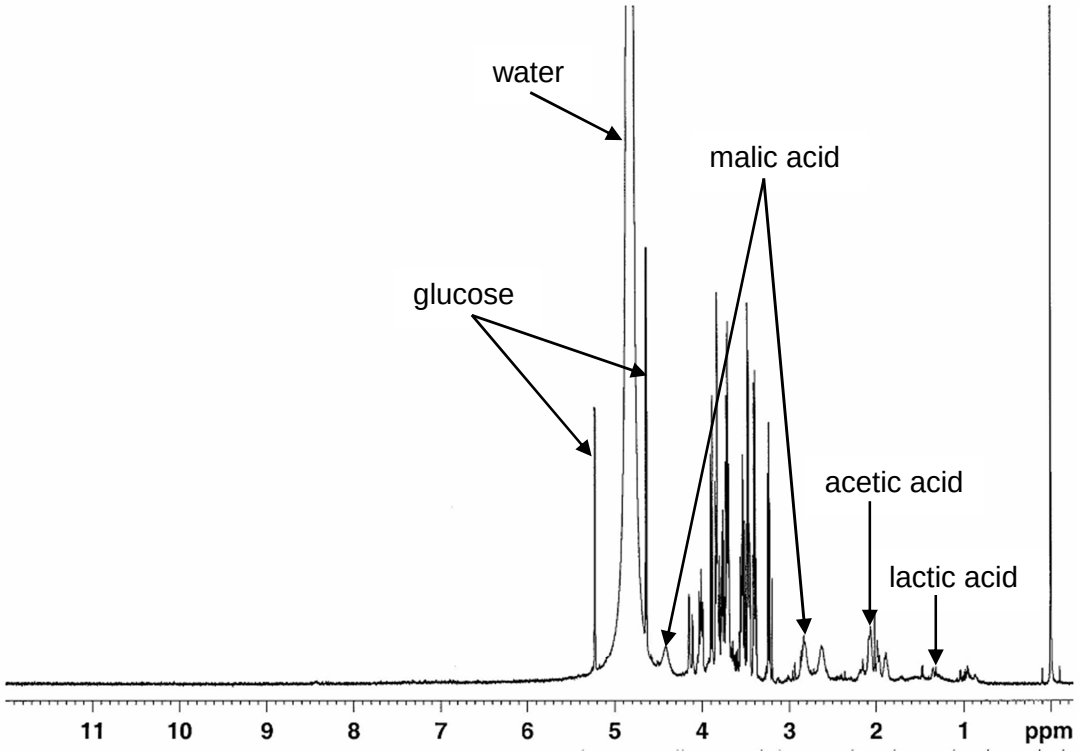
The three marker molecules for identifying *A. vera* gel material include aloverose (or partly acetylated polymannose or acemannan), glucose and malic acid (Jambwa *et al.*, 2011:435), which were all detected by ^{1}H-NMR spectroscopy in the *A. vera* gel material. Aloverose was not detected in the *A. marlothii* (Spectrum A.2) or *A. ferox* (Spectrum A.3) precipitated gel materials, although glucose and malic acid were present as found previously (Beneke *et al.,* 2012).



Spectrum A.1: ^{1}H-NMR spectra of *A. vera* gel materials



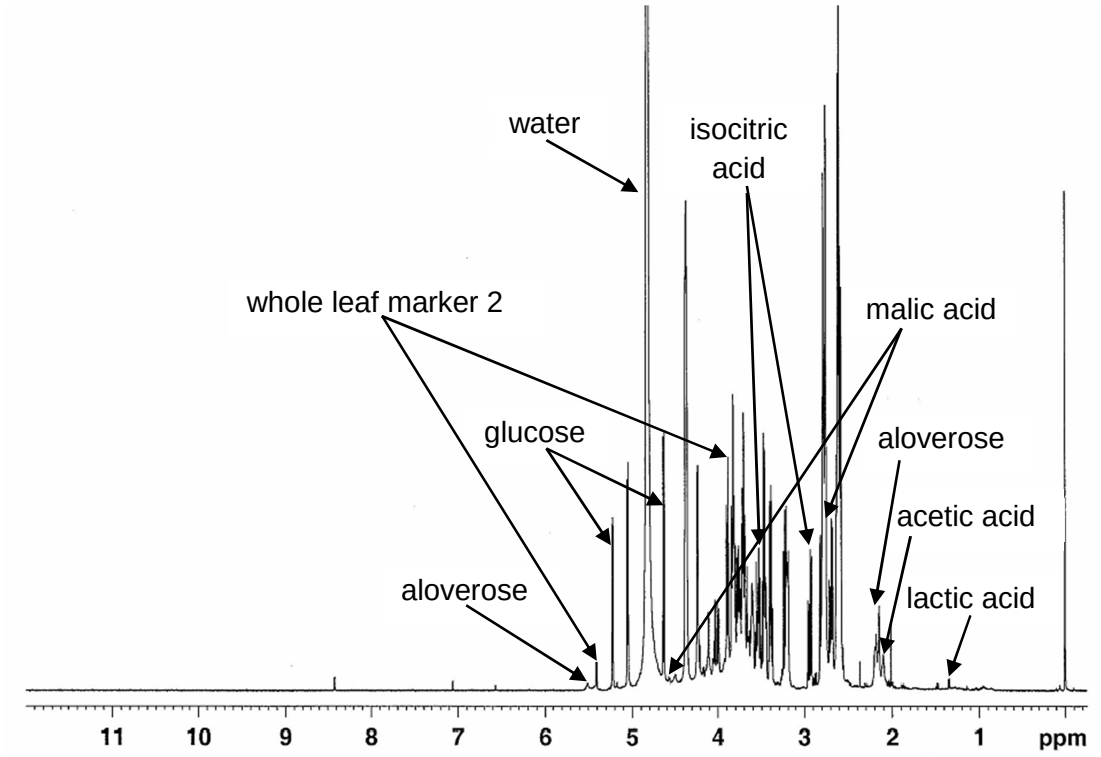
Spectrum A.2: ^{1}H-NMR spectra of *A. marlothii* gel materials



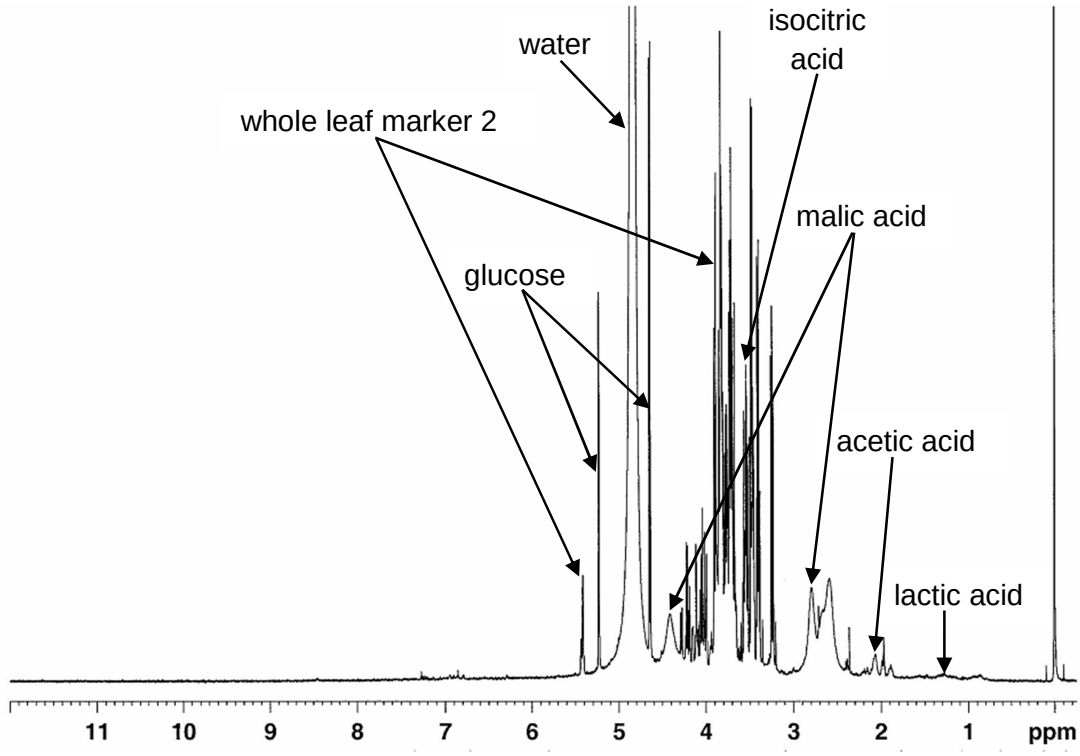
Spectrum A.3: ^{1}H-NMR spectra of *A. ferox* gel materials

A.3.2.2 Aloe whole leaf materials

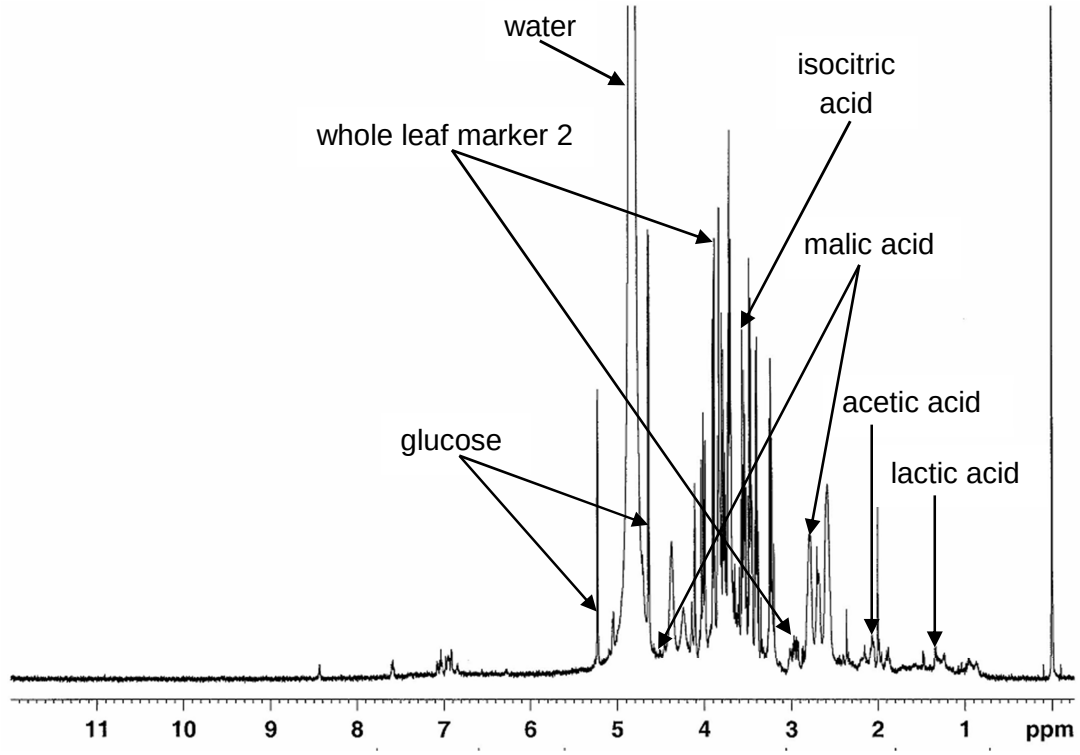
Spectrum A.4, A.5 and A.6 show the ^{1}H-NMR spectra for *A. vera*, *A. marlothii* and *A. ferox* whole leaf materials, respectively. Aloverose was detected in the whole leaf material of *A. vera* (Spectrum A.4), but not in the whole leaf materials of *A. marlothii* (Spectrum A.5) and *A. ferox* (Spectrum A.6). All the whole leaf materials also contained the other marker compounds, i.e. glucose and malic acid. An additional whole leaf marker (i.e. iso-citric acid) was detected in all the whole leaf materials and is characteristic of fresh aloe whole leaf extract material (Chen *et al.*, 2009:589; Jambwa *et al.*, 2011:436).



Spectrum A.4: ^{1}H-NMR spectra of *A. vera* whole leaf materials



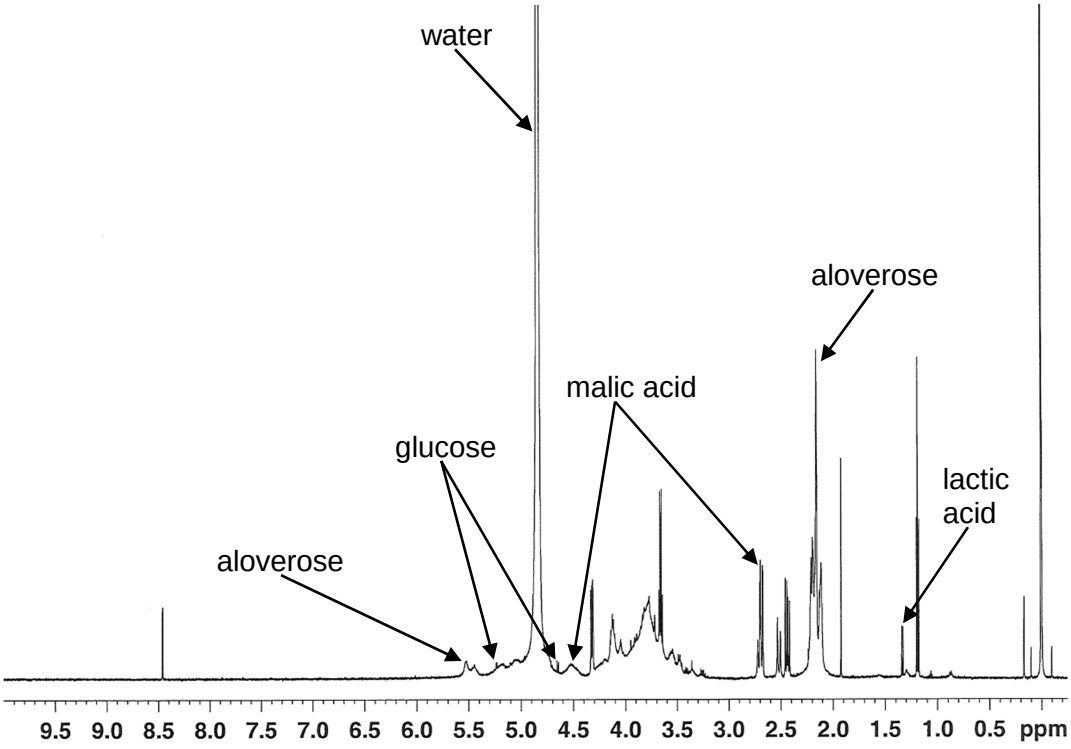
Spectrum A.5: ^{1}H-NMR spectra of *A. marlothii* whole leaf materials



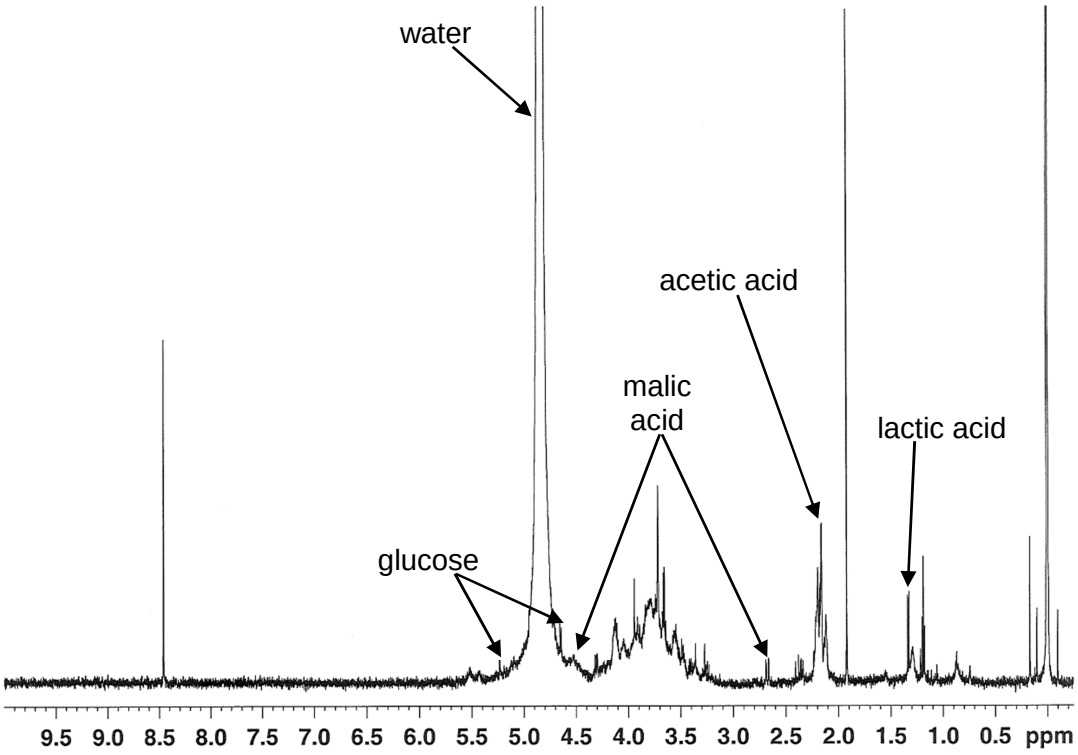
Spectrum A.6: ^{1}H-NMR spectra of *A. ferox* whole leaf materials

A.3.2.3 Polysaccharidic fraction of the aloe gel materials

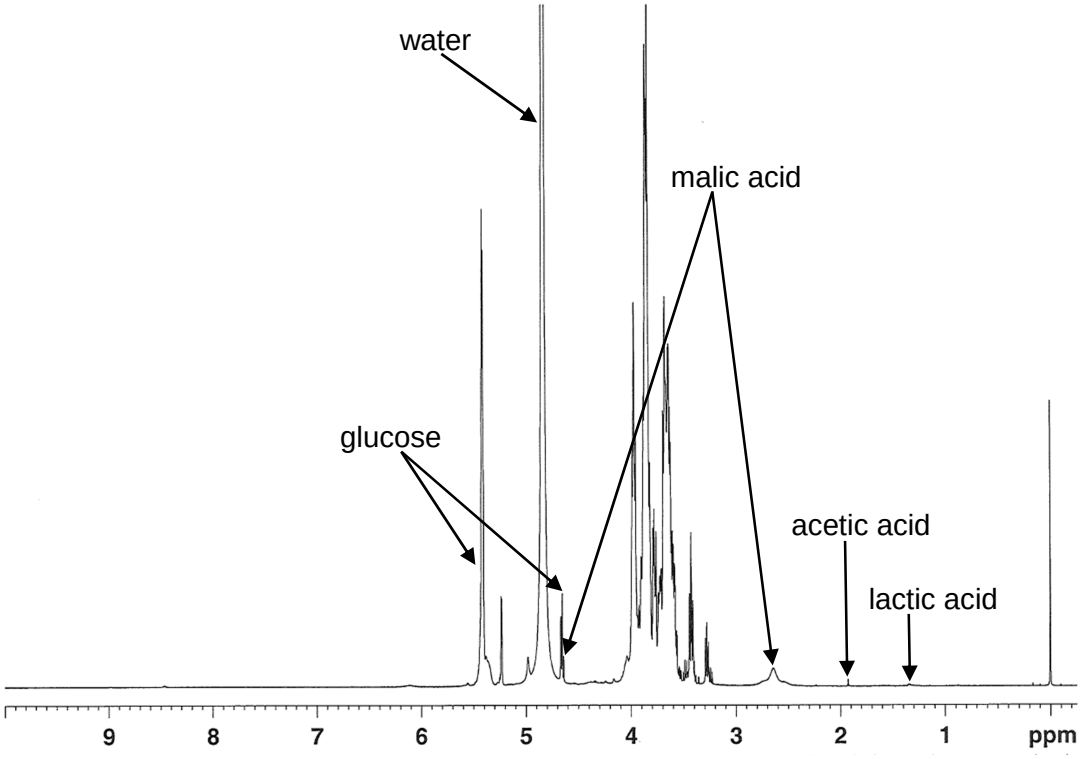
The ^{1}H-NMR spectra for the ethanol insoluble residues (or precipitated polysaccharidic fraction) of *A. vera*, *A. marlothii* and *A. ferox* gel material are given in Spectrums A.7, A.8 and A.9, respectively. Aloverose (or partly acetylated polymannose or acemannan), glucose and malic acid were detected by ^{1}H-NMR spectroscopy in the *A. vera* precipitated gel material. Glucose and malic acid were present in the *A. marlothii* (Spectrum A.8) and *A. ferox* (Spectrum A.9) precipitated gel materials, but aloverose was not detected as found previously (Beneke *et al.,* 2012).



Spectrum A.7: ^{1}H-NMR spectra of *A. vera* ethanol insoluble residues or precipitated polysaccharides



Spectrum A.8: ^{1}H-NMR spectra of *A. marlothii* ethanol insoluble residues or precipitated polysaccharides



Spectrum A.9: ^{1}H-NMR spectra of *A. ferox* ethanol insoluble residues or precipitated polysaccharides

A.4 Conclusion

This appendix describes the harvesting, processing and chemical fingerprinting procedures of the aloe leaf materials investigated during this study. As previously mentioned, it is of utmost importance to harvest and process the aloe leaves in such a way as to ensure that the essential bioactive components are preserved. From the ^{1}H-NMR spectra it is evident that all the aloe leaf materials contained the marker compounds necessary to identify them positively as aloe leaf materials.

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Appendix B

Hydration and anti-erythema effects of Aloe vera, Aloe ferox and Aloe marlothii gel materials after single and multiple applications

B.1 Introduction

The SC, also known as the horny layer, is the outermost layer of the skin (Rieger, 2002:121) and serves as a barrier to prevent the loss of internal body components (predominantly water) to the external environment (Roberts & Walters, 1998:5). A well-hydrated SC is vital for preventing scaly, rough, dry skin (Brewster, 2006:377) and to resist the penetration of irritants and allergens (Cork & Danby, 2009:4). The corneocytes, in a normal healthy skin barrier, contain water which causes them to swell and form a smooth skin barrier with no openings between the corneocytes. They also contain high levels of natural moisturising factor (NMF) which is necessary to attract water (Cork & Danby, 2009:4).

Xerosis (dry skin) is widespread, particularly in people past middle age and is commonly caused by frequent bathing with detergent containing soap and cold weather. Dry skin may rub off in small scales or flakes and can become irritated and itchy, therefore keeping the skin moist with moisturising ointments or creams is fundamental in treating dry skin conditions (McCoy, 2006:1). As far back as ancient times, people made use of topical products on the skin (Roberts & Walters, 1998:1), such as moisturisers to enhance the skin's ability to absorb moisture and also act as a barrier against moisture loss (Bazin & Fanchon, 2006:453). The hydration balance and the retention of water in the superficial skin layers ensures the skin's elasticity and flexibility (Darlenski & Fluhr, 2011:124-5) as dehydration of the skin causes a decrease in skin elasticity (Bazin & Fanchon, 2006:453).

Many commercial products such as cosmetics, lotions, sun screens, shampoos, etc. contain whole leaf extracts and pulp (or gel) of *A. vera* due to its soothing, astringent and healing properties (Morton, 1961:311; Choi *et al.*, 2001:535). Numerous studies on *A. vera* show its effectiveness in hydrating the skin for example, Dal'Belo *et al.* (2006:245) found that freeze-dried *A. vera* extract improved skin moisture by significantly increasing the water content of the SC although it did not alter the TEWL. This indicates that *A. vera* moisturises the skin by a humectant mechanism (Dal'Belo *et al.*, 2006:245). In a different study, gloves treated with *A. vera* gel were found to reduce the appearance of fine wrinkling and erythema, whilst also improving skin integrity (West & Zhu, 2003:42).

Aloe vera gel has shown potential anti-inflammatory activity and the results of Vázquez *et al.* (1996:74) suggest it has inhibitory action on the arachidonic acid pathway via cyclooxygenase. The long-term use of topical corticosteroids to treat chronic inflammatory skin conditions is associated with side-effects such as dyspigmentation, telangiesctasia and skin atrophy, hence mild substances such as aloe leaf materials, without these side-effects, are more desirable to treat inflammatory diseases (Reuter *et al.*, 2008:109). Reuter *et al.* (2008:107) tested the anti-inflammatory potential of a concentrated *A. vera* gel (97.5%) *in vivo* and found that *A. vera* gel did not show any anti-inflammatory effect after 24 h although, significant effect could be detected after 48 h. Onset of the effect was therefore delayed, but was stronger than that of the 1% hydrocortisone in placebo gel, although weaker compared to the commercially available corticosteroids. The *A. vera* gel was well tolerated with no side effects, although no conclusions could be made with regard to the sensitisation potential of *A. vera* gel, due to the rarity of an allergy after a single application (Reuter *et al.*, 2008:109).

Quantification of water in SC is a useful measurement which gives valuable information pertaining to the biophysical properties and the barrier function of the skin (Bazin & Fanchon, 2006:453). In this appendix, the *in vivo* moisturising and anti-erythema effects of *A. vera*, *A. ferox* and *A. marlothii* gel materials in human subjects are reported. The moisturising efficacy of the aloe materials was investigated after single (short-term study) and multiple applications (longer-term study); whereas the anti-erythema efficacy of the aloe materials was investigated on sodium lauryl sulphate (SLS) irritated skin over a period of seven days.

The instruments used during this study are considered to be non-invasive and accurate, causing no harm or discomfort during the *in vivo* investigation of the skin parameters (Darlenski *et al.*, 2009:296). The following instruments, with their corresponding parameters, were used to investigate the skin hydration properties of the aloe leaf material during the short and long term study:

- Skin hydration as measured by the Corneometer® CM 825 (Courage-Khazaka Electronic GmbH, Cologne, Germany).
- NRJ, ENT and HOM parameters as measured by the Visioscan® VC 98 (Courage-Khazaka Electronic GmbH, Cologne, Germany).
- R2-, R6-, R7- and R8-parameters as measured by the Cutometer® dual MPA 580 (Courage-Khazaka Electronic GmbH, Cologne, Germany).

The following instruments were used to investigate the anti-erythema activity of the aloes:

- TEWL as measured by the VapoMeter® (Delfin Technologies Ltd., Finland).
- Skin pH as measured by the Skin-pH-Meter® PH 905 (Courage-Khazaka Electronic GmbH, Cologne, Germany).
- Skin erythema (haemoglobin) as measured by the Mexameter® MX 18 (Courage-Khazaka Electronic GmbH, Cologne, Germany).

B.2 Materials and methods

B.2.1 Materials

The starting material for *A. vera* was dehydrated gel powder (Daltonmax 700®) obtained from Improve USA, Inc. (Texas, United States of America). The starting material for the *A. marlothii* was harvested sustainably from natural populations near Koster, in the North-West Province of South Africa. The *A. ferox* 200:1 gel powder containing methanol insoluble residues or polysaccharidic fraction (Campestrini *et al.*, 2013:512) was donated by Organic Aloe (Albertina, South Africa) produced from *A. ferox* leaves collected from natural populations in the Western-Cape Province (South Africa). The method for the preparation of the leaves, the precipitation of the organic solvent insoluble residues (or polysaccharidic fractions) (Campestrini *et al.*, 2013:512) and the proton nuclear magnetic resonance (^{1}H-NMR) fingerprinting spectra of the aloes are incorporated in Appendix A.

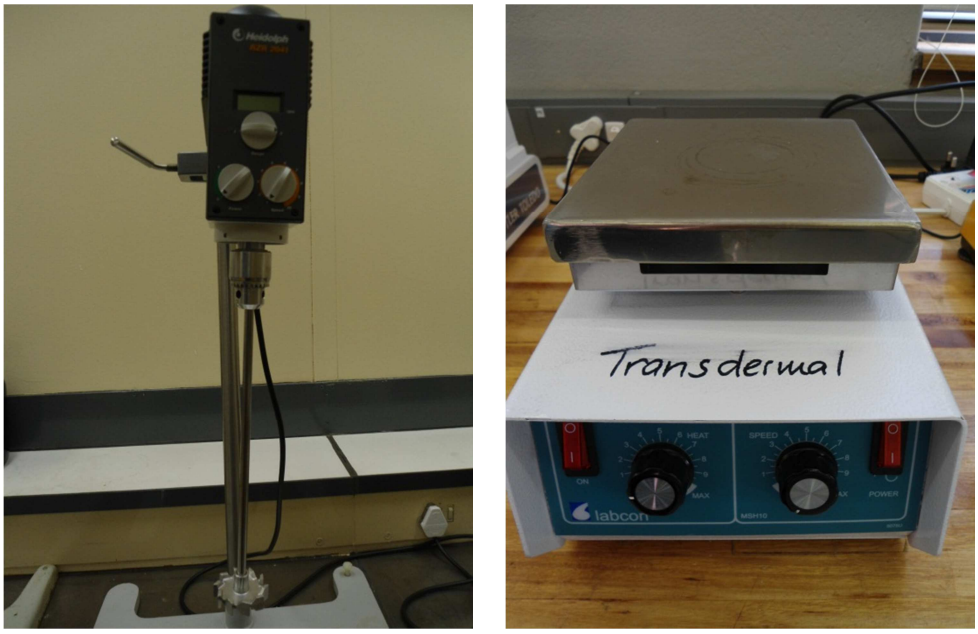
B.2.2 Aloe and hydrocortisone gel preparations for application to the skin

To obtain a 3% (w/v) solution with a gel structure, each of the selected aloe gel materials (i.e. *A. vera, A. ferox and A. marlothii*) were dissolved in ultrapure deionised water. A 1% (w/v) hydrocortisone gel was used as the positive control during the erythema study and its composition is given in Table B.1.

Table B.1: Hydrocortisone gel formulation (positive control group)

Components	Concentration
Hydrocortisone acetate	1% (w/v)
Ethanol (96% (v/v))	15% (v/v)
Polyethylene glycol	15% (w/v)
Carbopol Ultrez 20	1% (w/v)
Distilled water	Up to 100% of preparation
Tri-ethanol amine	Enough to adjust pH to approximately 6.8

Carbopol Ultrez 20 was homogenised together with distilled water at approximately 536 rpm with a Heidolph® Diax 600 homogeniser (Heidolph, Germany; Figure B.1a) for approximately 30 min. A Labcon® (South Africa) hotplate with stirrer (Figure B.1b) was used to melt the polyethylene glycol after which hydrocortisone acetate was slowly added together with ethanol. This mixture was slowly added to the Carbopol and distilled water mixture whilst homogenising. Subsequently the pH was adjusted to approximately 6.8 with tri-ethanol amine.



(a) (b)

Figure B.1: Apparatus used during preparation of hydrocortisone gel included a (a) Heidolph® Diax 600 homogeniser (Heidolph, Germany) and (b) Labcon® hotplate and magnetic stirrer

The gels provided as treatment groups were code named and neither the subjects, nor the technical assistant, knew the content of these groups and therefore a double-blinded study was conducted.

B.2.3 Non-invasive skin measurements

The different instruments used during this study for the skin measurements will be discussed in this section. The Corneometer®, Mexameter® MX 18 and Skin-pH-Meter® probes of Courage-Khazaka Electronic GmbH (Courage-Khazaka, Cologne, Germany) was connected to a computer with Multi Probe Adapter (MPA) software. The Visioscan® and Cutometer® (Courage-Khazaka, Cologne, Germany) probes were used with VisioscanFW and CutometerQ software systems, respectively. The VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland) was operated with DelfWin 4 software system.

B.2.3.1 Skin hydration

The Corneometer® (Courage-Khazaka Electronic GmbH, Cologne, Germany) (Figure B.2) was utilised during the short and longer term study. It operates at a low frequency (40-75 Hz) and measures the moisture content of the skin by a capacitance method. This method is based on water's much higher di-electrical constant (81) in the skin when compared to other substances (mostly < 7) (Berardesca, 1997:128; Holm *et al.*, 2006:774; Courage & Khazaka, 2010:5; Darlenski & Fluhr, 2011:128).

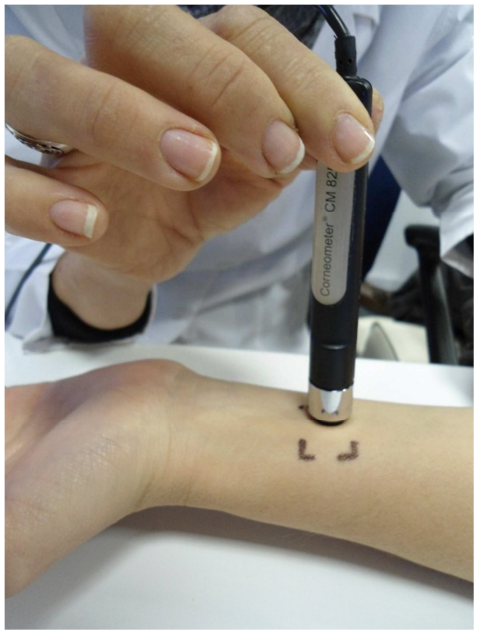


Figure B.2: The Corneometer® CM 825 (Courage-Khazaka Electronic GmbH, Cologne, Germany)

The probe head contains metallic tracks, which are separated from the skin by a glass lamina to prohibit current conduction in the sample. An electric field, with alternating attraction, develops between the tracks with one track obtaining a minus charge (surplus electrons) and the other a plus charge (lack of electrons). The scatterfield penetrates the first layer of the skin then the capacitance can be determined (Courage & Khazaka, 2010:5). An increase in water content/skin hydration will cause capacitance values to increase. The mean of three measurements are displayed in arbitrary units ranging from 0-130 (Berardesca, 1997:128, Holm *et al.*, 2006:774; Courage & Khazaka, 2010:5; Darlenski & Fluhr, 2011:128).

The Corneometer® only measures the moisture of the upper layers of the epidermis to an approximate depth of 10 µm, due to the very small penetration depth of the electrical scatterfield (Bazin & Fanchon, 2006:455; Courage & Khazaka, 2010:5). The short measuring time prevents occlusion effects that can ultimately influence the results (Courage & Khazaka, 2010:5). In order for the aloe to have a hydrating effect on the skin, the value should increase.

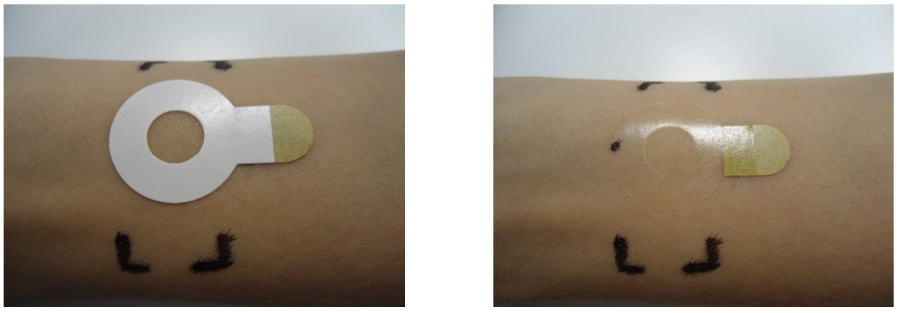
During the present study the probe head was placed vertically on the skin. Measurement started when the probe was in contact with the skin and a beep sound signified the measurement was carried out successfully. The average of three measurements was obtained.

B.2.3.2 Skin topography

The skin topography was analysed with the Visioscan® (Courage-Khazaka Electronic GmbH, Cologne, Germany) (Figure B.3) by taking an image (6 x 8 mm) of the skin by a built in CCD-camera during the short and longer term study.

Figure B.3: The Visioscan® VC 98 (Courage-Khazaka Electronic GmbH, Cologne, Germany)

In order to ensure the apparatus was ideally positioned, double-sided sticking rings (Figures B.4a and B.4b) were placed on the marked skin areas and their cover foils were removed. The camera's measuring head was removed and placed on the ring, where after the camera was placed back on the measuring head (Courage & Khazaka, 2009:24).



(a) (b)

Figure B.4: The double sided sticking rings before (a) and after (b) removal of the cover foil

The Visioscan® is connected to a computer by means of an image digitalisation unit which configures the image in 256 grey levels pixel by pixel, where black is resembled by 0 and white by 255 (Courage & Khazaka, 2009:11). Texture parameters are used to analyse differences in colours of neighboured pixels and those in this study were as follows:

- NRJ: this parameter indicates the homogeneity of an image. When moisturising or anti-aging agents are applied it is expected for this value to increase (Courage & Khazaka, 2009:37).
- ENT: this parameter indicates the "mess" of an image and the value is higher for highly hydrated skin than for a very dry one (Courage & Khazaka, 2009:37).
- HOM: this parameter indicates the uniformity of an image and the value is higher for highly hydrated skin than for a very dry one (Courage & Khazaka, 2009:38).

An increase in these parameter values indicates an increase in skin hydration (Courage & Khazaka, 2009:37-38).

B.2.3.3 Skin elasticity

The Cutometer® (Courage-Khazaka Electronic GmbH, Cologne, Germany) (Figure B.5) was used to assess the skin's viscoelastic properties (which indirectly relate to skin hydration) in the longer term study (Darlenski & Fluhr, 2011:130). Skin viscoelasticity is the ability of the skin to return to its original position, after a certain delay, once a force is removed (Courage & Khazaka, 2012a:5).

Figure B.5: The Cutometer® dual MPA 580 (Courage-Khazaka Electronic GmbH, Cologne, Germany)

The Cutometer® measures the elasticity of the upper skin layer through a suction method, whereby the skin is drawn into the aperture of the probe due to a negative pressure created in the probe and released again after a specific time. A non-contact optical measuring system determines the penetration depth and comprises a light source and a light receptor along with two prisms opposite each other, which project the light from transmitter to receptor. Due to the penetration depth of the skin, the light intensity differs. Throughout the measurements, the skin's resistance to the negative pressure (firmness) and its potential to return to its original position (elasticity) is shown as curves (penetration depth in mm/time). A typical skin deformation curve can be seen in Figure B.6. From these curves, certain measurement parameters can be calculated, of which the R-parameters were used during this study (Courage & Khazaka, 2012a:7, 25).

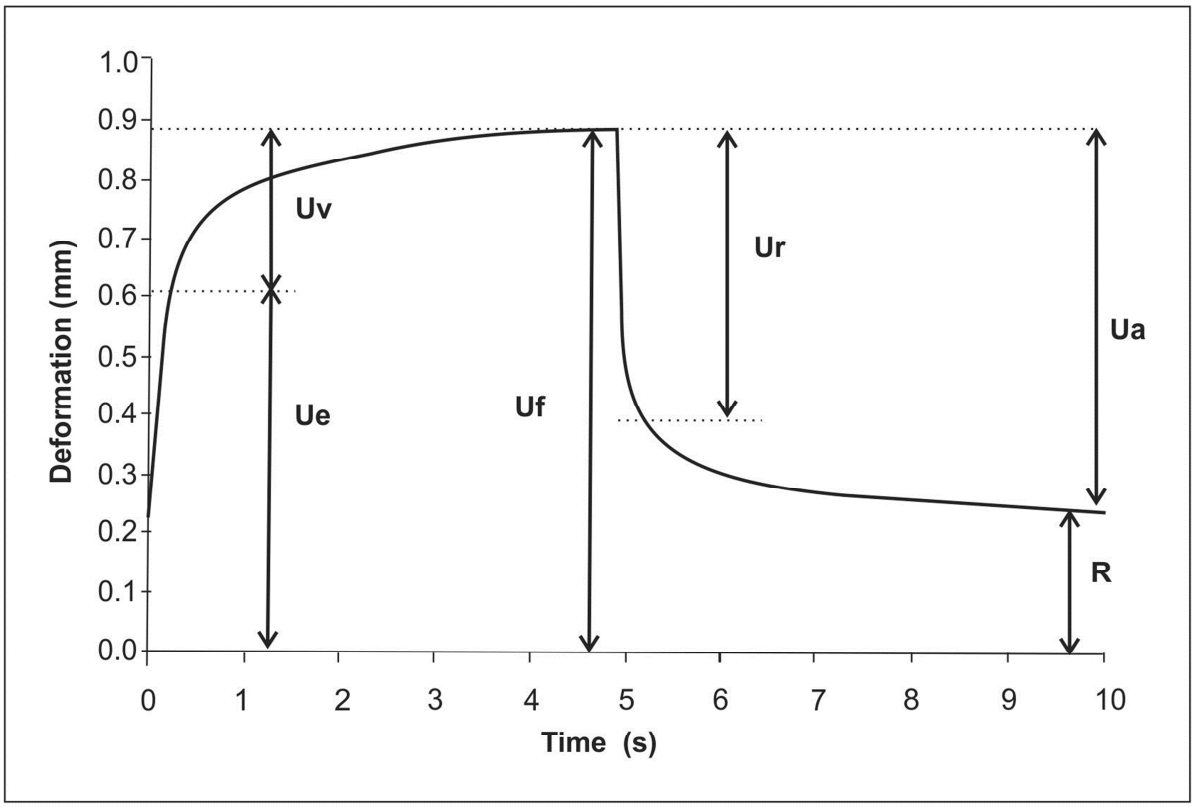


Figure B.6: A typical skin deformation curve obtained with the Cutometer® (Dobrev, 2000:240; Courage & Khazaka, 2012a:22).

The curve consists of two phases, suction and relaxation phase, both of which consist of two parts. During the first part of the suction phase, the skin enters the probe straight and instantaneously. The immediate elastic deformation/distension of the skin is shown as Ue on the curve. Uv represents the second part (the viscoelastic suction part), when the skin "creeps" into the probe (delayed distension). Uf represents the maximum penetration after suction time (skin distensibility/final distension). The complete relaxation (Ua) can be divided into two parts: the immediate elastic return/retraction (Ur) and the flat, viscoelastic part (Ua-Ur). The overall

capability of the skin to return to its original shape is shown by Uf – Ua. R (resilient distension) is the residual deformation at the end of the measuring cycle (Courage & Khazaka, 2012a:22; Dobrev, 2000:240).

Mode 1 was used for the duration of this study, meaning measurements were taken with a constant negative pressure. The skin was sucked into a probe with a 2 mm diameter aperture and an applied pressure of 350 mBar. The measurement consisted of two cycles with 5 s suction followed by 5 s of relaxation. The following parameters were investigated in this study:

- **R0 = Uf** - this parameter is the highest point/maximum amplitude of the first curve and indicates the firmness of the skin or the passive behaviour of the skin to an applied force and is given as distance in mm (Courage & Khazaka, 2012a:26).
- **R2 = Ua/Uf** - this indicates the ratio between the maximum amplitude and the ability of the skin to return to its original position (gross-elasticity). This is a very important parameter and the closer the value is to 1 (100%) the more elastic the curve is (positive percentage change as a function of treatment) (Dobrev 2000:240; Courage & Khazaka, 2012a:26).
- **R6 = Uv/Ue** - is the ratio of the viscoelastic to the elastic distension and results are obtained in percentages and the smaller this value is, the higher the elasticity (negative percentage change as a function of treatment) (Dobrev, 2000:240; Courage & Khazaka, 2012a:27).
- **R7 = Ur/Uf** - represents the ratio of the elastic recovery (immediate retraction) to the total distension with results obtained as percentages and the closer the value is to 1 (100%), the more elastic the skin is (positive percentage change as a function of treatment) (Dobrev, 2000:240; Courage & Khazaka, 2012a:27).
- **R8 = Ua** - this is the area under the suction part of the deformation curve. When Ua is closer to R0, it indicates the greater ability of the skin to return into its original state (positive change as a function of treatment). Results are obtained in distance (mm) (Dobrev 2000:240; Courage & Khazaka, 2012a:27).

In the course of the present study the probe was applied and held steadily at a right angle whilst measurements were taken. Care was taken not to press the probe too tightly onto the skin as this may cause disturbed blood circulation (influencing the measurements) or cause the skin to be pressed into the probe and touch or grease the glass prisms (Courage & Khazaka, 2012a:16).

B.2.3.4 Haemoglobin content of skin

The Mexameter® MX 18 (Courage-Khazaka Electronic GmbH, Cologne, Germany) (Figure B.7), which measures the content of melanin and haemoglobin (erythema) in the skin, the two components primarily responsible for skin colour, was used during the erythema study. Measurements are based on the absorption principle where the probe of the Mexameter® emits light of three defined wavelengths and the light reflected by the skin is measured by a receiver. The placement of the emitter and receiver ensures that only diffused and scattered light is measured. The quantity of the light absorbed by the skin can be determined as the quantity of emitted light defined (Courage & Khazaka, 2012b:1).

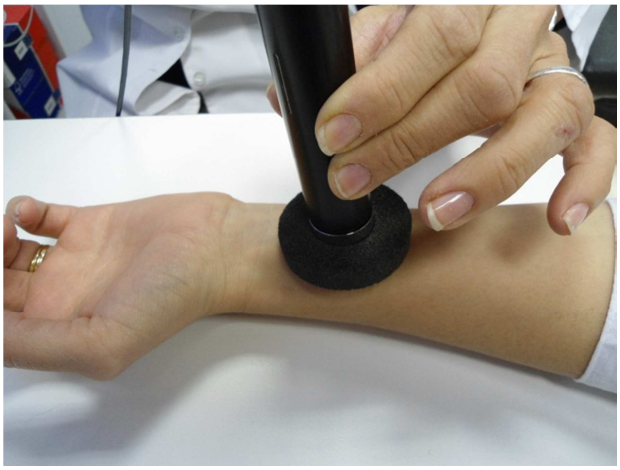


Figure B.7: The Mexameter® MX 18 (Courage-Khazaka Electronic GmbH, Cologne, Germany)

When erythema is measured, two different wavelengths are utilised to measure the absorption capacity of the skin. One of the wavelengths was chosen to avoid other colour influences (e.g. bilirubin) whilst the other corresponded to the spectral absorption peak of haemoglobin. Results are shown as indices on a scale from 0-999, which guarantees that even the smallest changes in colour are detected (Courage & Khazaka, 2012b:1).

During this study the lights in the laboratory was dimmed so as not to influence the results. The probe head was placed straight on the skin area to be measured and held still. Measurement was initiated by contact on the skin, after which the results for the erythema and melanin were displayed on the computer screen, accompanied by an acoustical signal. After irritation with SLS, the haemoglobin content values were expected to be higher than the baseline readings to indicate erythema. For the test materials to be effective as anti-erythema agents, the haemoglobin content values should decrease after treatment.

B.2.3.5 Skin pH

The ‘acid mantle’ of the skin appears to play a key role in the anti-microbial defence and formation of the permeability barrier of the skin (Fluhr *et al.*, 2001:44; Schmid-Wendtner & Korting, 2006:296). Overall pH can be described as the negative logarithm (base ten) of the concentration free hydrogen ions in aqueous solution. The buffering capacity of the skin and skin pH can be ascribed to secretions from the sweat and sebaceous glands and all the constituents of the SC (Schmid-Wendtner & Korting, 2006:297).

Measurements with the Skin-pH-Meter® (Courage-Khazaka Electronic GmbH, Cologne, Germany) (Figure B.8) are performed with a glass electrode that is filled with an inner buffer and separated from the solution to be measured by a special glass membrane which removes the potential of the internal side of the glass membrane. The potential of the external side of the glass membrane in contact with the measuring solution is carried away by a reference electrode, which is filled with electrolyte and fitted with a diaphragm to allow the ions to be transported between the inner buffer and the measuring solution whilst preventing these two substances from mixing (Courage & Khazaka, 2012c:1).

Figure B.8: The Skin-pH-Meter® (Courage-Khazaka Electronic GmbH, Cologne, Germany)

The Skin-pH-Meter® measures pH directly on the skin as the excretions of the skin are almost an aqueous solution (Courage & Khazaka, 2012c:1). In the present study, the probe was washed with distilled water. A moist probe is vital when taking measurements, although excess water should be shaken off as this may influence the results (Courage & Khazaka, 2012c:4). The Skin-pH-Meter® was used during the erythema study and after irritation with SLS, which causes disruption of the skin barrier, the pH values should initially increase with regards to the baseline readings (Voegeli, 2008:87).

B.2.3.6 Vapour loss

During the erythema study, the VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland) (Figure B.9) was used to measure TEWL values. The VapoMeter® forms a closed chamber after touching the skin and measures the relative humidity inside the capsule with an electronic hygro sensor (Honeywell humidity sensor HIH 3605-B). Water vapour from the skin surface is collected in the chamber and with time causes the humidity to rise. From the linearly rising part of the curve, the flux density is calculated (Roelandt *et al.*, 2011:257). The numerical values obtained for the TEWL are generally given as g/m^2.h (Darlenski *et al.*, 2009:297). It was found that TEWL measurement is the most appropriate non-invasive method to observe the impaired barrier function of the skin after irritation with SLS (Fluhr *et al.,* 2001:700). TEWL measurements, after the skin has been damaged by SLS, showed a wide inter-individual range of variation (Berardesca, 2011:95) with values increasing after SLS application. In order for the aloe materials to be effective as an anti-inflammatory, these values should decrease after treatment.

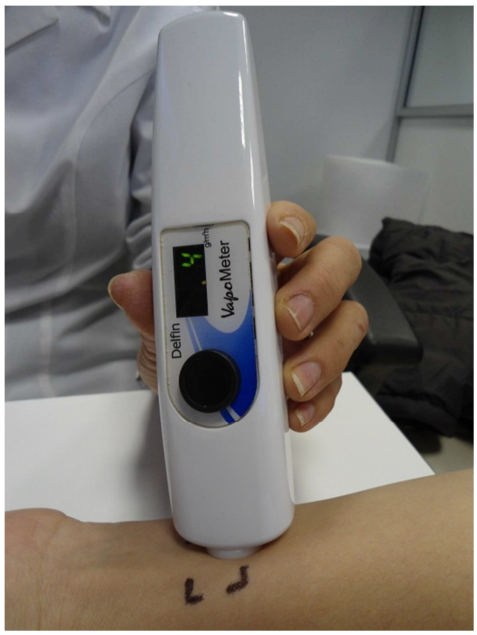


Figure B.9: The VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland)

B.2.4 Subject selection and ethical considerations

This study was carried out according to the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of the North-West University, South Africa under the title of "(*In vivo)* Cosmetic efficacy studies" (NWU-0097-10-A5).

Age, sex and race are thought to be key variables that can affect skin function and biophysical measurements. As a consequence these variables should be standardised or controlled. It has

been suggested that studies should be designed within the same age range, ethnic group and sex (Berardesca, 2011:93). Therefore, volunteers were selected by strict inclusion/exclusion criteria in this study. Female volunteers, in a good state of health, between 20 and 40 years of age and Fitzpatrick skin types II and III (based on Mexameter® readings on untreated skin) were included in the study. Exclusion criteria were as follows: psoriasis within six months prior to study, history of eczema, allergic skin reaction 30 days prior to the study, recent treatment with aloe containing products, having undergone cosmetic surgery within previous 12 months, pregnant or lactating woman, uncontrolled systemic disease, dermatological illnesses or conditions that may interfere with neuromuscular function such as myasthenia gravis, recent history of intolerance to drugs and/or cosmetic products and treatment with topical or systemic drugs that may influence the test results.

The study population included a total number of 59 subjects with 19, 23 and 17 volunteers that participated in the short term, longer term and erythema studies, respectively. Informed consent was obtained and the volunteers were informed about the aims, methods, risks and anticipated benefits of participation prior to the onset of the study.

B.2.5 Treatment protocol

Seven days prior to the onset of the study, the volunteers underwent a washout phase. Throughout this time and for the remainder of the study, the volunteers were only permitted to wash with Dove® soap; the use of other skin products, body powders, moisturisers and perfume on or near the test areas were not allowed for the duration of the study. The use of alcohol, caffeine and a number of vasoactive medications were prohibited on the day of the measurements as they alter skin microcirculation, which can indirectly influence the skin hydration profile (Darlenski & Fluhr, 2011:136).

The volar forearm, due to the relatively large available skin surface area, its hairlessness and the fact that it contains only a small number of sebaceous glands, was selected as the anatomical test site (Bazin & Fanchon, 2006:453, 458). The wrist and cubital fossa (anatomic occlusion zone) were avoided (Darlenski & Fluhr, 2011:135).

The hydration level of the SC between individuals differs, therefore the baseline hydration levels, before the topical application of the aloe gels, were measured to serve as an internal control (Darlenski *et al.*, 2009:299). Each volunteer served as her own control, which was achieved by measuring an untreated test field at each time point (Darlenski & Fluhr, 2011:131). A glove-covered finger was used to apply the test materials to ensure that interference with sebum and sweat secretion was prevented (Darlenski & Fluhr, 2011:137).

B.2.5.1 Single- (short-) and multiple applications (longer-term) hydration study

The guidelines for the assessment of SC hydration by The European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO) were followed throughout this study (Berardesca*,* 1997:130). The volar forearm skin of the dominant arm (short term study) and non-dominant arm (longer term study) was divided into five sites of 6 cm^2 each, bordered with a cosmetic pencil. To prevent any cross-contamination, space was left open between the sites. The first three sites on the forearm were treated with 0.5 ml of aloe leaf gel material (i.e. 3% (w/v) *A. ferox*, *A. marlothii* and *A. vera* gel solution), the fourth site was treated with the placebo, deionised ultrapure water and the fifth was the control which was left as 'untreated skin'.

In order to investigate the short-term hydration effects of the aloe gel materials, a single application study was carried out before the longer term, multiple application study started (Berardesca*,* 1997:130, Li *et al.*, 2001:32). A baseline reading (T_0) was taken followed by measurements at 30 (T_1), 90 (T_2) and 150 (T_3) min after application of the test materials (Berardesca*,* 1997:130).

In the course of the long term study, the aloe gel solutions were each applied twice daily (i.e. in the morning and in the evening). A baseline reading (T_0) was performed, followed by measurements after 1 (T_1), 2 (T_2), 3 (T_3) and 4 (T_4) weeks following the start of treatment. Measurements were taken 12 to 20 h following application, in the evening preceding the day of measurements.

The following instruments were used to measure the hydration effect of the test materials on the skin during the short and longer term studies: a Corneometer® CM 825 and Visioscan® VC 98 (Courage-Khazaka Electronic GmbH, Germany). The Cutometer® dual MPA 580 (Courage-Khazaka Electronic GmbH, Germany) was utilised during the longer-term study.

B.2.5.2 Erythema study

The guidelines on SLS exposure tests from the Standardization Group of the European Society of Contact Dermatitis (Tupker *et al.,* 1997) were followed during this study. Prior to the application of the Finn Chambers® (Figure B.10) (with an internal diameter of 8 mm containing filter papers) on Scanpor® (SmartPractice®, Mednom, Cape Town, South Africa) to the volar forearm, the baseline readings (T_0) were taken. A 1% (w/v) solution of SLS (99% purity, Merck, South Africa) in distilled water was prepared.

(a) (b)

Figure B.10: Finn-Chambers® (b) on Scanpor® (a) (SmartPractice®, Mednom, Cape Town, South Africa)

The negative control (untreated skin) was the application of one Finn Chamber® containing no solution. The remainder of the Finn Chambers® were filled with 20 µl of the 1% (w/v) SLS solution and in order to induce erythema all chambers were applied on the volar forearm skin of the dominant arm under occlusion for a period of approximately 22.5 h.

A certain time period is required after the skin has been irritated with SLS before the first measurement can be performed. Related studies where SLS was applied under occlusion for 24 h revealed an initial exsiccation of the SC, followed by hyperhydration (swelling of corneocytes). Hence, the first measurement (T_1) was performed 24 h after the Finn Chambers® were removed (Aramaki *et al.*, 2001; Arsic *et al.*, 2012:239). The aforementioned prevented false readings due to the occlusive effect of the Finn Chambers® and the initial hyperhydrating effect of SLS (Gloor *et al.*, 2004:147; Darlenski & Fluhr, 2011:133). To ensure that erythema was induced T_1 was compared to T_0. The aloe leaf gel materials (i.e. 3% (w/v) *A. ferox*, *A. marlothii*, and *A. vera* gel solutions) and the positive control, 1% (w/v) hydrocortisone gel, were applied to the volunteers where erythema was induced. From then on the test materials were applied twice daily (morning and evening) for the remainder of the study period. The second measurement (T_2) was made on the second day following one day of treatment and the final measurement (T_3) was on the seventh day following six days of treatment.

The Mexameter® MX 18 and Skin-pH-Meter® (Courage-Khazaka Electronic GmbH, Germany) were used during the erythema study to measure the haemoglobin content and pH of the skin, respectively. The VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland) was used to measure TEWL values.

B.2.6 Environmental conditions

Measurements were performed in the Cosmetic Efficacy Laboratory (CEL) of the North-West University, Potchefstroom Campus, South Africa. The temperature was controlled at 20 to 25 °C with 50 ± 10% relative humidity (Darlenski & Fluhr, 2011:137). To ensure full skin

adaptation, volunteers acclimatised in the CEL for at least 30 min prior to measurement (Courage & Khazaka, 2010:11). Direct air flow and sun light were avoided and measurements were performed at the same time of day to exclude the effect of circadian rhythms (Darlenski & Fluhr, 2011:137).

B.2.7 Data analysis

The effects of the test material are presented as percentage change, as calculated using Equation B.1, relative to the initial conditions (T_0) and to untreated values ($T_{0\ (untr)}$ and $T_{n\ (untr)}$) in terms of all the parameters measured in each part of the study.

$$\% \text{ Change} = \left[\frac{T_n - T_0}{T_0} \times 100\right] - \left[\frac{T_{n\ (untr)} - T_{0\ (untr)}}{T_{0\ (untr)}} \times 100\right] \qquad \textbf{Equation B.1}$$

Where T_n represents the value for n = 30, 90 and 150 min in the short term hydration study; n = 1, 2, 3 and 4 weeks in the long term hydration study.

Equation B.2 was utilised in the erythema study.

$$\% \text{ Change} = \left[\frac{T_n - T_1}{T_1} \times 100\right] - \left[\frac{T_{n\ (untr)} - T_{1\ (untr)}}{T_{1\ (untr)}} \times 100\right] \qquad \textbf{Equation B.2}$$

Where T_n represents the time of measurement after skin irritation and n = 1 at 24 h after removal of Finn chambers®, n = 2 on the second day (i.e. one day after application of test materials) and n = 3 on the seventh day (i.e. six days of application of test materials).

B.2.8 Statistical data analysis

Statistical analyses for the short and longer term studies were carried out by employing IBM SPSS Statistics Version 20 (SPSS Inc., 2011:1). A 2-way repeat measures ANOVA (analysis of variance) design was followed in this study as measurements were repeated over time and every subject was exposed to each of the different treatments. The basic method generally used for this type of design is repeated measures analysis of variance (ANOVA), which assumes independent data (compound symmetry). However, given the dependence structure in the data, this assumption was violated. Consequently, mixed models were used to investigate the influence of treatment and time on the different measures observed. Mixed model analysis allows a variety of variance-covariance structures (Seltman, 2012:357) and in this study, unstructured or first-order autoregressive (AR(1)) covariance structures were used. The two covariance structures were compared using -2 Restricted Log likelihood and Akaike's Information Criterion (AIC) measures. Mixed models employ both fixed and random effects. Fixed effects, such as treatment and time, have levels of primary interest; random effects, such as subjects, are not of primary interest (Seltman, 2012:358). In order to test for significant

differences between the fixed effects, test statistics (F) and probability (p), values were obtained by the Type III Test for Fixed Effects.

Statistical analysis for the erythema study was carried out using Microsoft Excel 2010. The Student *t*-test was performed to test for statistical significant differences between the different treatments and the different times. Statistical significance was tested at a 10% (0.10) level of significance. A p-value < 0.1 indicates statistically significant differences between results that were compared.

B.3 Results

B.3.1 Short-term study

B.3.1.1 Skin hydration and skin topography

The results for the Corneometer® measurements and the Visioscan® parameters (ENT, HOM, NRJ) can be seen in Table B.2, as the mean values with standard deviation. When inspecting the results obtained with the Corneometer® it is clear there was a positive percentage change in skin hydration after a single application of *A. marlothii* and *A. vera* gel materials after 30 (T_1), 90 (T_2) and 150 (T_3) min. Conversely, *A. ferox* gel material exhibited a dehydrating effect on the skin as indicated by the negative percentage change. However, this effect became less after 30 min (T_1). Initially, deionised water dehydrated the skin (30 and 90 min after application), but increased skin hydration at 150 min after application.

Table B.2: Short-term measurements of skin hydration (%change ± SD)

Treatment	Time	Corneometer®	ENT	HOM	NRJ
A. vera	**T1**	4.39 ± 15.29	2.11 ± 3.14	4.9 ± 7.9	22.1 ± 40.0
	T2	0.48 ± 13.64	1.02 ± 3.76	2.3 ± 9.5	13.9 ± 38.7
	T3	4.47 ± 17.35	0.79 ± 4.40	2.0 ± 11.5	12.1 ± 43.1
A. marlothii	**T1**	3.86 ± 14.72	3.35 ± 2.93	6.9 ± 8.2	41.3 ± 38.0
	T2	1.32 ± 13.72	1.16 ± 4.56	3.1 ± 12.1	20.3 ± 46.1
	T3	4.85 ± 16.25	1.59 ± 4.58	4.7 ± 11.8	25.3 ± 46.4
A. ferox	**T1**	-10.77 ± 9.42	0.91 ± 3.24	2.0 ± 9.4	15.6 ± 34.2
	T2	-6.05 ± 10.84	0.52 ± 3.97	0.5 ± 11.2	9.9 ± 38.1
	T3	-1.33 ± 11.15	0.55 ± 3.82	0.8 ± 11.0	11.0 ± 39.3
Deionised water	**T1**	-2.05 ± 8.31	1.41 ± 2.85	2.9 ± 9.5	11.2 ± 28.1
	T2	-2.42 ± 9.39	1.00 ± 3.79	0.7 ± 10.2	7.2 ± 35.6
	T3	3.03 ± 10.39	1.92 ± 3.98	4.8 ± 11.2	19.9 ± 44.4

Results in Table B.2 indicate that *A. marlothii* gel material improved skin ENT, HOM and NRJ to a greater extent than the deionised water and the gel materials of *A. vera* and *A. ferox* at 30 and 90 min after application. *Aloe vera* gel material did however increase skin ENT, HOM and NRJ more than *A. ferox* and deionised water at 30 and 90 min after application. Skin NRJ was improved more by *A. ferox* gel material than deionised water 30 and 90 min after application.

B.3.1.2 Statistical data analysis

Table B.3 shows the results obtained from the Fixed Effects Type III Test. From this test it was determined whether the fixed effects (time, treatment, interaction between time and treatment) were statistically significant or not.

Table B.3: Fixed Effects Type III Test for short-term measurements of skin hydration (red numbers indicate statistically significant differences)

	Statistical values	Effect		
		Time	Treatment	Time-Treatment Interaction
Corneometer®	p-Value	0.196	0.00001	0.017
	F-value	1.788	10.07600	3.540
ENT	p-Value	0.305	0.03600	0.154
	F-value	1.269	3.51600	1.811
HOM	p-Value	0.339	0.06600	0.084
	F-value	1.148	2.85500	2.262
NRJ	p-Value	0.434	0.00001	0.483
	F-value	0.875	9.84100	0.954

Statistically significant effects are revealed (as indicated by the red numbers in Table B.3) for the treatment and the time-treatment interaction of the HOM parameter and the Corneometer® measurements. The p-values revealed that treatment had a statistical significant effect for the NRJ and ENT. It was found that the effect of the treatment on the HOM parameter and the Corneometer® measurements depends on time, as was reflected by the significant interaction between time and treatment. Nevertheless, it is essential to notice that the significance of the interaction effect of the Corneometer® measurements may be induced by the dominant influence of treatment given its F-value of 10.076, which is 5.6 times larger than that of time (F = 1.788).

Comparing the different treatments and the different levels of time yielded a vast number of results (p-values) of which only the statistically significant values will be mentioned in the discussion below to ease reading and minimise the number of tables needed to show the data. Pairwise comparisons with a Bonferroni adjustment between the different treatments showed a

statistical significant difference between *A. ferox* and *A. vera* gel materials (p = 0.023), between *A. ferox* and deionised water (p = 0.016) and between *A. ferox* and *A. marlothii* gel materials (p = 0.007) with the Corneometer® measurements. With p-values of 0.067 and 0.068, respectively, skin HOM and ENT showed a statistical significant difference between *A. ferox* and *A. marlothii*. A statistical significant difference was seen between *A. marlothii* and deionised water (p = 0.003) and between *A. ferox* and *A. marlothii* (p = 0.003) for the measured NRJ parameter. Pairwise comparisons with a Bonferroni adjustment showed no significant difference between the levels of time for any of the skin hydration parameters investigated.

B.3.2 Longer term study

B.3.2.1 Skin hydration

The percentage change in skin hydration relative to the initial conditions (T_0), as measured by the Corneometer® after 1 (T_1), 2 (T_2), 3 (T_3) and 4 (T_4) weeks of treatment, are given in Table B.4. The results indicate that over the 4 week period of treatment, *A. vera* and *A. marlothii* gel materials had a predominantly dehydrating effect on the skin. *Aloe marlothii* gel material dehydrated the skin the most from week 1 to week 4. A 1.1% increase in skin hydration with *A. ferox* gel material was observed after one week of treatment, thereafter it exhibited a dehydrating effect on the skin, but the dehydration was less than with the other two aloe gel materials. In contrast to the test materials, the placebo (i.e. deionised water) increased the level of skin hydration over the four week time period.

Table B.4: Long-term Corneometer® measurements of skin hydration (%change ± SD)

	T1	T2	T3	T4
A. vera	-3.9 ± 23.0	-1.6 ± 22.2	-10.0 ± 20.6	-5.6 ± 21.2
A. marlothii	-4.1 ± 21.8	-3.5 ± 19.8	-10.4 ± 20.3	-7.1 ± 18.4
A. ferox	1.1 ± 19.8	-0.6 ± 17.8	-3.8 ± 21.5	-2.5 ± 18.4
Deionised water	8.3 ± 19.2	9.1 ± 18.3	9.7 ± 31.9	12.1 ± 30.2

B.3.2.2 Skin topography

Investigation of the skin's topography with the Visioscan® served as additional support for the findings obtained with the Corneometer®. Results are given in Table B.5 as the mean with standard deviation. It can be seen the ENT, HOM and NRJ parameters followed a similar pattern. It is clear the aloes and deionised water initially showed a positive effect on the skin's appearance thereafter a downward trend was observed in these parameters, with most of the treatments indicating none of the treatments improved the skin topography.

Table B.5: Long-term hydration of skin by investigating Visioscan® parameters (%change ± SD)

Treatment	Time	ENT	HOM	NRJ
A. vera	**T1**	0.35 ± 3.41	1.30 ± 8.10	6.06 ± 27.36
	T2	0.42 ± 3.35	1.58 ± 9.05	9.74 ± 34.16
	T3	-0.40 ± 3.85	-0.06 ± 10.92	-0.37 ± 38.58
	T4	-0.51 ± 3.57	-1.60 ± 10.45	4.02 ± 36.76
A. marlothii	**T1**	0.80 ± 3.16	2.21 ± 6.56	13.10 ± 26.05
	T2	-0.22 ± 3.88	-1.27 ± 10.77	1.86 ± 37.12
	T3	-0.56 ± 3.72	-0.85 ± 10.89	-0.76 ± 38.99
	T4	-0.58 ± 4.18	-2.84 ± 11.08	1.73 ± 40.31
A. ferox	**T1**	0.85 ± 4.13	1.95 ± 10.53	9.51 ± 35.91
	T2	-0.65 ± 3.72	-1.75 ± 9.85	-3.48 ± 34.20
	T3	-0.48 ± 4.28	-0.75 ± 12.49	0.80 ± 41.96
	T4	-0.96 ± 3.08	-3.29 ± 9.03	-6.83 ± 28.42
Deionised water	**T1**	0.65 ± 3.18	1.39 ± 9.59	6.93 ± 28.35
	T2	-0.32 ± 2.64	-0.58 ± 8.21	-6.63 ± 28.21
	T3	-0.35 ± 3.83	-0.84 ± 11.50	-2.24 ± 35.38
	T4	-1.15 ± 2.45	-3.92 ± 7.38	-11.99 ± 25.23

B.3.2.3 Skin elasticity

As previously stated, the Cutometer® indirectly indicates skin hydration by measuring the skin's viscoelastic properties. Certain R-parameters, which are highly dependent on the moisture content (hydration) of the skin, were determined to support the findings of the Corneometer®. Results, as the mean with standard deviation, are given in Table B.6. All the aloe gel materials and the placebo displayed a negative percentage change from the baseline value for the R2-parameter, indicating decrease in gross-elasticity. The lowest point in percentage decrease of the gross-elasticity of the skin was seen after 2 weeks of treatment with the deionised water, *A. marlothii* and *A. ferox* gel materials.

The R6-parameter is indicative of the stretch capacity of the skin. Negative values reflect improved skin condition (Berndt & Elsner, 2002:94). A positive percentage change in this parameter was seen for all the test materials after the first two weeks, thereafter the trend was downwards. The R7-parameter measures the elastic portion of the skin with negative values reflecting a decrease in biological elasticity (Berndt & Elsner, 2002:94). The highest percentage decrease was seen after 2 weeks of treatment. The complete relaxation (R8) of the skin also indicated a decrease in skin elasticity and followed almost the same pattern as R7.

Table B.6: Long-term Cutometer® measurements of skin hydration (%change ± SD)

Treatment	Time	R2 ± SD	R6 ± SD	R7 ± SD	R8 ± SD
A. vera	**T1**	-1.26 ± 8.81	-1.83 ± 28.54	-1.2 ± 17.8	-0.7 ± 19.4
	T2	-3.86 ± 7.84	3.21 ± 25.65	-7.2 ± 19.1	-12.1 ± 24.1
	T3	-3.99 ± 6.59	2.47 ± 22.35	-8.2 ± 13.8	-10.3 ± 12.3
	T4	-1.29 ± 6.98	-2.19 ± 27.28	-1.9 ± 15.3	-8.5 ± 16.3
A. marlothii	**T1**	-1.75 ± 6.69	-3.11 ± 31.46	-5.0 ± 12.6	-1.0 ± 14.9
	T2	-5.00 ± 9.61	6.98 ± 27.88	-10.7 ± 22.9	-16.4 ± 26.8
	T3	-3.42 ± 5.81	6.77 ± 26.59	-9.0 ± 11.9	-14.2 ± 17.4
	T4	-1.55 ± 9.37	0.04 ± 34.89	-3.2 ± 19.9	-10.0 ± 20.6
A. ferox	**T1**	-0.88 ± 6.73	1.47 ± 28.42	-3.4 ± 12.9	-5.5 ± 15.9
	T2	-3.35 ± 9.14	10.56 ± 26.10	-6.9 ± 22.0	-12.5 ± 24.6
	T3	-1.55 ± 5.93	5.42 ± 28.98	-7.0 ± 11.9	-8.4 ± 15.0
	T4	0.22 ± 8.83	-4.64 ± 33.90	-2.1 ± 19.7	-6.7 ± 18.3
Deionised water	**T1**	-0.52 ± 9.65	0.93 ± 42.42	-4.6 ± 16.5	-5.8 ± 22.0
	T2	-2.57 ± 7.26	19.00 ± 38.29	-6.8 ± 19.2	-15.6 ± 25.1
	T3	-0.90 ± 7.69	17.37 ± 37.64	-3.7 ± 14.2	-6.7 ± 17.1
	T4	-1.01 ± 9.61	10.93 ± 38.02	-4.5 ± 19.4	-8.6 ± 21.7

B.3.2.4 Statistical data analysis

Table B.7 shows the results for the Fixed Effects Type III Test with red p-values indicating statistically significant differences. When investigating the Corneometer® results the p-values obtained revealed statistical significant differences between the treatments. The stretch capacity (R6) and the HOM parameter of the skin showed a significant difference between the times of treatment. The gross-elasticity (R2) and complete relaxation (R7) of the skin revealed statistical significant differences for the interaction between time and treatment. Statistical significant effects for time and interaction between time and treatment were observed for R8.

Table B.7: Fixed Effects Type III Test for long-term measurements of skin hydration (red numbers indicate statistically significant differences)

	Statistical values	Effect		
		Time	Treatment	Time x Treatment
Corneometer®	p-Value	0.527	0.001	0.757
	F-value	0.763	7.318	0.633
ENT	p-Value	0.117	0.904	0.593
	F-value	2.202	0.187	0.835
HOM	p-Value	0.045	0.366	0.153
	F-value	3.147	1.110	1.682
NRJ	p-Value	0.188	0.207	0.104
	F-value	1.743	1.650	1.916
R2	p-Value	0.187	0.396	0.009
	F-value	1.746	1.036	3.447
R6	p-Value	0.091	0.267	0.153
	F-value	2.446	1.413	1.688
R7	p-Value	0.112	0.526	0.032
	F-value	2.241	0.765	2.637
R8	p-Value	0.067	0.840	0.074
	F-value	2.754	0.279	2.114

Due to the large number of comparisons made between groups, only the statistically significant p-values (i.e $p < 0.1$) of the Fixed Effects Type III test with Bonferroni adjustments are mentioned in the discussion below. Pairwise comparisons with a Bonferroni adjustment between the levels of time revealed that the time of treatment had no statistical significance, except for the HOM parameter, where statistical significant difference was seen between one and four weeks ($p = 0.037$) of treatment. Pairwise comparisons with a Bonferroni adjustment between the different treatments revealed statistical significant differences in the Corneometer® measurements between the placebo and *A. ferox* gel material ($p = 0.003$), *A. marlothii* gel material ($p = 0.001$) and *A. vera* gel material ($p = 0.007$) gel materials.

B.3.3 Erythema study

B.3.3.1 Skin erythema

The percentage change in skin erythema, as expressed by haemoglobin content from irritation (T_1) to two time intervals (T_2 and T_3), after treatment with test materials are given in Table B.8.

Hydrocortisone gel showed a 13.1% decrease in erythema after one day of treatment (T_2), followed by deionised water and *A. ferox* gel material. The lowest percentage decrease in erythema at T_2 was demonstrated by *A. vera* and *A. marlothii* gel materials.

Table B.8: Percentage change in skin erythema (haemoglobin) from irritation (T_1) to two time intervals (T_2 and T_3) after treatment (with standard deviation)

Treatment	T_2	T_3
Irritated	-4.5 ± 21.3	-13.1 ± 18.3
A. vera	-1.8 ± 19.4	-17.0 ± 19.1
A. marlothii	-1.8 ± 19.3	-9.0 ± 21.2
A. ferox	-7.0 ± 14.3	-15.2 ± 20.4
Deionised water	-7.8 ± 15.7	-13.0 ± 26.6
Hydrocortisone	-13.1 ± 21.6	-18.8 ± 26.0

On the seventh day, after six days of treatment (T_3), hydrocortisone gel decreased erythema by 18.8% followed by *A. vera* (17.0%), *A. ferox* (15.2%) and *A. marlothii* (9.0%). Statistically significant differences were found between *A. marlothii* and *A. vera* gel materials ($p = 0.051$), as well as between *A. marlothii* gel material and hydrocortisone gel ($p = 0.046$). A statistically significant difference ($p = 0.0196$) exists between T_2 and T_3 for *A. vera* gel material, indicating its anti-erythema effect is time dependent. As mentioned before, only the statistically significant p-values were mentioned for reasons of clarity and to minimise the number of tables needed to show the data.

B.3.3.2 Skin pH

Table B.9 shows the percentage change in skin erythema as expressed by skin pH from irritation (T_1) to two time intervals (T_2 and T_3) after treatment with test materials.

Table B.9: Percentage change in skin pH from irritation (T_1) to two time intervals (T_2 and T_3) after treatment (with standard deviation)

Treatment	T_2	T_3
Irritated	-1.5 ± 3.0	-2.4 ± 4.0
A. vera	-2.5 ± 4.1	-3.5 ± 4.1
A. marlothii	0.1 ± 4.8	-2.3 ± 5.8
A. ferox	-0.2 ± 3.5	-2.0 ± 5.9
Deionised water	-0.8 ± 4.4	-3.1 ± 5.0
Hydrocortisone	1.0 ± 7.2	-1.5 ± 6.5

At T_2 all the treatments except *A. marlothii* and hydrocortisone gel decreased the pH, which was elevated after irritation with SLS. *Aloe vera* lowered the pH most of all the treatments followed by the irritated untreated skin. A statistical significant difference was found between the effects of *A. vera* and *A. marlothii* ($p = 0.0159$) gel materials and between the effects of *A. vera* and *A. ferox* ($p = 0.0276$) gel materials.

After six days of treatment (T_3), all treatments lowered the pH of the skin with the positive control, hydrocortisone, showing restoration of the acid mantle of the skin the least. *Aloe vera* decreased the pH of the skin the most, although it was not statistically significant. Time was not a factor, as there was no statistical significant difference between T_2 and T_3 for any of the treatments.

B.3.3.3 Vapour loss

The percentage change in skin erythema as shown by TEWL from irritation (T_1) to two time intervals (T_2 and T_3) after treatment with test materials are given in Table B.10. As mentioned previously, only the statistically significant differences will be discussed.

Table B.10: Percentage change in TEWL from irritation (T_1) to two time intervals (T_2 and T_3) after treatment (with standard deviation)

Treatment	T_2	T_3
Irritated	-13.0 ± 26.4	-53.3 ± 36.3
A. vera	-7.3 ± 18.1	-43.4 ± 31.2
A. marlothii	3.0 ± 23.7	-30.5 ± 32.3
A. ferox	1.9 ± 28.3	-35.5 ± 43.0
Deionised water	5.2 ± 28.1	-35.2 ± 39.7
Hydrocortisone	-6.9 ± 26.8	-49.1 ± 39.8

Initially an increase in the TEWL values were observed for the skin exposed to SLS. After one day of treatment (T_2) with the different test materials the untreated irritated skin decreased the TEWL values the most, followed by *A. vera* and hydrocortisone gel. Deionised water, *A. ferox* and *A. marlothii* gel materials appeared to increase TEWL rather than decrease it. Statistical significant differences were observed between the untreated irritated skin and *A. marlothii* ($p = 0.0213$) and between the untreated irritated skin and deionised water ($p = 0.0526$). Statistical significant differences were found between *A. vera* and *A. marlothii* ($p = 0.029$), *A. ferox* ($p = 0.097$) and deionised water ($p = 0.089$). No statistical significant difference was found between *A. vera* and hydrocortisone gel. *Aloe vera* significantly proved a decrease in TEWL after one day of treatment when compared to the other aloe species and the placebo.

On the seventh day, after six days of treatment (T_3), TEWL was reduced the most with the untreated irritated skin. This was followed by hydrocortisone and *A. vera* materials. Deionised water and *A. ferox* reduced TEWL by almost the same percentage, whereas *A. marlothii* reduced TEWL even less than the placebo (deionised water). Statistical significant differences are observed between the untreated irritated skin and *A. marlothii* ($p = 0.0000114$) and *A. ferox* ($p = 0.00158$) gel materials. There was also a statistical significant difference between *A. marlothii* and hydrocortisone and between deionised water and hydrocortisone with p-values of 0.0212 and 0.0376, respectively. No statistical significant difference was found between *A. vera* and the other two aloe species in terms of vapour loss.

Time was found to be a factor, as a statistical significant difference was observed between T_2 and T_3 for all treatments. The p-values between T_2 and T_3 were 5.328×10^{-5} for untreated irritated skin, 1.22×10^{-4} for *A. vera*, 3.02×10^{-3} for *A. marlothii*, 6.20×10^{-4} for *A. ferox*, 2.07×10^{-5} for deionised water and 4.60×10^{-5} for hydrocortisone.

B.4 Discussion

The effects of formulations containing 5% (w/w) trilaureth-4 phosphate-based blend supplemented with 0.10%, 0.25% or 0.50% (w/w) *A. vera* gel extract on the volar forearm of volunteers, after single and multiple applications (1- and 2-week daily application), has previously been investigated by Dal'Belo *et al.* (2006:244). During the single application study, only the 0.50% (w/w) formulation significantly increased the Corneometer® measurements (SC water content) after an hour when compared to the vehicle. The SC water content was significantly increased 2 h and 3 h after application of the 0.25% and 0.50% (w/w) formulations, compared to the vehicle. An increase in skin hydration was observed for all three concentrations when compared to the vehicle after one and two weeks of application. However, when the formulations were compared with each other after two weeks of application the formulation containing 0.50% (w/w) *A. vera* extract proved to be significantly better than the 0.10% and 0.25% formulations (Dal'Belo *et al.*, 2006:244).

The Corneometer® values obtained in this study showed that *A. vera* and *A. marlothii* gel materials had a larger hydrating effect than deionised water on the skin at 30 (T_1), 90 (T_2) and 150 (T_3) min after a single application. Both these aloe materials exhibited a higher percentage increase in skin hydration at 30 and 150 min after application, with the lowest percentage increase observed at 90 min after application. At 90 and 150 min after application, *A. marlothii* gel material caused a slightly higher hydration effect than *A. vera* gel material. The short term hydrating effects of *A. vera* and *A. marlothii* were not statistically significantly different from that of deionised water. The skin hydration effect of *A. ferox* gel material differed significantly from the other aloe materials and the placebo, as it showed a dehydrating effect

(negative percentage change) over the short-term study. This dehydrating effect of *A. ferox* gel material became less, with time, after application. Initially deionised water dehydrated the skin at 30 and 90 min after application; thereafter it showed to hydrate the skin at 150 min after application. This increase in skin hydration, 150 min after application with deionised water, (placebo) was however less than that obtained with *A. vera* and *A. marlothii* gel materials.

Statistical analysis of the skin ENT and HOM values indicated only a statistically significant difference between *A. ferox* and *A. marlothii* gel materials. Skin NRJ showed a statistically significant difference between *A. marlothii* and *A. ferox* gel materials and in this case *A. marlothii* gel material exhibited a higher statistically significant skin NRJ value compared to the placebo. This indicates that *A. marlothii* gel material improved the general state of the skin more than deionised water.

The Corneometer® readings from the longer-term multiple application study indicated that *A. marlothii* gel material dehydrated the skin the most of all the aloe materials investigated from weeks 1 to 4. Results obtained from the multiple application study were in contrast with the results of the single application study as *A. marlothii* and *A. vera* gel materials did not hydrate the skin more than deionised water (placebo).

Aloe ferox gel material initially hydrated the skin after the first week of treatment; thereafter it also had a dehydration effect on the skin after two, three and four weeks of treatment. No statistical significant differences existed between the different aloe gel materials, although statistical significant differences were observed between the placebo and the different gel materials. Thus deionised water was statistically better than the aloes in improving skin hydration.

Investigation of the skin's topography with the Visioscan® supported the findings obtained with the Corneometer®. Skin ENT was slightly increased by the three aloe gel materials as well as the placebo after the first week of treatment, with *A. ferox* gel material showing the highest percentage increase; *A. marlothii*, *A. ferox* gel and the placebo decreased skin ENT after 2, 3 and 4 weeks of treatment. After two weeks of treatment *A. vera* gel material showed a small percentage increase in skin ENT, thereafter also decreasing after three and four weeks of treatment. Skin HOM almost followed a similar pattern, except that initially *A. marlothii* gel material increased this parameter the most after one week of treatment.

Following one week of treatment, an increase in skin NRJ was observed with all the aloe gel materials with *A. marlothii* gel material showing the highest increase. Both *A. marlothii* and *A. vera* gel materials decreased skin NRJ after 3 weeks of treatment, an increase was observed after 4 weeks of treatment. *Aloe ferox* gel material exhibited an increase in skin NRJ after 1 and 3 weeks, and a decrease in skin NRJ after 2 and 4 weeks of treatment. Deionised water

increased skin NRJ after the first week, thereafter decreasing it after two, three and four weeks of treatment. None of the differences between the treatments were found to be statistically significant.

The R-parameters obtained with the Cutometer® were highly dependent on moisture content (hydration) of the skin. *Aloe marlothii* gel material demonstrated the largest negative effect on the skin's gross elasticity (R2) compared to the other aloes and the placebo. These findings are comparable with the Corneometer® values, which indicated that *A. marlothii* gel material had the most dehydrating effect on the skin.

The R6-parameter correlates with the other R-parameters in showing that skin conditions did not improve (positive percentage change). The only negative percentage changes (indicating improved skin conditions) were after one week and four weeks of treatment with *A. vera*, one week of treatment with *A. marlothii* and after four weeks of treatment with *A. ferox*. Deionised water exhibited the highest percentage positive change indicating the least improvement of the R6-parameter.

The R7- and R8-parameters also showed a decrease in skin elasticity with *A. marlothii* gel material having the most negative effect on these parameters after two, three and four weeks of treatment. These results verify the findings of the Corneometer® that *A. marlothii* dehydrated the skin the most, as it had the most negative effect on the elasticity parameters. Nevertheless, statistically the Cutometer® results suggest that none of the treatments seemed to significantly alter the R2-, R6-, R7- and R8-parameters.

Previous research by Dal'Belo *et al.* (2006:244) found that *A. vera* extract moisturised the skin by significantly increasing the SC water content, whilst not altering TEWL. It was proposed that the rich composition of the *A. vera* extract, consisting of hygroscope mono- and polysaccharides (Femenia *et al.*, 1999; Dal'Belo *et al.*, 2006:244), together with the presence of the amino acids alanine, arginine, glycine, histidine, serine and threonine contributed to SC hydration (Rieger, 1992:90; Dal'Belo *et al.*, 2006:244).

Humectants promote the retention of water within the SC by attracting water from the outside in (from environment to skin) and from the inside out (from dermis to epidermis/SC) (Rawlings *et al.*, 2004:50). Even though it was not the aim of the study to determine the mechanism of skin dehydration by the aloe materials, a possible explanation should be further investigated in future studies. The dehydration of the skin by the aloe gel materials after multiple applications may be attributed to it attracting water not only from the dermal/epidermal layers of the skin, but also the SC. The dehydration effect may have occurred due to absorption of moisture from the skin into the aloe gel layer, which dried by evaporation of the water in which the gel materials were dissolved after application to the skin. The findings of the present multiple application study

differ from the results of other studies and may be ascribed to differences in the formulations tested; previous studies incorporated excipients, whilst in this study only the aloe materials were dissolved in water and the composition of the aloe materials may differ due to different growth conditions and harvesting processes (Capasso *et al.*, 1998:S125).

Numerous tests have been used to evaluate the anti-inflammatory efficacy of aloe gel or its various components and generally involve some kind of intentional wounding (Reynolds & Dweck, 1999:5). The first step in wound healing is anti-inflammation (Jia *et al.,* 2008:188) as the products of vasoactive amine and kinin systems modulate the inflammatory response in the early burn phase (0-72 h) (Duansak *et al.,* 2003:244).

In the present study, the SLS exposure test was performed to compare the anti-erythema efficacy of the aloe gel materials to the positive control group (hydrocortisone gel). As a topical glucocorticoid, hydrocortisone is considered as the basis of therapy for various inflammatory skin diseases (Wyatt *et al.*, 2001:1798). According to Tupker *et al.* (1997:66), SLS is a model irritant, although it cannot be uncritically projected to other categories of irritation as irritant substances commonly differ considerably in mode of responses and skin penetration capabilities.

Results obtained with the Mexameter® indicated that hydrocortisone gel decreased erythema (as measured by haemoglobin content) to a greater extent than the other treatments at T_2. However, no statistical significant differences were obtained between hydrocortisone gel, *A. ferox* gel material, deionised water and the untreated irritated skin in terms of anti-erythema effect at T_2. On the other hand, hydrocortisone gel performed statistically significantly better than *A. vera* and *A. marlothii* gel materials in reducing skin erythema at this time point. *Aloe ferox* gel material statistically significantly reduced erythema to a greater extent than *A. marlothii* gel material at T_2.

Although not statistically different from *A. vera* and *A. ferox* gel materials, hydrocortisone gel continued to decrease erythema (as measured by haemoglobin) to the greatest extent of all the treatment groups on the seventh day, after six days of treatment (T_3). Untreated irritated skin and deionised water showed a similar decrease in skin erythema at T_3 with a percentage decrease of 13.1% and 13.0%, respectively. The anti-erythema effect of *A. marlothii* gel material was less than deionised water and untreated irritated skin, although this was not statistically significant. A statistically significantly higher effect was seen with *A. vera* gel material and hydrocortisone gel when compared with *A. marlothii* gel material. The anti-erythema effect of *A. vera* gel material was found to be time dependent as a statistical significant difference was observed between T_2 and T_3 with this test material. These findings

coincide with a previous study where a lag phase was observed and the onset of *A. vera* gel's anti-inflammatory activity was found to be delayed (Reuter *et al.,* 2008:109).

Skin pH was elevated after the skin was exposed to SLS indicating a disturbance in the 'acid mantle' of the skin. After one day of treatment with the test materials *A. vera* reduced the pH of the skin significantly more than *A. marlothii* and *A. ferox*, although not statistically significantly better than hydrocortisone. The aloes proved to restore the 'acid mantle' of the skin more than hydrocortisone, although *A. marlothii* and *A. ferox* reduced the pH of the skin to a lesser extent than deionised water and untreated irritated skin after six days of treatment.

After one and six days of treatment, the untreated irritated skin showed the highest percentage reduction in TEWL values, indicating that the skin's natural ability to restore its own barrier function was better than with the different test materials. However, *A. vera* did statistically significantly reduce TEWL more than the other two aloes after one day of treatment, although there was no statistical significant difference between *A. vera* and hydrocortisone. *Aloe marlothii* indicated the least reduction in TEWL of all the test materials (including the placebo) after six days of treatment. *Aloe vera* reduced TEWL the most of all the aloe species although it was not statistically significant different.

From the anti-erythema results of these three non-invasive methods it can be concluded that *A. vera* performed better than the two aloe species indigenous to South African, namely *A. ferox* and *A. marlothii,* in reducing erythema (haemoglobin), pH and TEWL after skin was exposed to SLS. The effect of *A. vera* was also found to be dependent on time when reducing erythema (haemoglobin) and TEWL. Its effect was not statistically significantly different from that of hydrocortisone.

The differences obtained in the anti-erythema results for the different aloe gel materials could be explained by differences in their chemical compositions as confirmed with ^{1}H-NMR. As mentioned before, differences in their chemical compositions may be attributed to species and growth condition differences and after harvesting material processing procedures.

In general, the external use of aloe gel on intact skin is regarded as safe, as it is not associated with adverse reactions (Poppenga, 2002:8). This is in agreement with the findings of other studies (Reuter *et al.*, 2008:109; Eshghi *et al.*, 2010:649), though final conclusions on the overall safety in the study conducted by Reuter *et al.* (2008:109) could not be made due to the rarity of an allergy after a single application. In a previous study by Akhtar *et al.* (2011:178), a cream (w/o-emulsion) containing *A. vera* gel as well as the base cream produced a mild skin irritation and a slight increase in erythema level. These effects were however insignificant and as the *A. vera* containing cream did not significantly alter the skin pH levels it was considered as being safe to use.

In contrast to this, case studies on the topical application of aloe-derived products indicated some adverse reactions which included contact urticaria, dermatitis and acute eczema (Cosmetic ingredient review panel, 2007:45). In a study performed by Heggie *et al.* (2002:445), two out of 115 subjects treated with topical *A. vera* gel had an allergic reaction. It has been proposed that the presence of apoptosis-inducing anthraquinones in *A. ferox* may be responsible for the adverse effects (such as hypersensitivity) (Chen *et al.*, 2012:2).

In the present study, weekly questionnaires indicated that 11 (18.64 %), 14 (23.73 %) and two (3.39 %) out of the 59 volunteers reported a mild allergic reaction after topical application of *A. vera*, *A. marlothii* and *A. ferox* gel materials, respectively. Two volunteers were withdrawn from the study due to severe allergic reactions. The volunteers experienced a red rash, especially when the aloe gel started to dry on the skin, with either a burning or itching sensation.

B.5 Conclusion

The Corneometer® measurements, which are regarded as the most important indicator of skin hydration, indicate that *A. vera* and *A. marlothii* gel material did improve the hydration of the skin after a single application, although it was not statistically significantly different from the placebo (i.e. deionised water). *Aloe ferox* gel material dehydrated the skin after a single application when compared to the other aloe gel materials and the placebo. After multiple applications, the aloe gel materials had a dehydrating effect on the skin as opposed to deionised water, which significantly improved skin hydration. Mexameter® readings showed that *A. vera* and *A. ferox* gel materials were similar in their erythema reducing effects after six days of treatment to that of hydrocortisone gel. The anti-erythema effect of *A. vera* gel material was found to be dependent on time as there was a statistical significant difference between the second day of treatment and seventh day of treatment. *Aloe marlothii* gel material dehydrated the skin to the largest extent during the longer-term study and was less effective than deionised water and untreated irritated skin in decreasing erythema. It also caused the highest number of mild skin reactions in the volunteers. Whilst this study has elucidated the effects of gel materials from different aloe species on skin hydration and erythema in human subjects, the mechanisms of action should be investigated in future studies.

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Appendix C

Forms used in clinical cosmetic efficacy study of the aloe leaf materials

This appendix contains all the forms given to the volunteers prior to, as well as during the clinical cosmetic efficacy study with the aloe leaf materials.

The different forms include:

- Informed consent.
- Pre-treatment questionnaire – in order to include or exclude volunteers according to the criteria mentioned in Appendix B the volunteers had to complete this form before commencement of the study.
- Information form – this form gave information to the volunteers with regards to the purpose of the study, as well as possible risks and benefits.
- Instructions – this form gave general instructions to be followed during the study.
- Weekly questionnaire – this questionnaire was completed by the volunteers at each measurement. The volunteers were also encouraged to indicate on this form any allergic reactions experienced or any problems which may have occurred.

ALOE STUDY PROTOCOL

NO	

INFORMED CONSENT

STUDY IDENTIFICATION
CLINICAL COSMETIC EFFICACY STUDIES WITH ALOE GEL MATERIALS
INSTITUTION: UNIT FOR DRUG RESEARCH AND DEVELOPMENT NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)
INVESTIGATOR – LIZELLE FOX (083 **** ***)

I, the undersigned _____ agree to take part in the experimental study that was described to me and which will be supervised by Professor J.H. Hamman.

I am aware that there are risks involved, such as adverse skin effects, that along with my role in the study, was exhaustively explained (orally and written) to me and that I am free to ask any questions regarding the study.

I am free to retire my participation before the study or to interrupt my participation during the study at any time. I also accept to inform the investigator of any change in my state of health or pharmacological treatment that might occur during the execution of the study.

I am free to ask for any further information regarding the study or to inform the study leader of any adverse effects at once, by contacting the investigator at cell phone number: **083 **** ***.

I have read and signed this consent form, fully aware of the test procedures and other information such as risks, benefits, confidentiality and voluntary participation as indicated on the information form.

I accept that the data recorded during the study could be subjected to computerised analysis by the investigator, but I am also aware that any information that can identify me will remain confidential throughout the study recordings.

☐ I agree
☐ I do not agree

_____	_____	_____
SUBJECT NAME AND SURNAME	**SIGNATURE**	**DATE**

OFFICE USE ONLY

I confirm that the volunteer has read the title and the purpose of the clinical study and the informative form. The volunteer has had the opportunity to ask questions to which was given an exhaustive answer. The volunteer was explained the aim of the study, the method and the features of the clinical survey, benefits and possible discomforts. The informed participant, has agreed to take part in the test.

_____	_____	_____
INVESTIGATOR	**DATE**	**SIGNATURE**

ALOE STUDY PROTOCOL

NO	

PRE-TREATMENT QUESTIONNAIRE

INCLUSION CRITERIA

1	Age between 20 and 40 years	**YES**	**NO**
2	Good health state with no pathological events for the period immediately before tests [examples include cancer, diabetes, any infectious diseases (common cold, influenza, urinary tract infections, chickenpox, gastroenteritis, athlete's foot and malaria) or autoimmune disorders such as lupus or rheumatoid arthritis]	**YES**	**NO**
3	Understanding of both English and Afrikaans languages	**YES**	**NO**
4	Can be contacted at any time via cell phone or landline	**YES**	**NO**

EXCLUSION CRITERIA

1	Subject out of age bracket for specific test	**YES**	**NO**
2	Subject has a history of allergic reaction 30 days prior to test	**YES**	**NO**
3	Subject has a history of eczema	**YES**	**NO**
4	Subject had psoriasis within 6 months of enrolment	**YES**	**NO**
5	Subject is pregnant or lactating	**YES**	**NO**
6	Subject had prior cosmetic procedures or major surgery within the previous 12 months that could affect response evaluation	**YES**	**NO**
7	Subject had previous treatment with the products that are tested	**YES**	**NO**
8	Subject has uncontrolled systemic disease	**YES**	**NO**
9	Subject has any condition that could interfere with neuromuscular function (e.g. Myasthenia gravis/ Eaton-Lambert syndrome/ Amyotrophic lateral sclerosis)	**YES**	**NO**
10	Subject has a dermatological illness that could interfere with treatment or interpretation of results (for example vitiligo)	**YES**	**NO**
11	Subject is taking topical or systemic drugs that could influence the test results (e.g. topical anti-inflammatory / corticosteroids / etc.)	**YES**	**NO**
12	Subject has a **recent** history of intolerance to drugs and/or cosmetic products	**YES**	**NO**
13	Subject is currently involved in another clinical investigation or was involved within a period of 30 days prior to admission to this study	**YES**	**NO**
14	Subject is going to expose herself to intensive doses of UV radiation within period of study	**YES**	**NO**
15	Subject's race, please indicate (for purposes of keeping statistics on a database)	**Black**	**White**
		Coloured	**Indian**

_____ _____ _____

SUBJECT NAME AND SURNAME **SIGNATURE** **DATE**

OFFICE USE ONLY			
	The subject can be included in the test	**YES**	**NO**

ALOE STUDY PROTOCOL

INFORMATION FORM

STUDY IDENTIFICATION
CLINICAL COSMETIC EFFICACY STUDIES WITH ALOE GEL MATERIALS
INSTITUTION: UNIT FOR DRUG RESEARCH AND DEVELOPMENT NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)
INVESTIGATOR – LIZELLE FOX (083 **** ***)

Dear Miss/Mrs,

We invite you take part in a clinical study aimed to evaluate the moisturising effects as well as the soothing effects of two indigenous aloe species, *Aloe ferox and Aloe marlothii*, against the well-known and documented *Aloe vera*.

BACKGROUND

Aloe vera and other aloe species have a long history as traditional folk remedy and is most commonly used to treat conditions such as constipation, arthritis, blood pressure problems, burns, diabetes, eczema, psoriasis and skin cancer [1-2]. The whole leaf extracts and pulp (or gel) of *A. vera* are used in many commercial products such as cosmetics, lotions, sun screens, shampoos and various other products due to their soothing, astringent and healing properties [1; 3].

There are more than 360 species of the genus aloe known worldwide [4], of which 160 are indigenous to South Africa [5]. Therapeutic uses of aloe are based almost exclusively on research or subjective evidence obtained from *A. vera*; therefore it is vital for scientists to investigate and determine the medicinal uses and pharmaceutical applications of other aloe species [2].

RISKS AND BENEFITS

Risks – To the best of our knowledge no low, medium to serious side effects are expected to occur. The external use of aloe gel on intact skin is not associated with adverse reactions and is generally regarded as safe [6].

Benefits – It is possible that an improvement on the treated skin area will be noticed during the study period.

PRODUCTS USED / APPLICATION

The aloe gels will be applied twice daily (morning: 06:00 to 08:00 and evening: 18:00 to 20:00) to the volar forearm (inner arms) in clearly marked areas for the duration of the study.

CONFIDENTIALITY

All information gathered during the survey will remain strictly confidential and you will never be identified (data will therefore be kept anonymous). During or after the clinical tests, the promoters of the survey and/or health officers may have to examine your case file. The results of the study could also be published, but under no circumstance will your name be mentioned, unless absolutely necessary to personnel who are subject to professional secrecy.

PARTICIPATION

Your participation in this clinical trial must be absolutely voluntary and under no circumstances should you feel forced. If you decide not to take part, you will neither experience any inconvenience nor lose any benefit which you will enjoy at the inclusion time. If you decide to interrupt the treatment before the tests are finished, you can do so without any prior warning and you will neither experience any inconvenience nor lose any benefit. However, if you do decide to interrupt the study, you should inform the investigator immediately. The investigative panel also reserves the right to interrupt your study if we detect any adverse reactions that can cause harm.

MEDICAL ASSISTANCE

You will have access to a qualified medical practitioner should you need any medical assistance due to your participation in this project. Alcohol consumption, caffeine intake and some vasoactive medications alter skin microcirculation which indirectly influences skin hydration measurement. The use of such substances is prohibited on the day of skin measurements and should be reported if any such medicine was used.

If you have any questions or remarks before, during or after the study, you can contact your investigator at any time. The investigator will attend to you throughout the clinical study.

Regards,

LIZELLE FOX

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ALOE STUDY

INSTRUCTIONS

STUDY IDENTIFICATION
CLINICAL COSMETIC EFFICACY STUDIES WITH ALOE GEL MATERIALS
INSTITUTION: UNIT FOR DRUG RESEARCH AND DEVELOPMENT NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)
INVESTIGATOR – LIZELLE FOX (083 **** ***)

SHORT-TERM STUDY

A short-term study will be performed during the first week of the trial to investigate the short-term hydration effect of the aloe gel materials (measured at 1, 2 and 3 h after application). The investigators will apply the aloe gel materials to the volar forearm skin of the dominant arm; after which measurements will be taken.

LONG-TERM STUDY

The aloe gels will be applied twice daily (morning: 06:00 to 08:00 and evening: 18:00 to 20:00) by the volunteer to the volar forearm (inner arm) of the non-dominant hand in clearly marked areas for a period of four weeks. An amount of 0.05 ml of each product should be applied to their allocated areas twice daily. The volunteer should apply the different aloe products as indicated on the illustration given (colour-coded). The time at which the solutions were applied should be indicated daily on the program schedule given. On the day of the measurements no aloe product should be applied in the morning, but product can be applied again in the evening.

ERYTHEMA STUDY

The erythema study is a nine day study wherein Finn Chambers containing sodium lauryl sulphate will be applied on the volar forearm skin of the dominant arm for a period of approximately 22.5 h. The different gel solutions (0.05 ml of each to the allocated areas) will be applied by the investigator on the third day, followed by application by the volunteer (program schedule will be given) for the second time that day. On the fourth day of the study measurements will be taken; thereafter the gels should be applied twice daily until the ninth day, when the final measurements will be done.

GENERAL COMMENTS

Starting one week to the onset of the study as well as during the study, the volunteer must wash the skin of both forearms with Dove® soap only. Normal toiletry products may be used on the rest of the body and facial area.

On the day of the measurements the volunteers must please refrain from consuming alcohol, caffeine and some vasoactive medications as they may alter skin microcirculation which indirectly influences skin hydration measurements.

Following is a list of some vasoactive medications:

- Nasal decongestants:
  - Pseudoepedrine (Sudafed®, Flutex®, Actifed®, Demazin NS®, Nurofen® Cold & Flu tablets, Sinugesic®, Sinumal®, Sinutab®)
  - Phenylpropanolamine (Sinuclear®, Sinustat®)
- Vasodilators
  - Glyceryl trinitrate/nitroglycerin (Angised®, Nitrolingual Spray®)
  - Isosorbide dinitrate (Isordil®)
  - Isosorbide mononitrate (Elantan®, Imdur®, Ismo® 20)
- Agents acting on arteriolar smooth muscle:
  - Hydralazine (Apresoline®, Hyperphen®, Sandoz-Hydralazine®)
- Peripherally acting anti-adrenergic agents/alpha-adrenoceptor antagonists:
  - Prazosin (Pratsiol®)
  - Doxazosin (Cardura®, Adco-Doxazosin®, Cardugen®)
  - Terazosin (Hytrin®)
- Beta-blockers:
  - Propranolol (Inderal®, Prodolol®, Pur-Bloka®, Sandoz Propranolol®)
  - Atenolol (Tenormin®, Adco-Atenolol®, Bio-Atenolol®, Hexa-blok®, Sandoz-Atenolol®, Ten-bloka®, Tenoretic®, Tenoret 50®, Tenchlor®, Tenchlor HS®)
  - Bisoprolol (Concor®, Adco-Bisocor®, Bilocor®)
  - Nebivolol (Nebilet®)

- Calcium channel blockers:
  - Amlodipine (Norvasc®, Amloc®, Nortwin®)
  - Diltiazem (Tilazem®, Dilatam®, Sandoz Diltiazem®, Zildem®)
  - Felodipine (Plendil®, Felodipine-Hexal®)
  - Isradipine (Dynacirc®)
  - Nifedipine (Adalat®, Adalat XL®, Cipalat Retard®, Vascard SR®, Bio-Nifedipine®)
  - Nimodipine (Nimotop®)
  - Verapamil (Isoptin®, Calcicard SR®, Ravamil SR®, Sandoz Verapamil®)
  - Lercanidipine (Zanidip®)

The volunteer must please inform the investigator of any change in her state of health or pharmacological treatment that might occur during the execution of the study. The volunteer is also encouraged to report any side-effects that might occur during the study.

If you have any questions or remarks before, during or after the study, you can contact your investigator at any time. The investigator will attend to you throughout the clinical study.

Regards,

LIZELLE FOX

Investigator

Cell phone: **083 **** ***
Office telephone: **018 299 4373**
E-mail: **12815268@nwu.ac.za**

ALOE STUDY PROTOCOL

NO	

WEEKLY QUESTIONNAIRE

NAME AND SURNAME_____

STUDY IDENTIFICATION
CLINICAL COSMETIC EFFICACY STUDIES WITH ALOE GEL MATERIALS
INSTITUTION: UNIT FOR DRUG RESEARCH AND DEVELOPMENT NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)
INVESTIGATOR – LIZELLE FOX (083 **** ***)

1	Were there any problems with the application of the test substances?	**YES**	**NO**
2	If "Yes", what were the problems?		
	_____		
	_____		
	_____		
3	Did you experience any unusual emotional distress?	**YES**	**NO**
4	Deviation from protocol (e.g. use of vasoactive medicines)	**YES**	**NO**
	Kind of deviation (if present)		
	_____		
5	Do you use the Pill as a contraceptive measure?	**YES**	**NO**
6	Where in your menstrual cycle are you currently?		
	Before menstruation		
	During menstruation		
	After menstruation		

Appendix D

Validation of the HPLC experimental method for ketoprofen

D.1 Introduction

The objective of the validation of the analytical procedure, is to demonstrate that it is sensitive and reliable in the quantification of the concentration of the active ingredient (ketoprofen) present in the membrane release and skin diffusion samples (ICH, 2005:1). The analytical method described in this Appendix was created and developed, under the supervision, guidance and knowledge of Professor Jan du Preez. The conducting of all the procedures was in the Analytical Technology Laboratory of the North-West University (NWU), Potchefstroom, South Africa.

D.2 Chromatographic conditions

Analytical instrument: The HPLC analysis of ketoprofen was performed by using an Agilent® 1100 series HPLC with an isocratic pump, auto sampler injection mechanism, diode array detector and Chemstation Rev. A.10.01 software for data acquisition and analysis

Column: A Venusil XBP C18(2) column, 4.6 x 150 mm, 5 µm, 100 Å (Agela® Technologies, Newark, DE) was used during the analysis

Mobile phase: The mobile phase consisted of 70 volumes of acetonitrile (CH_3CN), 29 volumes of distilled water and 1 volume of acetic acid (CH_3COOH).

Flow rate: 1 ml/min

Injection volume: 50 µl

Detection: UV at 255 nm

Run time: Approximately 6 min

Retention time: The analyte eluted at approximately 3.03 min

Solvent: The solvent used was methanol (5% (v/v)) and phosphate buffer solution (PBS, pH 7.4). The PBS was prepared by mixing 250 ml 0.2 M potassium dihydrogen orthophosphate (KH_2PO_4) with 393.4 ml 0.1 M sodium hydroxide (NaOH). Subsequently the pH was adjusted with 10% phosphoric acid (H_3PO_4) (BP, 2013a:1).

D.3 Standard solution preparation

To generate a curve for testing the linearity of the analytical procedure, a sufficient number of standard ketoprofen solutions were used to efficiently define the relationship between concentration and instrument response (FDA, 2001:6). The solutions were prepared as follows:

- To make the mother solution (250 µg/ml) approximately 25 mg ketoprofen was accurately weighed off and dissolved in 5 ml methanol in a 100 ml volumetric flask. It was made up to volume with PBS (pH 7.4).
- A volume of 5 ml of the mother solution was diluted to 50 ml with PBS (pH 7.4) to obtain a 25 µg/ml ketoprofen solution.
- A volume of 5 ml of the 25 µg/ml ketoprofen solution was diluted to 50 ml with PBS (pH 7.4) to obtain a 2.5 µg/ml ketoprofen solution.
- A volume of 5 ml of the 2.5 µg/ml ketoprofen solution was diluted to 50 ml with PBS (pH 7.4) to obtain a 0.25 µg/ml ketoprofen solution.
- A volume of 5 ml of the 0.25 µg/ml ketoprofen solution was diluted to 50 ml with PBS (pH 7.4) to obtain a 0.025 µg/ml ketoprofen solution.

These solutions were injected at different injection volumes to obtain different concentrations (Refer to Section D.5.1). For the remainder of the validation parameters, the solutions were prepared as follows:

- To make the mother solution (250 µg/ml) approximately 25 mg ketoprofen was accurately weighed off and dissolved with 5 ml methanol in a 100 ml volumetric flask. The volumetric flask was subsequently filled up to volume with PBS (pH 7.4).
- Ten millilitres of the mother solution was diluted to 50 ml with PBS (pH 7.4) to obtain a 50 µg/ml ketoprofen solution.
- Ten millilitres of the 50 µg/ml ketoprofen solution was diluted to 100 ml with PBS (pH 7.4) to obtain a 25 µg/ml ketoprofen solution.
- Five millilitres of the 25 µg/ml ketoprofen solution was diluted to 100 ml with PBS (pH 7.4) to obtain a 12.5 µg/ml ketoprofen solution.

The concentrations of these solutions fell within the concentration range used for linearity. All samples were placed in HPLC vials and analysed using HPLC.

D.4 Samples from membrane release and skin diffusion studies

The samples collected from the Franz diffusion cells were placed in HPLC vials and analysed directly on the HPLC without any additional preparation.

D.5 Validation parameters

D.5.1 Linearity

The linearity of an analytical method is its capacity (within a given range) to elicit test results which are directly proportional to the concentration of analyte in the sample (USP, 2013a:986). The linearity of ketoprofen was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml) of the standards, prepared as described above. This plot should give a straight line ($r^2 = 1$) and can best be described by the following linear equation:

$y = mx + c$ **Equation D.1**

Where:

y = peak area of the analyte (ketoprofen)
m = slope
x = concentration of the analyte (ketoprofen)
c = y intercept

The standards prepared, as described in Section D.3, were injected in duplicate on the HPLC at different injection volumes to obtain a concentration range of between 0.006 and 63.75 µg/ml. The peak areas of ketoprofen were integrated from the chromatograms and can be seen in Table D.1. The regression value ($r^2 = 0.999$), obtained from the linear regression curve, (Figure D.1) indicates a high degree of linearity and therefore demonstrates a direct correlation between response and analyte concentration.

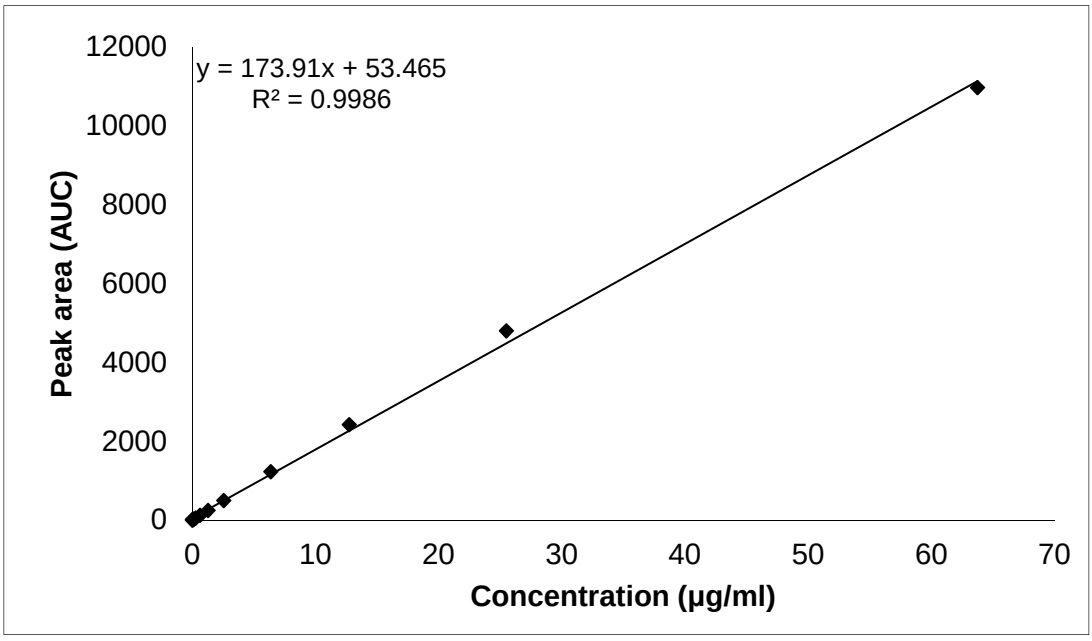

Figure D.1: Linear regression graph obtained for ketoprofen

Table D.1: Linearity results of ketoprofen standard solutions

Concentration (µg/ml)	Mean peak area (mAU)
0.00638	2.89
0.01275	5.20
0.02550	10.48
0.06375	13.49
0.12750	26.66
0.25500	53.06
0.63750	124.02
1.27500	246.05
2.55000	493.19
6.37500	1223.71
12.75000	2422.41
25.50000	4803.66
63.75000	10978.85
r^2	0.99
y-intercept	53.47
Slope	173.91

D.5.2 Accuracy and precision

According to the USP (2013a:984), the accuracy of an analytical procedure is the closeness of the test results attained by that procedure to the true value. Precision can be described as the degree of agreement between a series of measurements obtained from multiple sampling of the same homogeneous substance under prescribed conditions (USP, 2013a:985). Precision was investigated in terms of intraday (repeatability) variation and inter-day (reproducibility) under normal operating conditions.

D.5.2.1 Accuracy

It is recommended that accuracy of an analytical method should be investigated by employing a minimum of nine determinations over a minimum of three concentration levels covering the specified range (i.e. three concentrations and three replicates of each concentration) (ICH, 2005:10). Therefore, HPLC analysis was performed on the three standards (prepared as described in Section D.3) of known concentrations (i.e. 12.5, 25.0 and 50.0 µg/ml) of ketoprofen during the same day. The accuracy of an analytical procedure is reflected by the mean percentage recovery of the analyte and should be between 98 – 102% (APVMA, 2004:5). Table D.2 establishes that the method yielded an acceptable mean recovery of 98.88%. The standard deviation (SD) and percentage relative standard deviation (%RSD) is also given in the table.

Table D.2: Accuracy results of ketoprofen

Concentration spiked (µg/ml)	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
12.595	2386.61	2376.20	2381.41	12.25	97.29
12.595	2364.36	2371.14	2367.75	12.18	96.69
12.595	2378.96	2375.53	2377.24	12.23	97.11
25.190	4754.12	4774.95	4764.53	25.41	100.89
25.190	4764.82	4775.73	4770.27	25.45	101.01
25.190	4781.84	4767.65	4774.75	25.47	101.11
50.380	9171.74	9171.90	9171.82	49.75	98.75
50.380	9140.18	9137.03	9140.18	49.58	98.40
50.380	9161.16	9192.88	9161.16	49.69	98.63
				Mean	**98.88**
				SD	**1.74**
				%RSD	**1.76**

D.5.2.2 Inter-day precision

The precision of an analytical procedure is generally expressed as the variance, SD or coefficient of variation of a series of measurements (APVMA, 2004:5). HPLC analysis was performed on three different samples of a known concentration (25 µg/ml) ketoprofen, in triplicate on three different days. The percentage RSD should be equal to or less than 5% (APVMA, 2004:5). Table D.3 illustrates the results, which show the inter-day precision was acceptable with a percentage RSD of 0.93%.

Table D.3: Inter-day precision results of ketoprofen

Days	%Recovery			Mean	SD	%RSD
Day 1	100.89	101.01	101.11	101.00	0.09	0.09
Day 2	100.91	100.77	100.09	100.59	0.36	0.36
Day 3	98.86	98.86	98.86	98.86	0.00	0.00
		Between days:		**100.15**	**0.93**	**0.93**

D.5.3 Sensitivity

The sensitivity of an analytical method can be evaluated in terms of the limit of detection (LOD) as well as the limit of quantification (LOQ).

D.5.3.1 Limit of detection (LOD)

The LOD can be described as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified as an exact value, under the specified experimental conditions (USP, 2013a:986). In reference to a signal to noise ratio of 3:1, where the analyte peak is almost three times the height of the baseline noise (ICH, 2005:11), the LOD of ketoprofen was determined to be 0.00146 µg/ml.

D.5.3.2 Limit of quantification (LOQ)

The LOQ can be described as the lowest concentration of an analyte in a sample that can be determined quantitatively with acceptable precision and accuracy (percentage RSD < 15%) under specified conditions (USP, 2013a:986). Based on a signal to noise ration of 1:10 (ICH, 2005:12), the LOQ of ketoprofen was determined to be 0.005 µg/ml.

D.5.4 Ruggedness

A 50 µg/ml sample was prepared as described in Section D.3 and to test its stability, it was left on the autosampler tray and analysed with the HPLC at hourly intervals up to 24 h. Sample solutions should not be used for a period longer than it takes to degrade by 2%. As can be seen from Table D.4, ketoprofen was stable over a 24 h period.

Table D.4: The stability of ketoprofen over 24 h

Time (h)	Peak area of ketoprofen	Percentage (%)
0	9033.43	100.0
1	9002.35	99.7
2	9036.57	100.0
3	9020.13	99.9
4	8962.93	99.2
5	8924.97	98.8
6	8935.47	98.9
7	8896.73	98.5
8	8899.05	98.5
9	8926.39	98.8
10	8952.73	99.1
11	8932.27	98.9
12	8954.00	99.1
13	8923.54	98.8
14	8936.85	98.9
15	8966.64	99.3
16	8992.93	99.6
17	8968.02	99.3
18	9015.82	99.8
19	9001.66	99.6
20	9019.19	99.8
21	9031.78	100.0
22	9078.89	100.5
23	9068.27	100.4
24	9069.69	100.4
Mean	**8982.0**	**99.4**
SD	**53.12**	**0.59**
%RSD	**0.59**	**0.59**

D.5.5 System repeatability

In order to evaluate the repeatability of the peak area and retention time, a sample with a concentration of 25 µg/ml was injected six consecutive times on the HPLC (USP, 2013a:985). The peak area and retention times should have a percentage RSD of 2% or less (Du Preez, 2010:5) and results can be seen in Table D.5. The variation in response (percentage RSD) was within the accepted range with a value of 0.81% for the peak area and 1.10% for retention time.

Table D.5: Variations in response (percentage RSD) concerning the peak area and retention time of ketoprofen

Injection number	Peak area	Retention times (min)
1	4704.85	3.05
2	4665.81	3.04
3	4690.01	3.07
4	4642.26	3.06
5	4609.96	3.00
6	4604.30	2.98
Mean	4652.87	3.03
SD	37.78	0.03
%RSD	0.81	1.10

D.5.6 Specificity

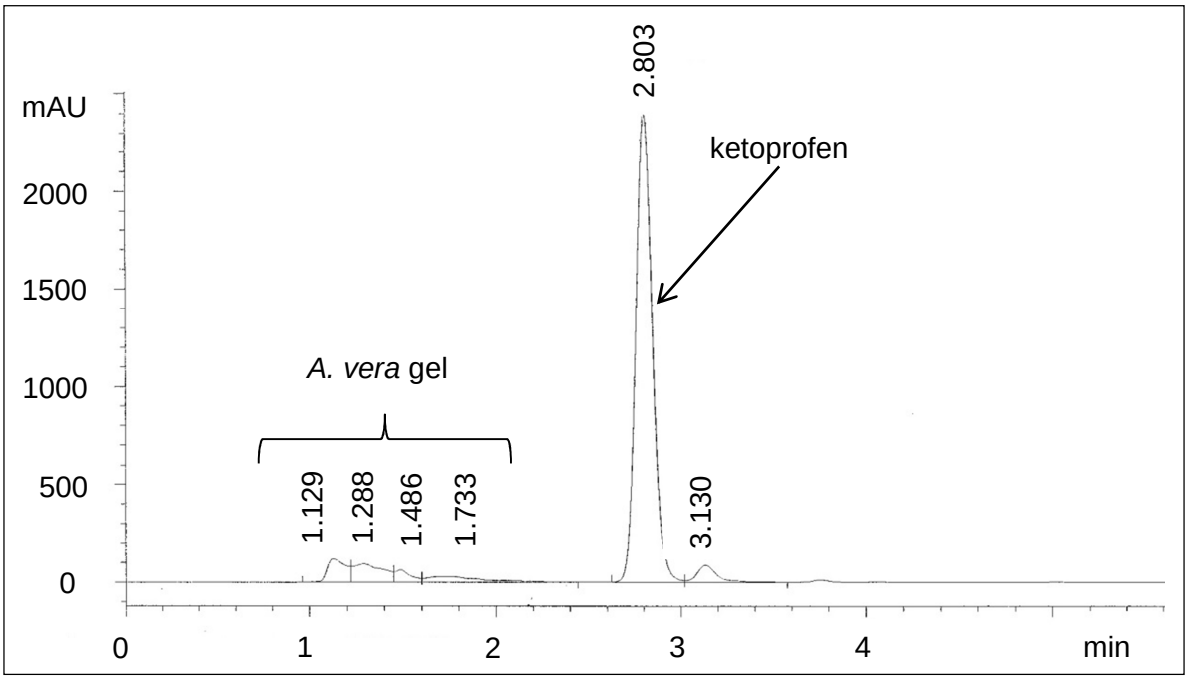

Figure D.2: HPLC chromatogram illustrating the retention time of ketoprofen in the presence of *A. vera* gel

Specificity is the ability to evaluate, unequivocally, the analyte in the presence of components that may interfere with the detection of the analyte (ICH, 2005:4). The method is specific when no interfering peaks with the same retention time as the drug are detected. Therefore, a solution was prepared which represents the donor solutions utilised during the membrane release and skin diffusion studies (Appendix E).

The solution was prepared with a ketoprofen concentration of 50 µg/ml, containing 0.5% (w/v) aloe leaf material, 10% (v/v) ethanol and PBS (pH 6.5; 11.99 g H_2NaO_4P in HPLC-grade water, pH adjusted with 2 M NaOH, BP, 2013b:1). It was subsequently filtered and transferred to HPLC vials. Figure D.2 illustrates the retention times and peaks obtained for ketoprofen in the presence of *A. vera* gel. Only one chromatogram was included in this appendix to serve as an example as the rest of the aloe leaf materials showed similar results/chromatograms.

D.6 Application in Franz cell diffusion studies

Methanol was replaced by ethanol, which is a more appropriate option for transdermal diffusion studies, as the solvent in the preparation of the standards and test samples during the membrane release and skin diffusion studies. During the membrane release and skin diffusion studies there was an interfering peak that eluded at approximately the same time as ketoprofen. Therefore the mobile phase was adjusted proportionally to yield a mixture of 60 volumes of acetonitrile, 39 volumes of HPLC-grade water and 1 volume of acetic acid.

According to the USP (2013b:273), it is permissible to adjust the mobile phase, although some limits apply: the amounts of the components in the mobile phase can be adjusted by ± 30% relative, although the change of any component may not exceed ± 10% absolute (i.e. in relation to the total mobile phase) (USP, 2013b:273). Therefore, it was acceptable to adjust the acetonitrile as mentioned because it was within the range of 10%. This adjusted mobile phase caused the ketoprofen peak to elute at approximately 4.2 min.

D.7 Conclusion

The developed HPLC method for ketoprofen was found to be reliable and sensitive enough for the determination of the concentration ketoprofen present in the membrane release and skin diffusion samples. The results of the various validation parameters contained in this appendix show that all the criteria for the validation parameters were met with the method used to analyse the ketoprofen.

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USP ***see*** UNITED STATES PHARMACOPEIA.

Appendix E

Membrane release and skin diffusion studies

E.1 Introduction

As the largest organ of the body, the skin functions as a protective barrier to the external environment (Black, 1993:145). The outer layer of the skin, known as the SC, serves as a physical barrier to both the permeation of xenobiotics into the body and the evaporation of water out of the body (Jain *et al.*, 2006:320). The delivery of drugs via the transdermal route of drug administration offers some advantages, which include avoiding first-pass metabolism, needing less frequent dosage regimens, availability of a relatively large surface area for absorption and increased patient acceptability due to availability of non-invasive delivery systems (Roy, 1997:139-140; Naik *et al,.* 2000:319). Therefore, in order to exploit these advantages of the transdermal route of drug administration, the barrier that the SC provides needs to be overcome (Jain *et al.*, 2006:320).

Penetration enhancers can be used to enhance the penetration rate of drugs across the skin (Barry, 2002:522) by means of two possible mechanisms of action. Firstly the penetration enhancer increases the solubility of the drug within the SC (in other words it alters partitioning of the drug into the SC) and secondly the enhancer can disrupt the ordered nature of the skin lipids (which consequently influences diffusion across the SC) (Thomas & Finnin, 2004:700; Behl *et al.,* 1993:250). The properties of an ideal penetration enhancer include the following: (1) it should be pharmacologically inert, (2) it should be non-allergenic, non-irritating and non-toxic, (3) it should be compatible with all excipients and drugs, (4) its action should be immediate and its effect should be predictable, (5) it should be colourless, inexpensive and odourless, (6) it should not instigate the loss of any endogenous materials such as fluids and electrolytes, (7) it should be a good solvent for drug molecules, (8) it should be cosmetically tolerable and (9) its action should be reversible (Barry, 2002:522).

The exploitation of natural products as safe and effective drug permeation enhancers has been receiving considerable attention (Fang *et al*., 2003:253). Due to the humectant and 'skin friendly' properties of aloe leaf materials, the use of this natural product can be considered as a promising prospective (Meadows, 1980:56; Cole & Heard, 2007:10). Numerous patents (US 20040219194, US 20040001882, CA 2534338) also claim that *A. vera* enhances the penetration of co-formulated drugs (Finckh *et al.*, 2004:2; Tisa-Bostedt *et al.*, 2004:5; Altenschoepfer *et al.,* 2005:1). *Aloe vera* has been extensively researched in general and it's skin penetrating enhancement effects were investigated to some extent, however, it is important

for researchers to investigate the possible pharmaceutical applications of other aloe species (Loots *et al.,* 2007:6891), such as *A. marlothii* and *A. ferox.*

In vitro models, to screen for penetration enhancing effects of compounds, have a major advantage over *in vivo* investigations as the experimental conditions can be controlled precisely with the only variables being the skin and the test material (Walters & Brain, 2008:38). A high correlation exists between *in vivo* and *in vitro* data for compounds penetrating and permeating human skin adding support to the usefulness of the more cost-effective *in vitro* techniques (Walters & Brain, 2008:43).

The aim of this study was to determine whether the whole leaf or gel materials of three selected aloe species (viz. *A. vera, A. marlothii* and *A. ferox*) enhanced the permeation of the marker compound ketoprofen across human skin and to compare the effects of the two aloe species indigenous to South Africa to that of *A. vera*. Prior to the *in vitro* diffusion studies across excised human skin, membrane studies were performed to determine the effect of different concentrations (3.00%, 1.50% or 0.75% (w/v)) of aloe leaf materials (i.e. the gel and whole leaf materials of the selected aloe species) on the release characteristics of the ketoprofen (Barry, 2002:518).

E.2 Materials and methods

E.2.1 Aloe leaf materials

The gel **(G)** and whole leaf **(WL)** materials of *A. vera* **(AV)**, *A. marlothii* **(AM)** and *A. ferox* **(AF)** were collected and processed as described in Appendix A. For aesthetic purposes and ease of reading, the abbreviations given above in the bold brackets will be used from here on in this appendix for the aloe leaf materials, e.g. **AVG** indicates to *A. vera* gel, etc.

E.2.2 Preparation of phosphate buffer solutions

The PBS (pH 7.4 and pH 6.5) used during this study was prepared according to the British Pharmacopoeia (BP, 2013a:1; BP, 2013b:1). PBS (pH 7.4) was prepared by mixing 250 ml of 0.2 M potassium dihydrogen orthophosphate (KH_2PO_4) with 393.4 ml of 0.1 M sodium hydroxide (NaOH). Subsequently the pH was adjusted with 10% phosphoric acid (H_3PO_4) (BP, 2013a:1). For the PBS (pH 6.5) 11.99 g sodium dihydrogen phosphate (H_2NaO_4P) was dissolved in 900 ml HPLC-grade water, the pH was adjusted with 2 M NaOH and thereafter diluted to 1000 ml with HPLC-grade water (BP, 2013b:1).

E.2.3 Sample analysis of ketoprofen by high performance liquid chromatography

A HPLC method was developed and validated for ketoprofen at the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom Campus to demonstrate that the method was sensitive and reliable in the determination of the amount of ketoprofen in the samples analysed (See Appendix D). The limit of detection was 0.001 µg/ml and the limit of quantification was 0.005 µg/ml. Samples were analysed using an Agilent® 1100 series HPLC equipped with an isocratic pump, an autosampler, a diode array detector and Chemstation Rev. A.10.01 software for data acquisition and analysis (Agilent Technologies, Palo, Alto, CA). An Agela® Venusil XBP C_{18} (2) column (4.6 x 150 mm) from Agela® Technologies (Newark, DE) with a 5 µm particle size was used.

The mobile phase was degassed using an ultrasonic bath prior to use and consisted of 600 ml acetonitrile (CH_3CN) and 10 ml acetic acid (CH_3COOH) filled up to 1000 ml with HPLC-grade water. The flow rate was set at 1 ml/min with an injection volume of 1 or 2 µl for the membrane release studies and 50 µl for the skin diffusion studies. The UV-detector was set at a wavelength of 255 nm to detect ketoprofen. The total running time for each sample was 6.5 min and the retention time of ketoprofen was approximately 4.2 min.

E.2.4 Standard preparation

During the membrane release studies as well as the skin diffusion studies standard solutions of ketoprofen was prepared and injected in the HPLC at different volumes in order obtain a calibration curve (used to calculate concentration values) prior to each HPLC analysis. Throughout the membrane release studies, approximately 25 mg ketoprofen was accurately weighed, dissolved in 5 ml ethanol and made up to volume with PBS (pH 7.4) in a 100 ml volumetric flask. Ten millilitres of this solution was diluted to 25 ml with the PBS (pH 7.4). This solution was injected at different injection volumes on the HPLC to obtain a calibration curve of between 250 and 4000 µg/ml.

Standard solutions of ketoprofen at different concentration ranges were used during the skin diffusion studies, as much lower flux values were obtained. The first (mother) solution was prepared by accurately weighing approximately 12.5 mg ketoprofen, which was transferred to a 100 ml volumetric flask, dissolved in 5 ml ethanol and subsequently made up to volume with the PBS (pH 7.4). For the second solution, 5 ml of mother solution was transferred and made up to a volume of 100 ml with PBS (pH 7.4). These two solutions were injected on the HPLC at different injection volumes in order to obtain a calibration curve with a concentration range of between 0.125 and 50.000 µg/ml.

E.2.5 Preparation of receptor and donor phase solutions

PBS (pH 7.4) containing 10% HPLC-grade ethanol was employed as the receptor phase for the membrane release as well as the skin diffusion studies. For relatively lipophilic compounds, such as ketoprofen (log P value of 1.0, pH 7.4) (Dollery, 1999:K18), it is recommended that a receptor fluid should be chosen into which the compound will freely partition (Collier & Bronaugh, 1991:47). Therefore 10% ethanol (v/v) was incorporated into the receptor fluid as it enhanced the solubility of ketoprofen and maintained sink conditions (Brain *et al.*, 1998:164). Prior to and during the membrane and skin diffusion studies, the receptor solution was kept at a temperature of 37 °C in a water bath.

The basic composition of the gel-like aloe-containing donor solutions can be seen in Table E.1. The solutions were adjusted to a pH of 6.5, similar to previous studies involving ketoprofen (Ceschel *et al*., 2002:39; Lodén *et al.*, 2004:24).

Table E.1: Composition of the gel-like aloe-containing donor solutions

Ingredients	Concentration
Ketoprofen	2.50% (w/v)
Ethanol (99%)	10.00% (v/v)
Aloe leaf material*	3.00% or 1.50% or 0.75% (w/v)
PBS (pH 6.5)	Up to 20 ml
2 M NaOH	Enough to adjust pH to approximately 6.5

*Either the gel or whole leaf materials of either *A. vera*, *A. marltohii* or *A. ferox*

The donor phase was prepared by dissolving the ketoprofen in HPLC-grade ethanol and adding a small volume of PBS (pH 6.5). Subsequently, the aloe leaf materials were added to obtain one of three possible concentrations: 3.00%, 1.50% or 0.75% (w/v). Next a small volume of PBS (pH 6.5) was added, the pH was adjusted to pH 6.5 with 2 M NaOH (as it was found that the ketoprofen tended to lower the pH of the solution) and the solution was made up to volume (20 ml) with PBS (pH 6.5).

All the solutions, with the various concentrations of the aloe leaf materials (i.e. 3.00%, 1.50% or 0.75% (w/v)), were tested for their release characteristics during the membrane release studies. Based on the results of the membrane release studies, only one concentration was chosen with which to conduct the skin diffusion studies. For the control study, ketoprofen (2.5% (w/v)) was dissolved in ethanol (10.0% (v/v)) and the solution was made up to volume (20 ml) with PBS (pH 6.5) and the pH was adjusted to 6.5 with 2 M NaOH (the addition of the ketoprofen lowered the pH of the solution). The donor solutions were pre-heated in a water bath at 32 °C prior to the membrane and skin diffusion studies.

E.2.6 Preparation of human skin membranes

Ethical approval for the procurement and exploitation of human skin was obtained from the Research Ethics Committee of the North-West University, under reference number NWU-00114-11-A5. Prior to the study, informed consent was obtained from all the donors. For the most relevant data, the use of human skin is recommended over animal skin (Bronaugh, 1998:158), therefore fresh, female Caucasian abdominal skin was collected after cosmetic surgery and frozen at -20 °C within 24 h after removal (Brain *et al.,* 1998:167). It is important that relatively 'hair-free' types of skin should be used in order to minimise damage to the hair follicles during processing, as severing of the hair follicles may lead to erroneously high penetration values (Brain *et al.,* 1998:167).

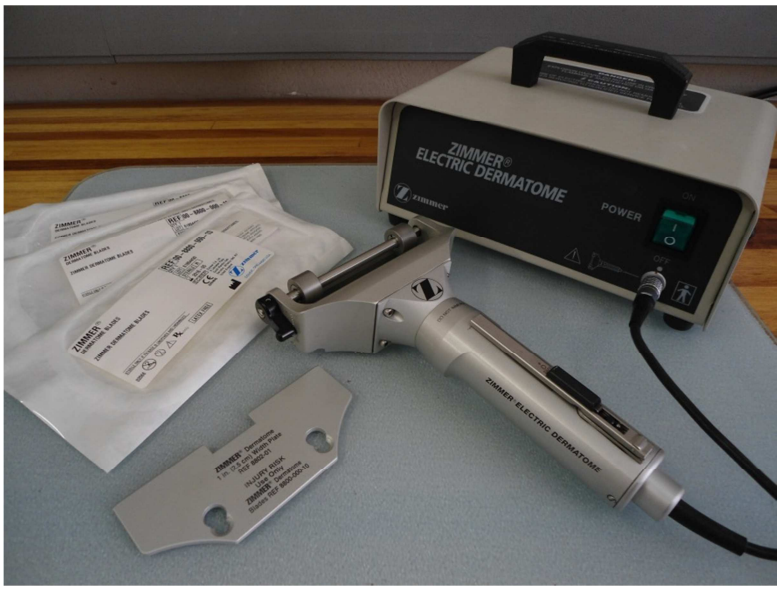


Figure E.1: Zimmer™ electric dermatome model 8821 with sterile blades and 2.5 cm width plate

Dermatomed skin was prepared according to a method previously described by Zender & Petruzzelli (2005:26) and Otto *et al.* (2008:328):

- Prior to processing, the skin (with attached subcutaneous fat layer) was thawed at room temperature.
- Tissue paper was laid on a glass surface and the skin was placed with the SC facing upwards.
- The surface of the skin was rinsed with deionised water to remove any blood or possible fat residue.
- The Zimmer™ electric dermatome model 8821 was fitted with a sterile blade and a 2.5 cm width plate (Figure E.1). It was set to cut the skin at a thickness of 400 µm to include the SC, viable epidermis and upper dermis.

- To harvest the skin, the dermatome was engaged at the donor site at a 30 – 45° angle while maintaining constant pressure and momentum throughout.
- When the required length of skin graft was cut, the hand piece was dropped parallel to the skin surface. If necessary the graft was cut with scissors from its attachment.
- All the harvested skin samples were examined for any defects (scars, stretch marks, etc.) and placed on Whatman® filter paper with the SC facing upwards.
- Subsequently the skin was wrapped in aluminium foil, placed in a Ziploc® bag and kept frozen (-20 °C) until used. The storage time did not surpass six months.
- Before the onset of the diffusion study, the frozen skin was thawed at room temperature and cut into circles with a diameter of approximately 15 mm.

E.2.7 Permeation experiments

Vertical Franz diffusion cells (Figure E.2 (a)) were used for the permeation experiments and consisted of a donor (top) and a receptor (bottom) compartment where the drug solution and receptor fluid were placed, respectively.

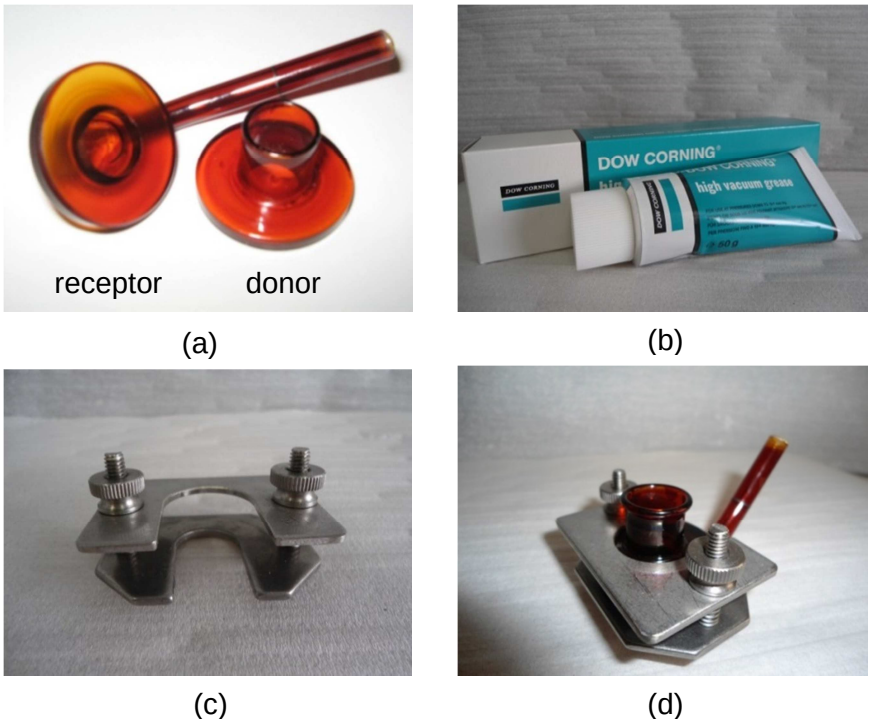


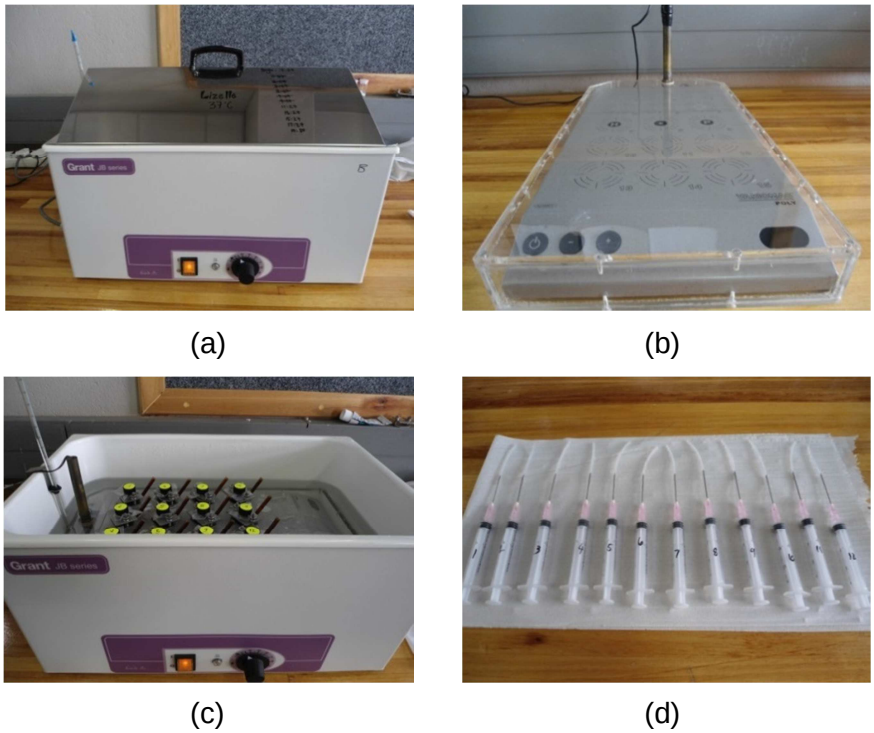
Figure E.2: Photographs illustrating (a) the donor and receptor compartments of a Franz diffusion cell, (b) Dow Corning® high vacuum grease, (c) horse-shoe clamp and (d) the assembled Franz diffusion cell

The donor compartments were filled with 1 ml of the drug solution. The receptor compartments, with a diffusional area of 1.13 cm^2, were filled with 2 ml of the receptor solution (prepared as

described in Section E.2.5). A small magnetic stirrer bar was placed in each receptor compartment to maintain stirring throughout the experiment. For the release and skin diffusion studies, cellulose nitrate membranes or dermatomed skin (SC facing upward) were respectively placed on the surface of the receptor compartment and covered by the donor compartment. In order to prevent any leakage, the Franz cells were sealed with Dow Corning® vacuum grease seen in Figure E.2 (b).

Horseshoe clamps (Figure E.2 (c)) were used to clamp the donor and receptor compartments together to ensure the skin/artificial membrane was firmly mounted. A picture of the assembled Franz cell can be seen in Figure E.2 (d). The receptor compartments were carefully filled with the receptor phase to avoid the entrapment of air bubbles under the artificial membrane or skin. The donor compartments were filled with 1 ml of the drug solution (32 °C) and subsequently covered with Parafilm® and a plastic cap to prevent evaporation for the duration of the experiment. Two Franz cells with donor solutions containing no ketoprofen were used as the negative control (PBS, pH 6.5 with 10% ethanol (v/v)).

The Franz cells were then positioned on a tray and placed in a Grant water bath (Figure E.3 (a)) on a submersible Variomag® magnetic stirrer plate (Figure E.3 (b)).



(a) (b)

(c) (d)

Figure E.3: Photographs illustrating (a) the Grant water bath, (b) Variomag® magnetic stirrer plate, (c) assembled Franz Cells in water bath and (d) syringes used to withdraw samples from the receptor compartments

The water bath was maintained at 37 °C to ensure that a skin temperature of 32 °C was attained. Figure E.3 (c) shows the assembled Franz cells on the magnetic stirrer plate in the

water bath. The entire receptor compartment fluid volume of each Franz cell was withdrawn and replaced with fresh receptor solution (preheated to 37 °C) at specific time intervals (see Sections E.2.7.1 & E.2.7.2). Syringes with plastic tubing over the needle (Figure E.3 (d)) were used for this purpose. The withdrawn samples were placed in vials and analysed by means of HPLC to determine the concentration ketoprofen that permeated the artificial membranes/skin.

E.2.7.1 Membrane release studies

Prior to the skin diffusion studies, membrane release studies were performed as described in Section E.2.7. The aim of the membrane release studies was to determine at which concentration (i.e. 3.00%, 1.50% of 0.75% (w/v)) aloe leaf material in solution (with a gel-like structure) released ketoprofen at the highest rate. Porafil® cellulose nitrate filters with a pore size of 0.20 µm (Separations, Johannesburg) were utilised during these studies. The entire receptor phase was extracted hourly for 6 h to be analysed for ketoprofen concentration and was replaced with fresh preheated (37 °C) receptor phase after each withdrawal (Section E.2.5).

E.2.7.2 Skin diffusion studies

The skin diffusion studies were also performed according to the method described in Section E.2.7. The aim of these studies was to investigate the penetration enhancing effects of the different aloe leaf materials across skin by determining the flux of the marker compound, ketoprofen. Following the results of the membrane release studies, only one concentration of the aloe leaf materials was selected for testing permeation enhancing effects across the skin. The entire volume of the receptor compartment was withdrawn and replaced with fresh preheated (37 °C) receptor phase (Section E.2.5, pH 7.4 containing 10% ethanol) after 20, 40, 60, 80 and 100 min, as well as 2, 4, 6, 8, 10 and 12 h. Tape stripping was performed after the completion of the skin diffusion study (Section E.2.5) to determine the content of ketoprofen in the different outermost layers of the skin.

E.2.8 Tape stripping

Tape stripping is a well-established technique used to examine the penetration of topically applied substances into the uppermost layers of the skin (Klang *et al*., 2012:604). It involves the removal of the outermost layer (SC) of the skin in a stepwise manner by adhesive films (Purdon *et al*., 2006:299). Tape stripping was performed according to a method previously described by Pellet *et al.* (1997:94). After the final 12 h withdrawal of the receptor phases and the removal of the donor phases, the cells were gently taken apart. The skin was pinned on a piece of Parafilm® to a solid surface and subsequently dabbed dry with tissue paper to remove any remaining drug solution present on the surface of the skin. 3M Scotch® Magic™ tape was

cut into pieces in order to cover the exposed diffusional area, whilst not overlapping the areas outside of the diffusion cell imprints. The first tape strip was disposed of as it was regarded as part of the cleaning procedure and may have been contaminated with the drug solution that adhered on the surface of the skin. The succeeding 15 tape strips (SC-epidermis) were placed in a polytop filled with 5 ml of a 40% ethanol in PBS (pH 7.4). An indication that the SC was completely removed is the glistening of the viable epidermal layer. To determine the ketoprofen content in the rest of the skin, excess skin was trimmed away from the flange imprints of the diffusion cells and the remaining skin (epidermis-dermis) was cut into small pieces to enlarge the surface area (Pellet *et al.,* 1997:94). It was then placed in a polytop containing 5 ml of a 40% ethanol in PBS (pH 7.4). The polytops containing the samples were kept overnight at 4 °C. The samples were filtered by utilising 0.45 µm syringe filters and were transferred to vials and assayed by HPLC to determine the concentration ketoprofen.

E.2.9 Data analysis

E.2.9.1 Transdermal data analysis

The cumulative amount of ketoprofen per membrane/skin surface area that permeated into the receptor chamber were plotted against time (h) for both the membrane release and skin diffusion studies, respectively. The average flux was obtained from the slope of the linear portion of the curve. The average percentage released/diffused from the total amount applied to the donor compartment of the Franz cells was determined for both the membrane release and skin diffusion studies. The enhancement ratio (ER) was obtained by dividing the average flux of ketoprofen from the aloe containing solution by the average flux of ketoprofen from the control group (Cole & Heard, 2007:12).

The lag time was determined from the data of the skin diffusion studies by extrapolating the steady-state portion of the line to the time axis (Martin, 1993:328). The lag time can be defined as the time that is needed for a penetrant to ascertain a uniform concentration gradient within the membrane (i.e. the skin), which is separating the donor and receptor compartments from one another (Martin, 1993:328).

The permeation profiles were further analysed as previously done by Otto *et al.* (2008:330) by applying the data to a non-linear curve fitting procedure described by Díez-Sales *et al.*, (1991:3). The following equation (Scheuplein, 1967:82) was used to fit the data:

$$Q(t) = AKhC_v \left[D\frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n-1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(\frac{-Dn^2\pi^2 t}{h^2}\right)\right] \qquad \textbf{Equation E.1}$$

Where Q(t) is the quantity of the active substance that permeates the skin within time t. A is the diffusion area (1.13 cm^2), K is the partition coefficient of the active between the skin and vehicle,

h is the diffusional path length, D is the diffusion coefficient of the active across the skin and C_v is the actual concentration of the active in the donor vehicle. As t approaches infinity, the exponential term becomes insignificant and Equation E.1 can be simplified to:

$$Q(t) = AKhC_v \left[D\frac{t}{h^2} - \frac{1}{6}\right] \qquad \textbf{Equation E.2}$$

As K and D are unknown, the products K x h and D/h^2 were replaced by α and β, respectively, which were determined by fitting Equation E.2 to the obtained permeation plots using a computerised non-linear least square method (EasyPlot, Spiral Software, Norwich, Vt., USA). The permeability coefficient (k_p) were calculated using Equation E.3.

$$k_p = \frac{KD}{h} \; (= \alpha\beta) \qquad \textbf{Equation E.3}$$

E.2.9.2 Statistical data analysis

Statistical analyses for the data obtained during the membrane release, skin diffusion and tape stripping studies were carried out using Statistica (data analysis software system), version 11 (StatSoft, Inc., 2013). Descriptive as well as inferential statistics were employed to analyse the data. Descriptive statistics consisted of methods and procedures used to summarise the data and involved the calculation of the average (with standard deviation) and median (middle score in a distribution) of the flux values (Sheskin, 2000:1, 4; Millar, 2001:23). Box-plots were used to depict the data graphically. The box-plots were constructed by using the median and the first and third quartiles of the distribution (Dawson & Trapp, 2004:38). The box-plots were drawn with the top at the third quartile and the bottom at the first quartile. The length of the box visually represents the middle 50% of the data. The whiskers (straight lines) extend 1.5 times the interquartile range above and below the 75^{th} and 25^{th} percentiles. Outliers (values above or below the whiskers) can be seen as circles (e.g. Figure E.4) (Dawson & Trapp, 2004:39). Inferential statistics were employed to make predictions or draw conclusions about the data (Sheskin, 2000:1) by applying analysis of variance (ANOVA) and non-parametric hypothesis testing.

A three-way analysis of variance (ANOVA) was carried out with the data from the membrane release studies to determine the effects of the different factors (concentration, species and type, i.e. whole leaf or gel) and their interactions on the flux values. One-way ANOVA was used to assess whether or not there was a difference between at least two averages in the data from the different aloe leaf material solutions (Sheskin, 2000:511). Post-hoc comparisons, to determine exactly between which average values the differences lay, were performed using Tukey's HSD (honestly significant difference) test. The Tukey's HSD test is generally performed for unplanned comparisons when all possible pairwise comparisons in a set of data are made (Sheskin, 2000:534).

The transdermal skin diffusion data (average flux, α, β and permeability coefficient (k_p) values, lag times and tape stripping data) of each aloe leaf material were compared by applying the parametric one way ANOVA and post-hoc Tukey's HSD and Dunnett's t-tests with a 0.05 significance level. Parametric statistical tests are based on certain assumptions or parameters, which include the data samples are randomly drawn from a normal population, are measured using an interval or ratio scale, comprise independent observations, are of an acceptable size and approximately resemble a normal distribution (Corder & Foreman, 2009:2).

Therefore, normal probability plots were constructed in order to evaluate the normality of the data (Christensen, 1996:45). A normal probability plot is used to compare the distribution of data to the (standard) Gaussian distribution (Emerson, 1991:187). Therefore it would be expected that a plot of the pairs would give a line that goes through the origin with a slope of about 1 (Christensen, 1996:45).

Since it was found the data was skew and not close to normal distribution, the equivalent non-parametric test (i.e. the Kruskal-Wallis *H*-test with multiple comparisons) was applied (Corder & Foreman, 2009:100). A p-value < 0.05 indicates statistically significant differences between the values compared. It is important to note that the results from the Kruskal-Wallis multiple comparisons table revealed less statistical significant differences than the Tukey post-hoc test. Consequently the more conservative Kruskal-Wallis multiple comparisons test results were preferred.

E.3 Results and discussion

E.3.1 Membrane release studies

Results from the membrane release studies (Table E.2) indicated that ketoprofen permeated through the cellulose nitrate membranes from all the aloe-containing solutions investigated in this study. This suggests that all the aloe material-containing solutions released a portion of ketoprofen from their gel-like structures to different extends. However, the average flux values are influenced by skewed distributions around the central location and could therefore give an inaccurate estimation of the true flux values. Therefore, calculation of the median flux would give a better representation of the true flux value of ketoprofen from the different aloe leaf material solutions, as it is not affected by outliers in the data (Dawson & Trapp, 2004:30). The aforementioned can be observed in Figure E.4. When comparing the median flux values of the different aloe leaf material concentrations (0.75, 1.50 and 3.00% (w/v)) it was evident that the highest median flux values were depicted at the 0.75% (w/v) (lowest concentration aloe) for most of the solutions tested, except for AFG.

When comparing the different concentrations of aloe leaf materials with each other (i.e. 0.75, 1.50 and 3.00% (w/v)) it is evident that the 0.75% (w/v) concentration had the highest average percentage released of all the concentrations, except for AMWL and AFG, which had lower ketoprofen release. The highest percentage ketoprofen released at 0.75% (w/v) (the lowest concentration of the aloe leaf materials tested) was obtained with AVWL, followed by AVG, AMG, AFG, AMWL and lastly AFWL.

Table E.2: Average flux (µg/cm^2.h), median flux (µg/cm^2.h) and average percentage ketoprofen diffused from the different aloe material solutions through cellulose nitrate membranes after 6 h

Aloe leaf material	Concentration(w/v)	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)	Average %released
AVG	3.00%	1694.8 ± 102.954	1775.17	26.733
	1.50%	2508.6 ± 209.908	2524.73	39.171
	0.75%	2572.4 ± 208.945	2699.76	43.961
AVWL	3.00%	3173.4 ± 375.626	2724.13	38.833
	1.50%	1753.8 ± 119.413	2454.30	34.014
	0.75%	2941.0 ± 297.825	2928.72	47.032
AMG	3.00%	1747.5 ± 178.909	1930.78	29.290
	1.50%	1729.9 ± 91.526	1866.01	29.077
	0.75%	2282.2 ± 90.555	2367.88	35.710
AMWL	3.00%	2180.2 ± 127.121	2206.38	33.887
	1.50%	2148.5 ± 204.894	2206.42	33.557
	0.75%	2216.1 ± 139.911	2224.37	32.962
AFG	3.00%	2275.4 ± 149.469	2356.74	35.320
	1.50%	2178.4 ± 86.286	2172.69	32.623
	0.75%	2219.5 ± 108.341	2249.41	33.419
AFWL	3.00%	1663.2 ± 201.892	1795.52	27.097
	1.50%	2490.2 ± 275.723	1956.75	24.042
	0.75%	2066.7 ± 177.705	2138.62	32.115

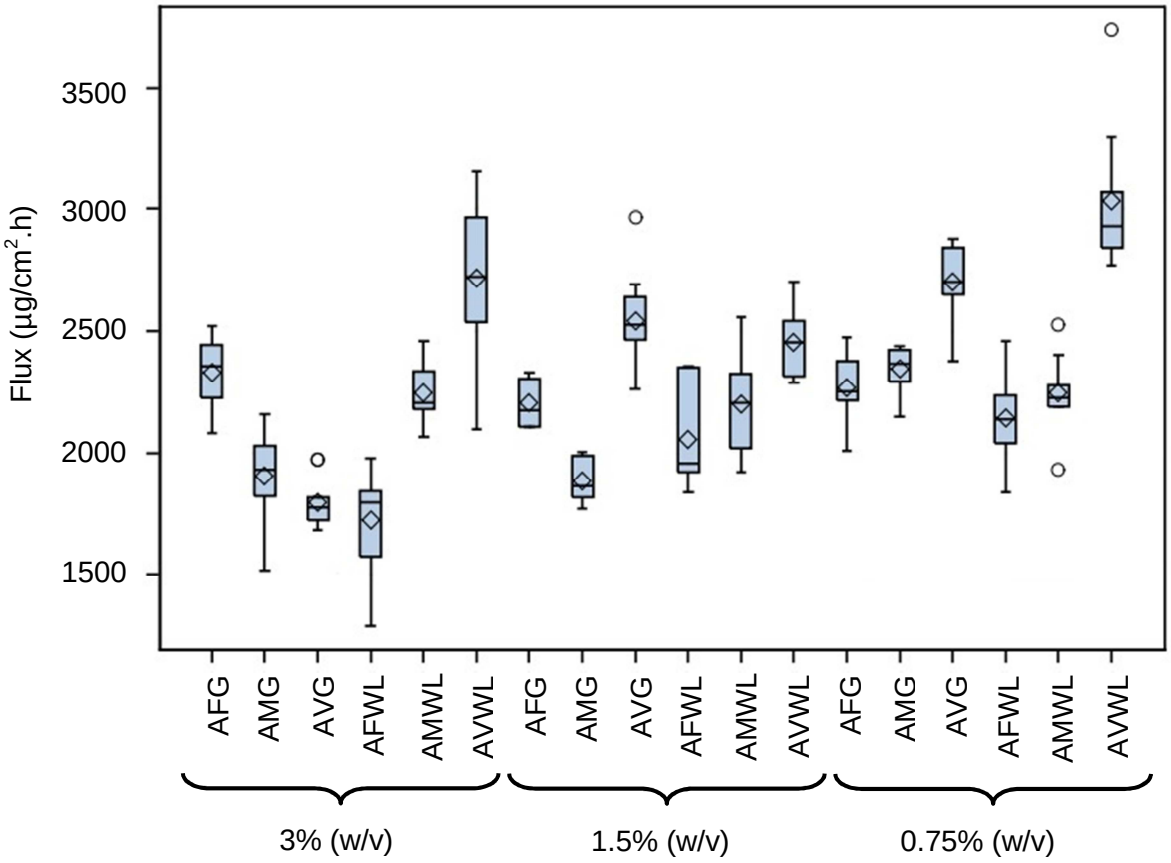


Figure E.4: Box-plots representing the cumulative concentration of ketoprofen of the different aloe leaf material solutions in the membrane diffusion studies. The average and median flux values are indicated by the diamond shape and line, respectively.

E.3.2 Transdermal skin diffusion studies

E.3.2.1 Flux, percentage ketoprofen diffused and enhancement ratio

Based on the results of the membrane release studies, it was decided to test the permeation enhancing effects of the various aloe leaf materials at a concentration of 0.75% (w/v). The average cumulative amount of ketoprofen per surface area (µg/cm^2) that permeated the skin was plotted against time and can be seen in Figures E.5, E.7, E.9, E.11, E.13, E.15 and E.17. The cumulative amount per area (µg/cm^2) of ketoprofen that permeated the skin for each individual Franz cell was plotted against time for all the aloe leaf materials and that of the control group and is seen in Figures E.6, E.8, E.10, E.12, E.14, E.16 and E.18. Table E.3 lists the average as well as the median flux values, average percentage diffused and the ER. The results can be seen graphically as comparative box-plots in Figure E.19 and illustrates the differences observed between the average (diamond shape) and median (line) flux values for the control group and each of the aloe leaf material solutions. The data will be investigated further in Section E.3.4.2 using statistical methods as described.

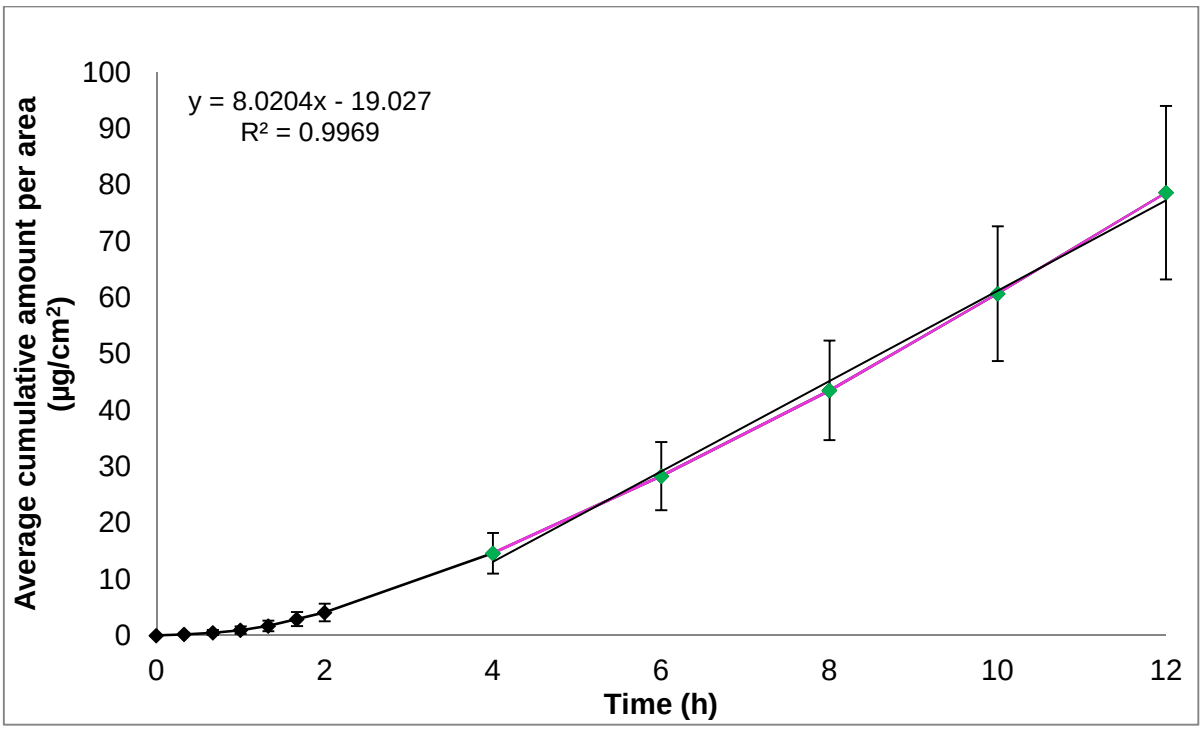


Figure E.5: Average cumulative amount per surface area (µg/cm^2) of ketoprofen alone (control group), which permeated the skin as a function of time to illustrate the calculation of the average flux from the linear part of the graph (4 – 12 h; n = 9)

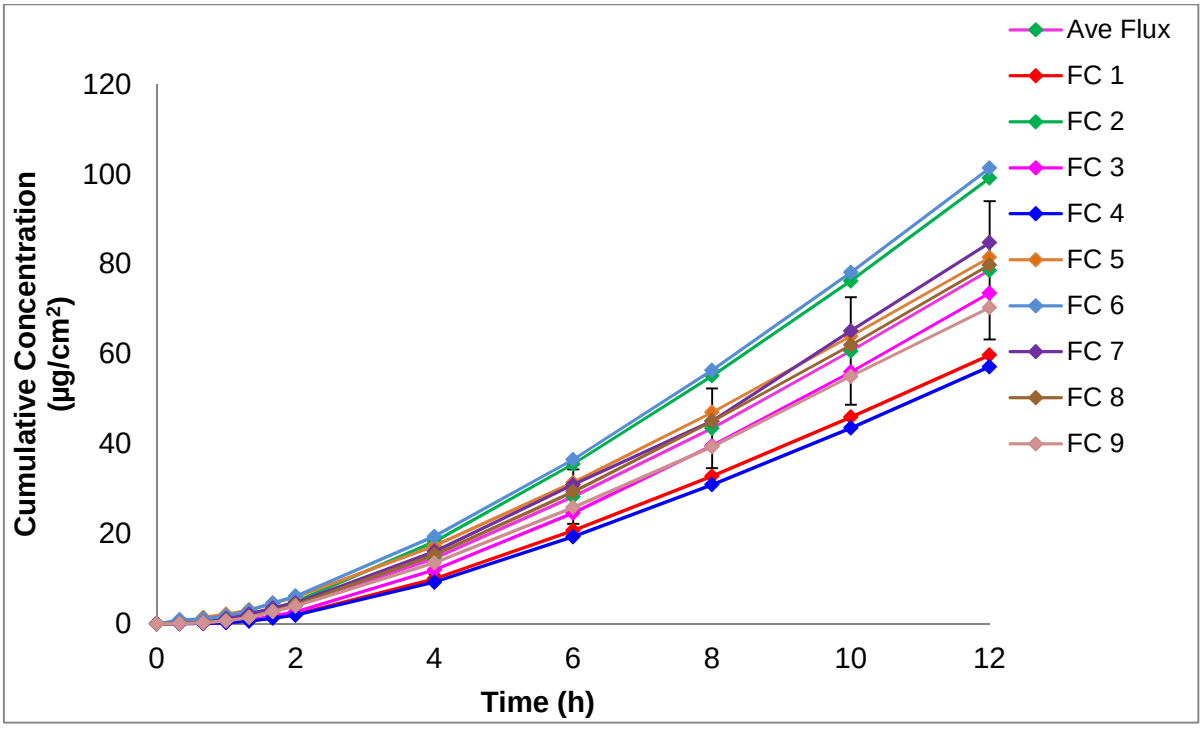


Figure E.6: Cumulative amount per surface area (µg/cm^2) of ketoprofen alone (control group), which permeated the skin as a function of time for each individual Franz cell

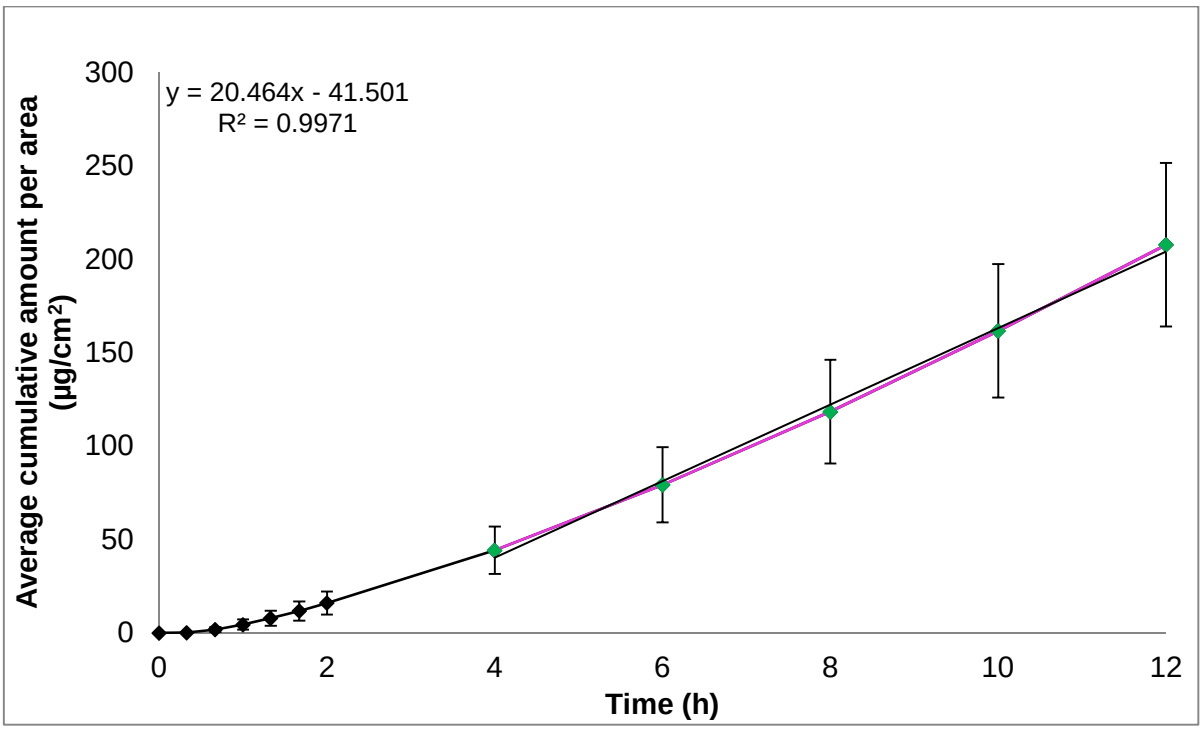


Figure E.7: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the calculation of average flux for the AVG solution from the linear part of the graph (4 – 12 h; n = 10)

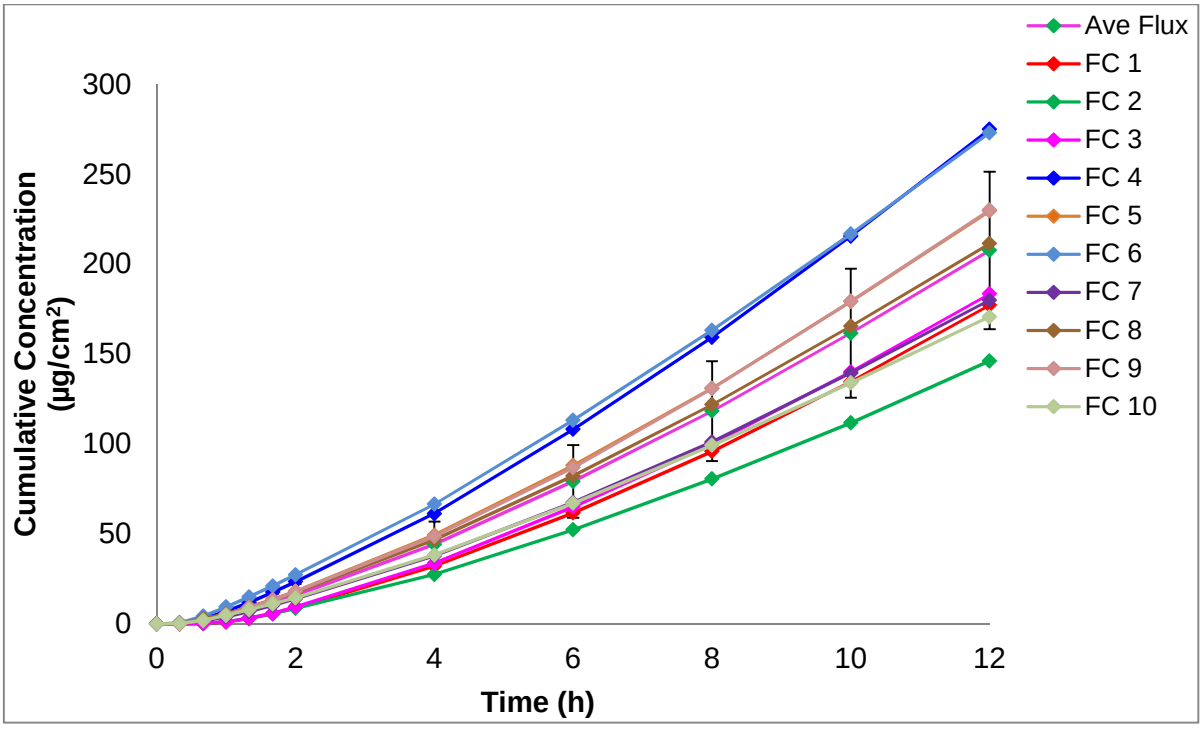


Figure E.8: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AVG solution

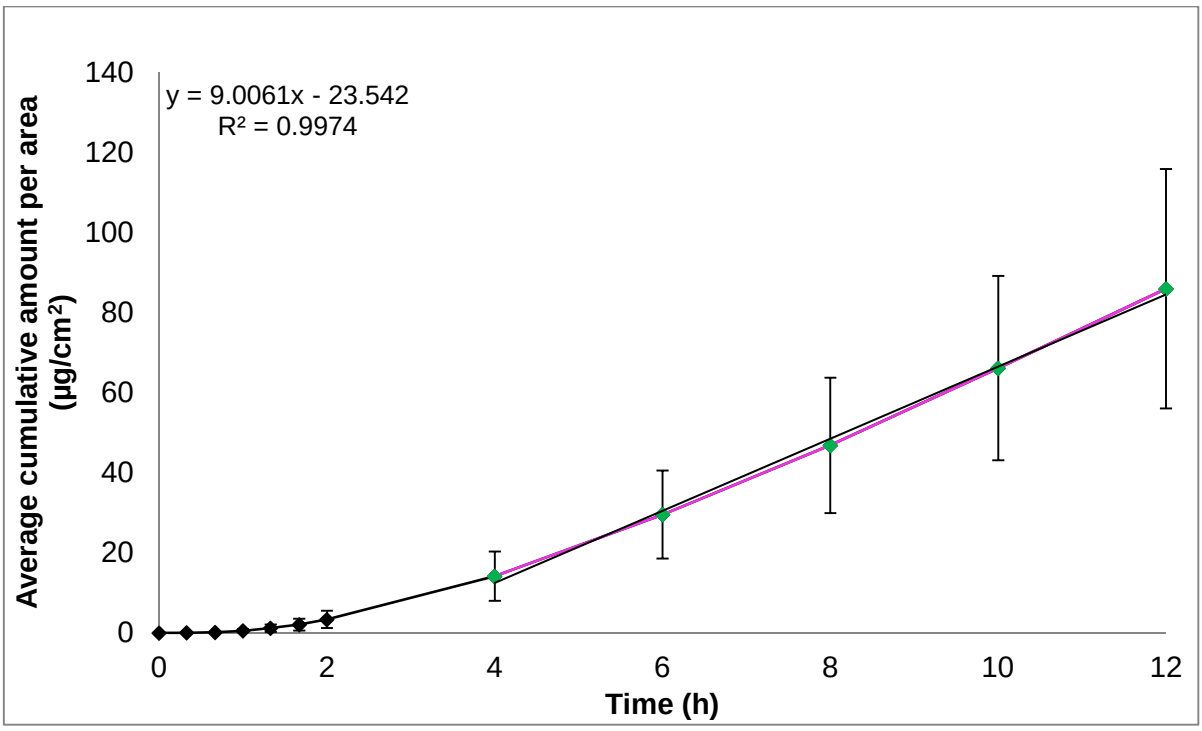


Figure E.9: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the calculation of average flux for the AVWL solution from the linear part of the graph (4 – 12 h; n = 10)

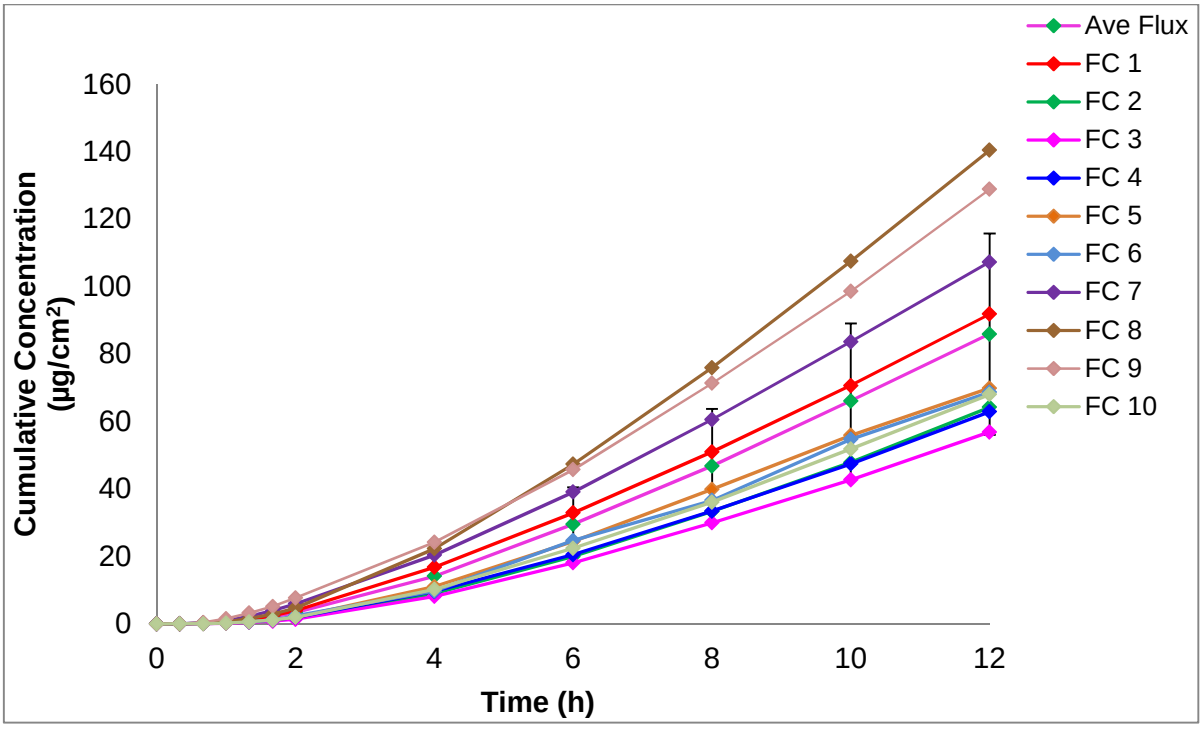


Figure E.10: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AVWL solution

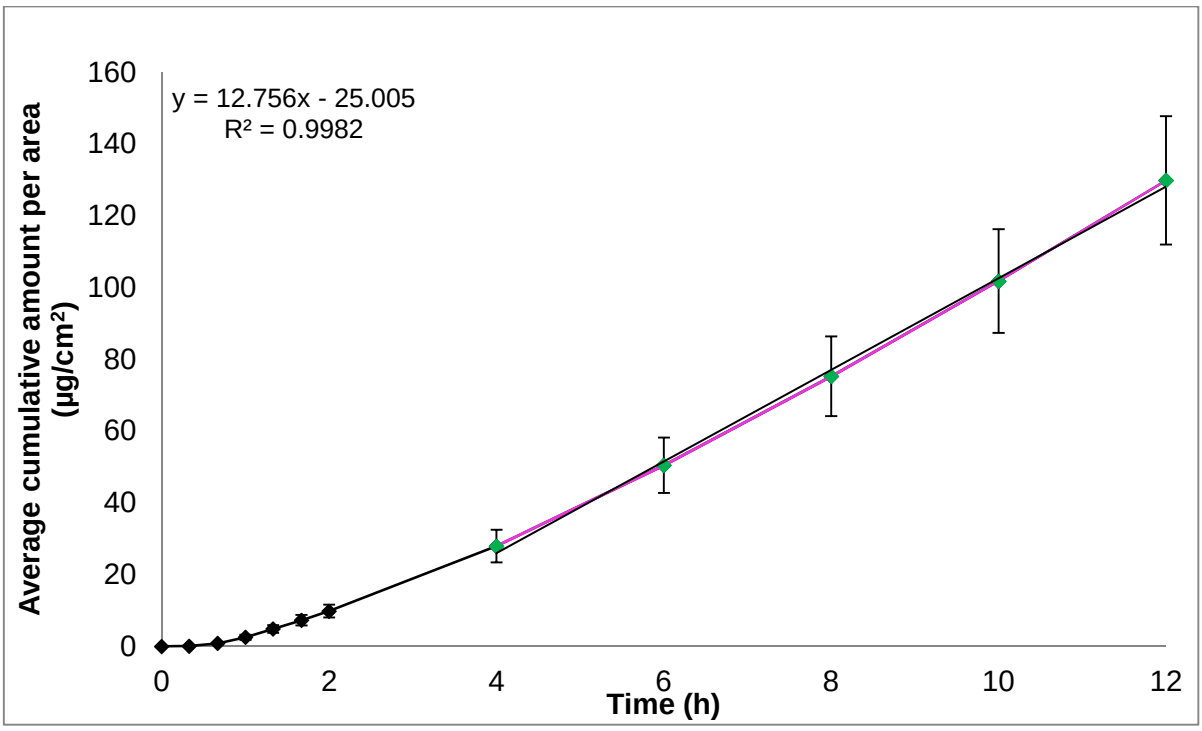


Figure E.11: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the calculation of average flux for the AMG solution from the linear part of the graph (4 – 12 h; n = 9)

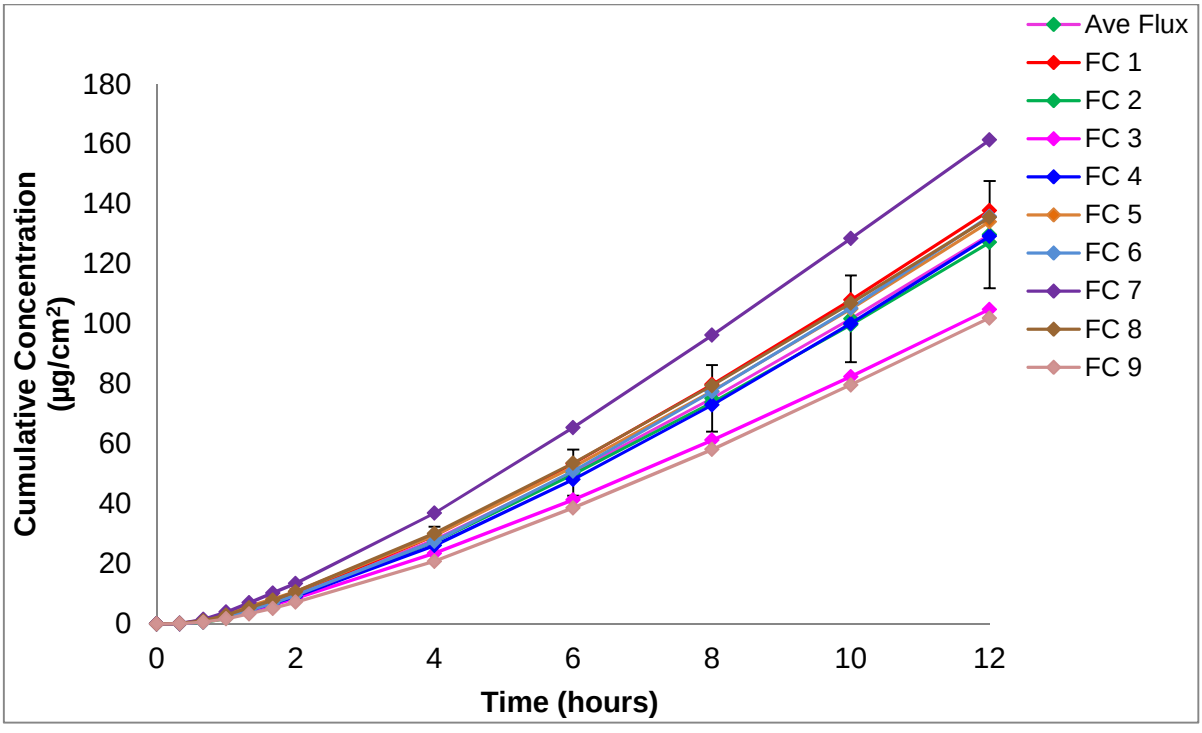


Figure E.12: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AMG solution

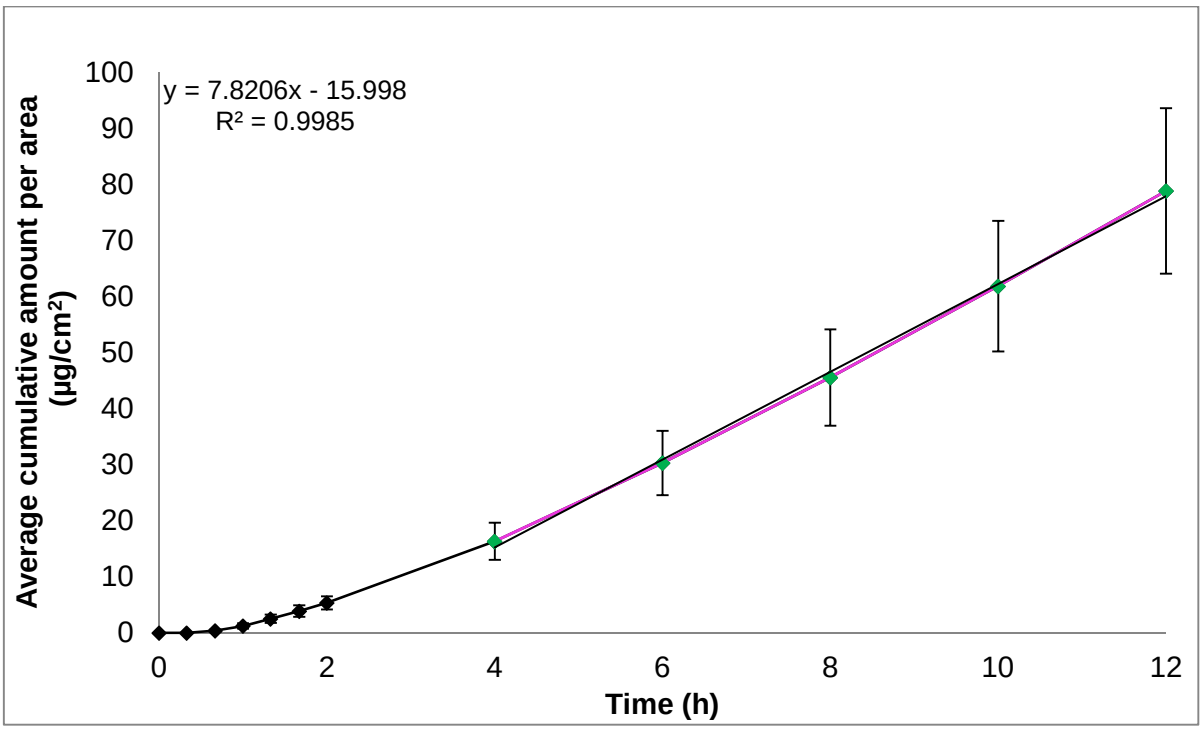


Figure E.13: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the calculation of average flux for the AMWL solution from the linear part of the graph (4 – 12 h; n = 10)

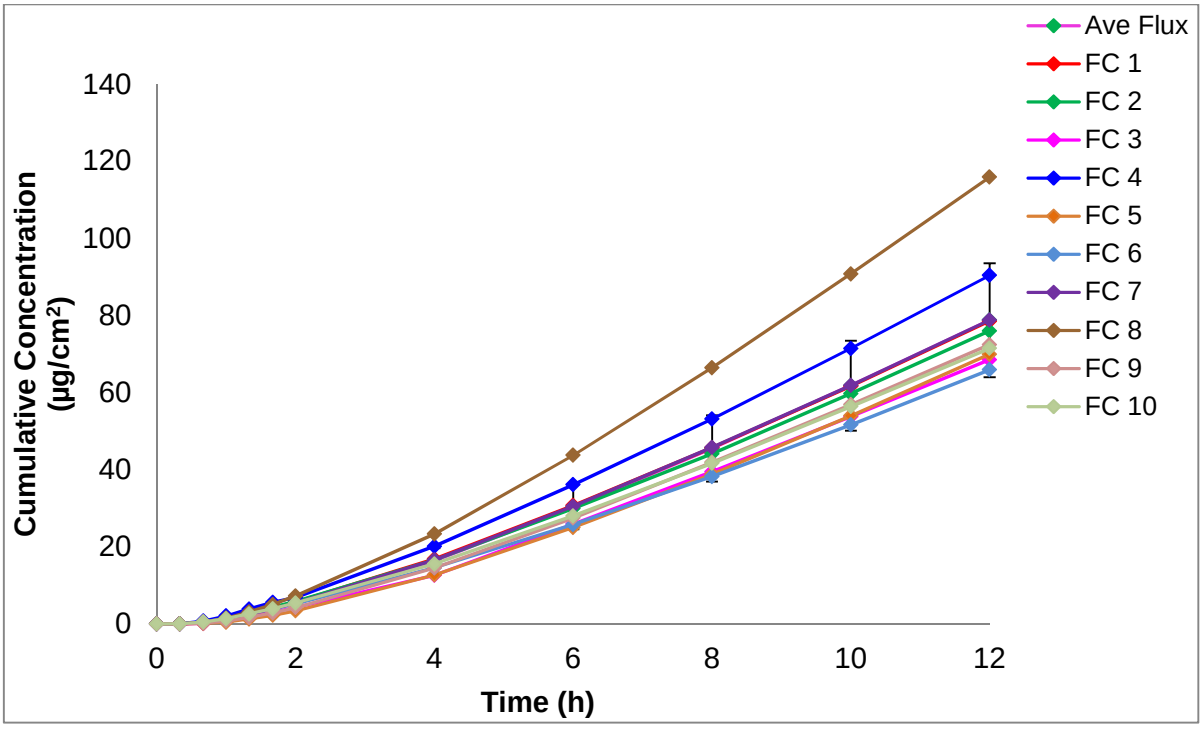


Figure E.14: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AMWL solution

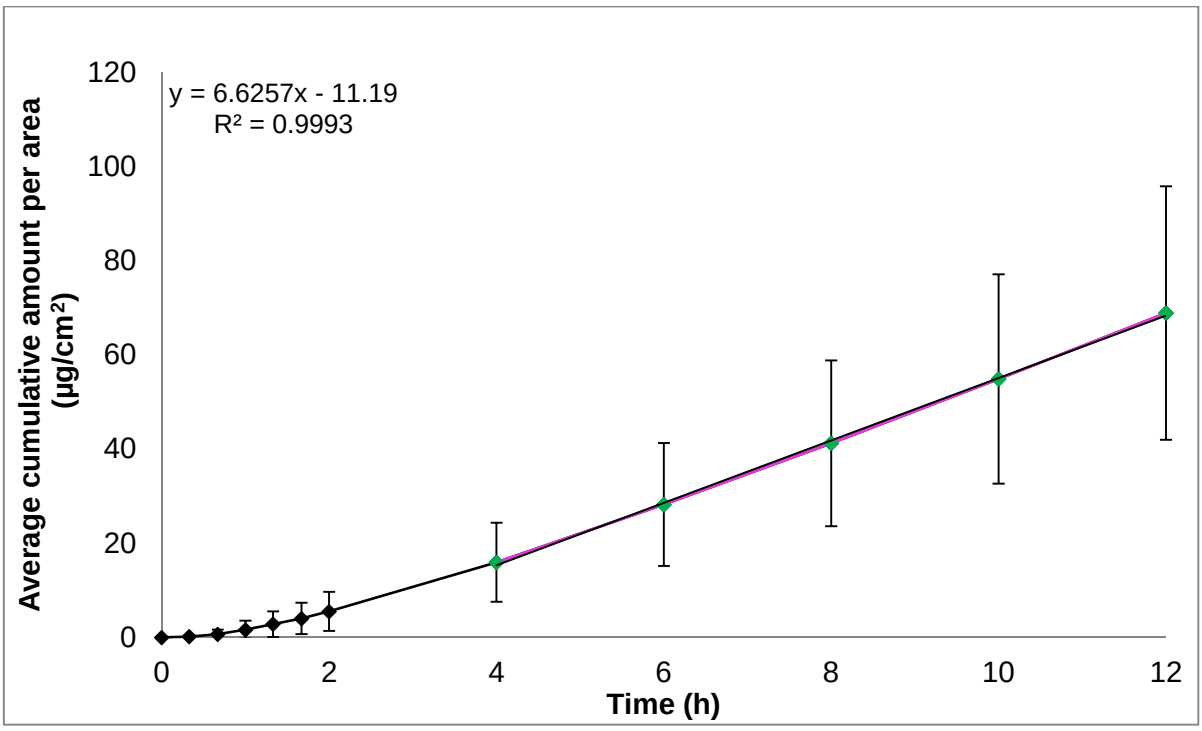


Figure E.15: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the average flux for the AFG solution from the linear part of the graph (4 – 12 h, n = 10)

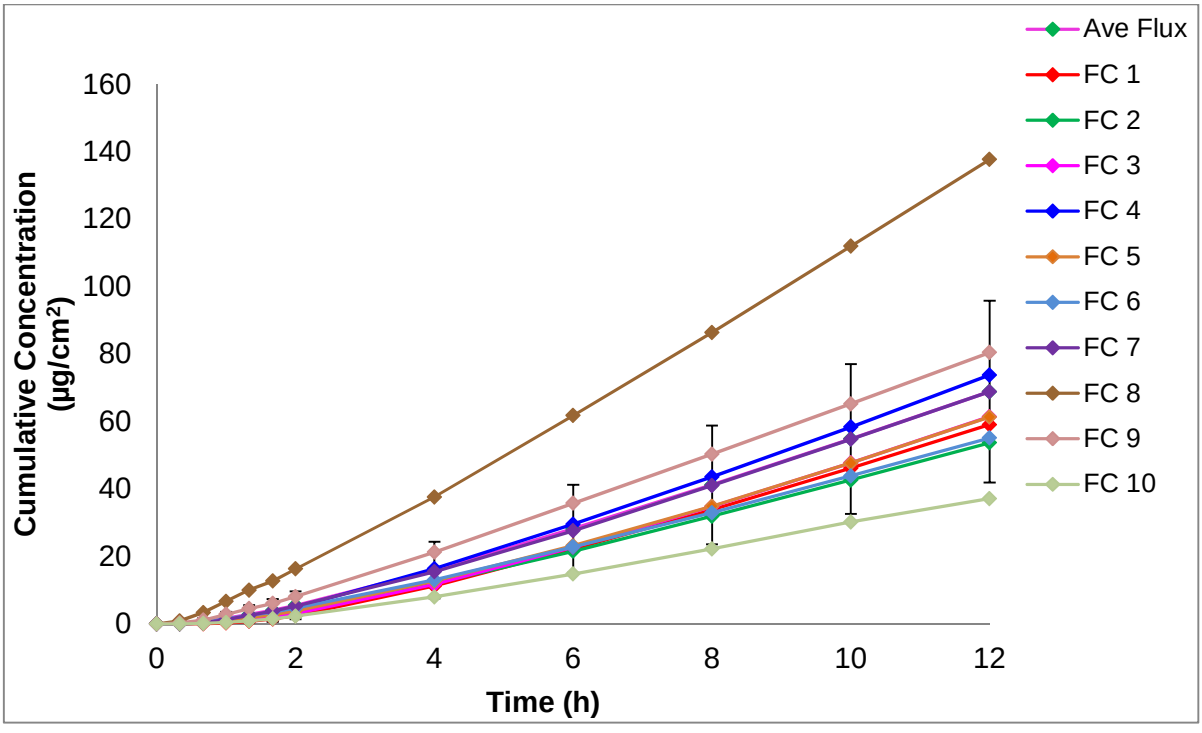


Figure E.16: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AFG solution

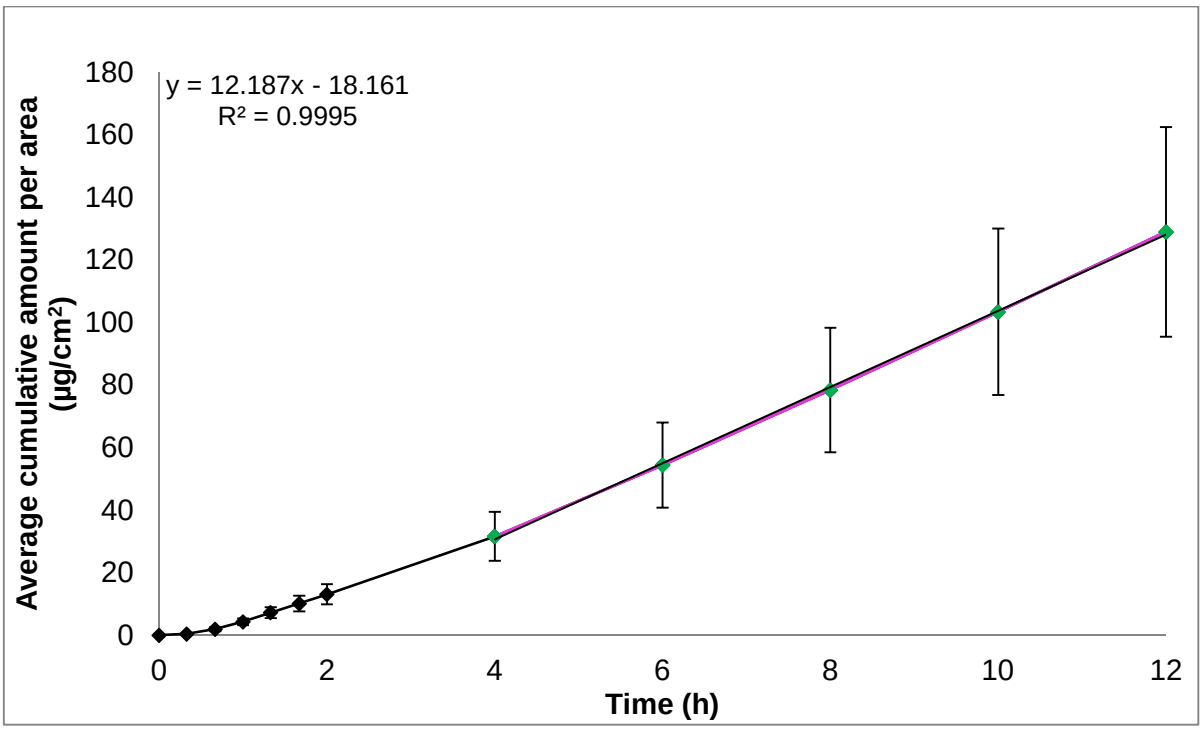


Figure E.17: Average cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time to illustrate the calculation of average flux for the AFWL solution from the linear part of the graph (4 – 12 h; n = 9)

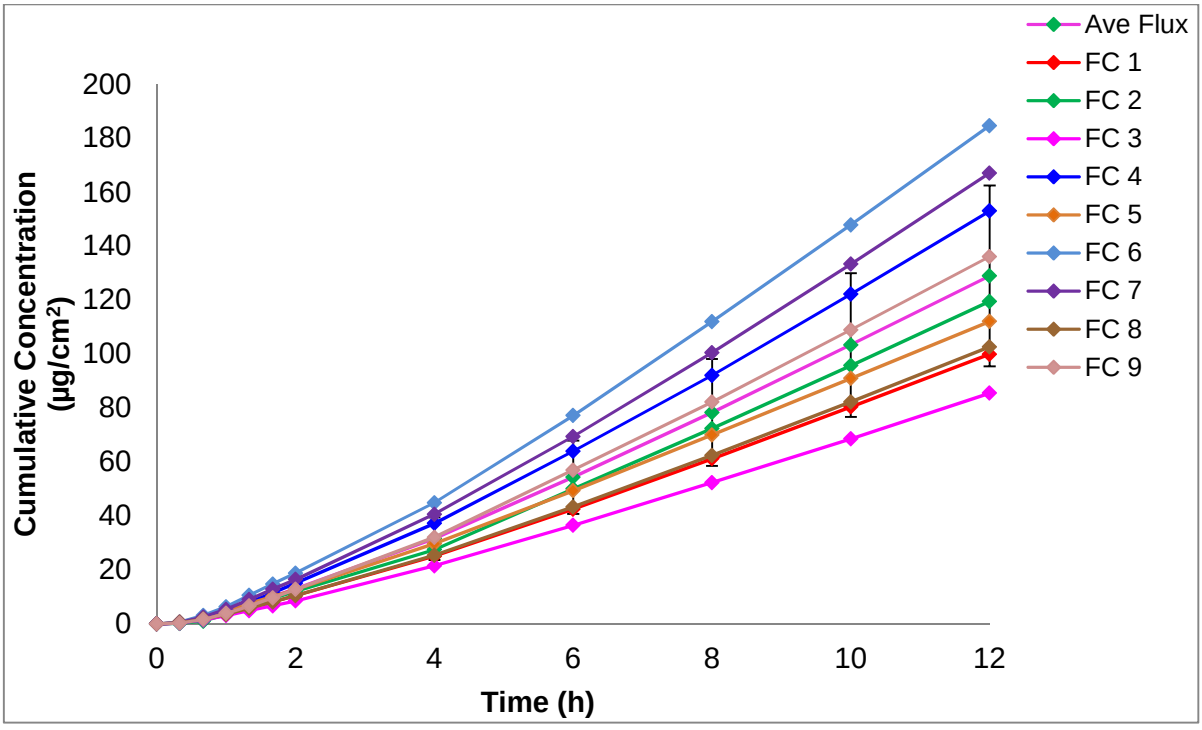


Figure E.18: Cumulative amount per surface area (µg/cm^2) of ketoprofen, which permeated the skin as a function of time for each individual Franz cell with the AFWL solution

Table E.3: Average flux (µg/cm^2.h), median flux (µg/cm^2.h), average percentage ketoprofen diffused and enhancement ratio (ER) values obtained from the different aloe leaf material solutions across skin over a 12 h period

Solution	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)	Average %diffused	ER
Control	8.020 ± 1.497	6.859	0.169	
AVG	20.464 ± 3.941*	16.776	0.446	2.551
AVWL	9.006 ± 2.997	6.040	0.185	1.123
AMG	12.756 ± 1.701	11.402	0.279	1.590
AMWL	7.821 ± 1.471	6.383	0.169	0.975
AFG	6.626 ± 2.332	5.261	0.148	0.826
AFWL	12.187 ± 3.229	10.237	0.277	1.520

*p < 0.05 (comparison to control)
Average flux values are with standard deviation

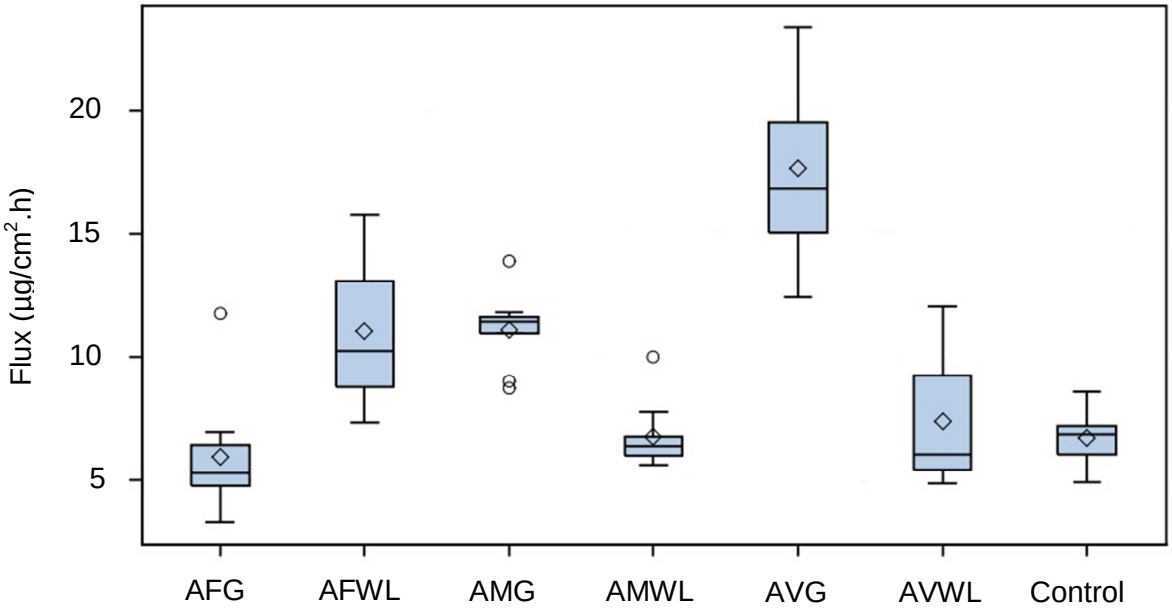


Figure E.19: Box-plots representing the cumulative concentration of ketoprofen of the various aloe leaf material solutions in the skin diffusion studies. The average and median flux values are indicated by the diamond shapes and lines, respectively.

AVG, AMG and AFWL had higher average flux values, median flux values, as well as a higher percentage ketoprofen diffused across the skin than the control group (ketoprofen alone) after 12 h as can be seen from Table E.3. AVWL, AMWL and AFG had similar or even slightly lower values (average flux, median flux and percentage diffused) than the control group. AVG (ER of 2.551) was superior to AMG (ER = 1.590) and AFWL (ER = 1.520) in enhancing the permeation of ketoprofen. These results were confirmed with the comparative box-plots (Figure E.19), where AVG showed the highest average and median flux values of ketoprofen through the skin

when compared to the other test materials. As can be seen, the distribution for most of the test materials (except AMWL) was distributed asymmetrical.

Cole & Heard (2007:11) previously investigated the *in vitro* skin permeation enhancement potential of *A. vera* juice by utilising porcine ear skin membranes and saturated solutions of various model drugs (i.e. 'within-vehicle') with different molecular weights and lipophilicities (i.e. caffeine, colchicine, mefenamic acid, oxybitynin and quinine). No link was found between the lipophilicity of the drug and the permeation enhancement effect of the *A. vera* juice, although *A. vera* juice had a higher permeation enhancement effect on drugs with a higher molecular weight (Cole & Heard, 2007:13). However, it was noticed that a significant proportion of certain *A. vera* constituents permeated the skin and the relative amount of permeation was inversely proportional to the drug's (administered with it) molecular weight (Cole & Heard, 2007:15).

Subsequently a mechanism was proposed whereby the smaller molecular weight drug has less opportunity to interact with the enhancing factor, as these smaller molecules are less efficient at blocking *A. vera* constituents from the permeation pathways, thereby causing the enhancing factor to permeate the skin. The penetration enhancing constituent(s) are therefore 'lost' from the solution. In contrast, a drug with a larger molecular weight will block the permeation pathways across the skin more effectively to allow increased possibility for the drug to interact with the enhancing factor and probably complex with it prior to being transported across the skin, i.e. permeation enhancement occurs by a 'pull effect' (Cole & Heard, 2007:15). It was also found that "double strength" *A. vera* juice, at a concentration of 3% (w/v), enhanced the permeation of quinine significantly higher when compared to the "standard" strength (Cole & Heard, 2007:15).

In another study, Ballam & Heard (2010:113) investigated the possibility of utilising *A. vera* juice as a pre-treatment to enhance the *in vitro* permeation of ketoprofen across pig ear skin. During their study, the *A. vera* juice was applied and subsequently removed after 1 h before dosing the skin with a saturated solution of ketoprofen in polyethylene glycol 400 (Ballam & Heard, 2010:114). The authors found that *A. vera* juice did not have penetration enhancing effects across the skin when used as a pre-treatment for the model drug ketoprofen. It was therefore suggested that the ketoprofen could not interact with the *A. vera* phytochemicals (i.e. enhancing factor) in the way described above by Cole & Heard (2007:11) when used 'within-vehicle' (Ballam & Heard, 2010:116). Results in this study showed the permeation enhancing effects of *A. vera* gel when ketoprofen was incorporated into the solution (i.e. 'within-vehicle'). It is possible that the ketoprofen could interact with the enhancing factor in the aloe-containing solutions in order to facilitate its transport across the skin.

In general, the 'pull' or 'drag' effect is due to the close relationship between the rates of permeation of the excipient and the solute (Heard *et al.*, 2006:167). The 'push-pull' mechanism has previously been described by Kadir *et al.* (1987:774) with the delivery of theophylline into excised human skin from alkanoic acid solutions. The 'pull' effect resulted from an increase in the solubility of theophylline in the membrane-propionic acid phase whereby it enhanced the drug's penetration (Kadir *et al.*, 1987:778). The effect where the permeation of the solute was facilitated by the permeation of the enhancer was also observed by Heard *et al.* (2006:167) when they investigated the skin penetration enhancement of mefenamic acid by 1,8-cineole and ethanol. Heard *et al.* (2003:168) suggested that the permeation rates observed are not only related to the permeant, but also to the permeation rate of the overall solvated complex formed by the solute molecule, which remains partially associated with its solvation cage.

The results in the present study showed that the gel materials (i.e. AVG and AMG) enhanced the permeation of ketoprofen, whereas the whole leaf materials (i.e. AVWL and AMWL) did not. Since the mucilaginous gel of the aloe leaves consists mainly of polysaccharides (Eshun & He, 2004:94), which are known skin penetration enhancers (Fox *et al.*, 2011:10532), it was expected that the gel materials of the different aloe species will have a greater permeation enhancing ability than the whole leaf materials. However, AFW had a much higher permeation ER (1.52) than the AFG (0.826) material.

These differences in the penetration enhancing ability of the different species of aloe leaf materials can possibly be linked to the differences in their chemical compositions, as verified with the ^{1}H-NMR spectra (Appendix A). Furthermore, there are certain known factors than may influence the composition of the aloe leaf materials and include location, soil, climate, species, different growth conditions and harvesting processes (Ballam & Heard, 2010:116; Capasso *et al.,* 1998:S125).

E.3.2.2 Curve fitting and lag times

The permeation profiles were analysed using a non-linear curve-fitting procedure (Díez-Sales *et al.,* 1991:3) to obtain α, β and k_p values which are presented in Table E.4. This data indicates how the aloe leaf materials possibly affected the permeability barrier of the skin (Hadgraft *et al.*, 2003:141). A change in α indicates an effect on the partition coefficient (K) and a change in β indicates an effect on the diffusivity (D) (with the assumption that *h*, the diffusional path length, is constant) (Otto *et al*., 2010:278).

Table E.4: Calculated α, β and the permeability coefficient (k_p) values after analysing the permeation profiles using a non-linear curve-fitting procedure as well the lag times of the different test materials (with standard deviation)

Solution	α	β	k_p (cm/h)	Lag time (h)
Control	0.0015 ± 0.0002	0.155 ± 0.016	0.00024 ± 0.00005	2.387 ± 0.214
AVG	0.0034 ± 0.0005	0.185 ± 0.028	0.00062 ± 0.00010	2.061 ± 0.290
AVWL	0.0018 ± 0.0006	0.139 ± 0.010	0.00026 ± 0.00009	2.655 ± 0.229
AMG	0.0021 ± 0.0003	0.183 ± 0.011	0.00039 ± 0.00005	1.965 ± 0.148
AMWL	0.0014 ± 0.0003	0.174 ± 0.014	0.00024 ± 0.00005	2.045 ± 0.185
AFG	0.0011 ± 0.0002	0.190 ± 0.061	0.00021 ± 0.00008	1.773 ± 0.411
AFWL	0.0016 ± 0.0005	0.244 ± 0.024	0.00039 ± 0.00010	1.474 ± 0.139

The higher α-values of AVG and AMG are a reflection that these two aloe leaf materials increased the skin partitioning of the ketoprofen when compared to the control group. Table E.4 shows that AVG and AMG gel had a higher permeability coefficient (k_p) than the control group which may be ascribed to the higher partitioning (higher α-value) of the ketoprofen into the outer layers of the SC.

AFWL had the highest obtained β-value (Table E.4) compared to the control group, followed by AFG and then AVG. This indicates these aloe leaf materials modified the diffusion characteristics of the skin toward ketoprofen (Hadgraft *et al*., 2003:141). The higher β-values also explain the higher permeability coefficient (k_p) obtained for AFWL and AVG. The k_p values obtained for AVWL and AMWL were similar to that of the control group.

AVG changed the values of both α- and β-values compared to those of the control group, therefore it had an effect on both the partitioning coefficient as well as the diffusion coefficient of ketoprofen. It is important to notice that the change in the α-value for AVG from that of the control group was much higher (more significant) than the β-value, indicating that AVG had a bigger effect on the partitioning of the drug than the diffusivity. The increase in β for AFG was counteracted by the reduced α (lower compared to the control group), which may be a possible explanation for why AFG did not enhance the flux of ketoprofen.

The lag times (Table E.4) of the different aloes and control group can be arranged in the following decreasing order: AVWL (2.655 h) > control group (2.387 h) > AVG (2.061 h) > AMWL (2.046 h) > AMG (1.965 h) > AFG (1.773 h) > AFWL (1.474 h). All the aloe leaf materials decreased the lag time, except for AVWL which had a slightly higher lag time than obtained with the control group. The AF species had the most significant effect/shortening of the lag time compared to the control group. No correlation can be seen between the lag time and the permeation enhancing effects of the aloe leaf materials.

E.3.3 Tape stripping

In order to facilitate reading, the SC-epidermis layer was referred to as **SCE** and the epidermis-dermis layer is referred to as the **ED** in the rest of the appendix. The average and median concentrations of ketoprofen in the epidermis and dermis for the various test solutions are shown in Table E.5.

Table E.5: The average and median concentration (µg/ml) ketoprofen present in the SCE and ED accumulated over a period of 12 h

Solution	Average concentration in SCE (µg/ml)	Median concentration in SCE (µg/ml)	Average concentration in ED (µg/ml)	Median concentration in ED (µg/ml)
Control	1.812	1.943	0.467	0.484
AVG	2.817	2.623	1.295	0.939
AVWL	2.292	2.053	1.382	1.070
AMG	2.107	1.865	1.505	0.926
AMWL	3.285	2.461	2.646	1.508
AFG	1.693	1.242	1.110	1.050
AFWL	1.709	1.565	1.125	1.0042

E.3.3.1 Ketoprofen concentration in the SCE for the different test solutions

To visually compare the average and median SCE concentrations for the various test solutions, a box-plot is given in Figure E.20. Comparison of the average SCE concentrations showed that AMWL (3.285 µg/ml) achieved the highest average concentration ketoprofen in the SCE, followed by AVG (2.817 µg/ml), AVWL (2.292 µg/ml), AMG (2.107 µg/ml), control group (1.812 µg/ml), AFWL (1.709 µg/ml) and finally AFG (1.709 µg/ml).

AMWL delivered ketoprofen into the SCE at the highest concentration, even though it did not significantly enhance the flux of ketoprofen across the skin. Conversely, AVG significantly enhanced the permeation of ketoprofen across the skin and also delivered a higher concentration ketoprofen into the SCE layer of the skin compared to the control group. A possible correlation could be the high α-value obtained for AVG, indicating the enhanced solubility of the active pharmaceutical ingredient (API) in the skin. Important to note is that AFG did not only have a lower flux value for ketoprofen than the control group, but also delivered ketoprofen into the epidermis at the lowest concentration.

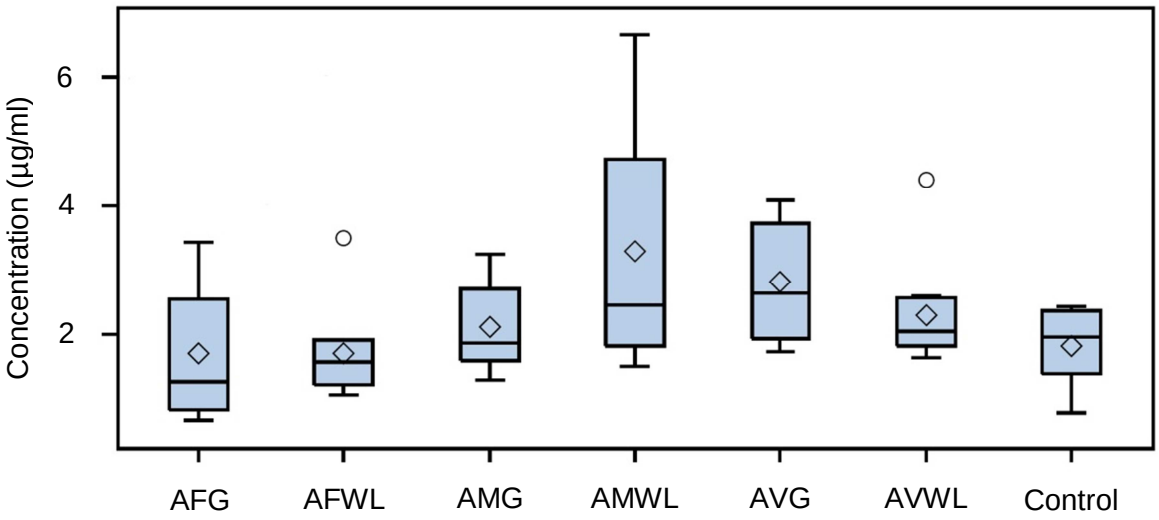


Figure E.20: Box-plots depicting the concentration (µg/ml) ketoprofen present in the SCE for the different aloe leaf material solutions after tape stripping. The average and median concentration values are indicated by the diamond shapes and lines, respectively.

No major differences were noted when the average and median concentrations were compared with each other, except for AMWL. Therefore, it is proposed that the median concentrations be used as it is unaffected by a distortion in the spread of the data (Dawson & Trapp, 2004:30).

E.3.3.2 Ketoprofen concentration in the ED for the different test solutions

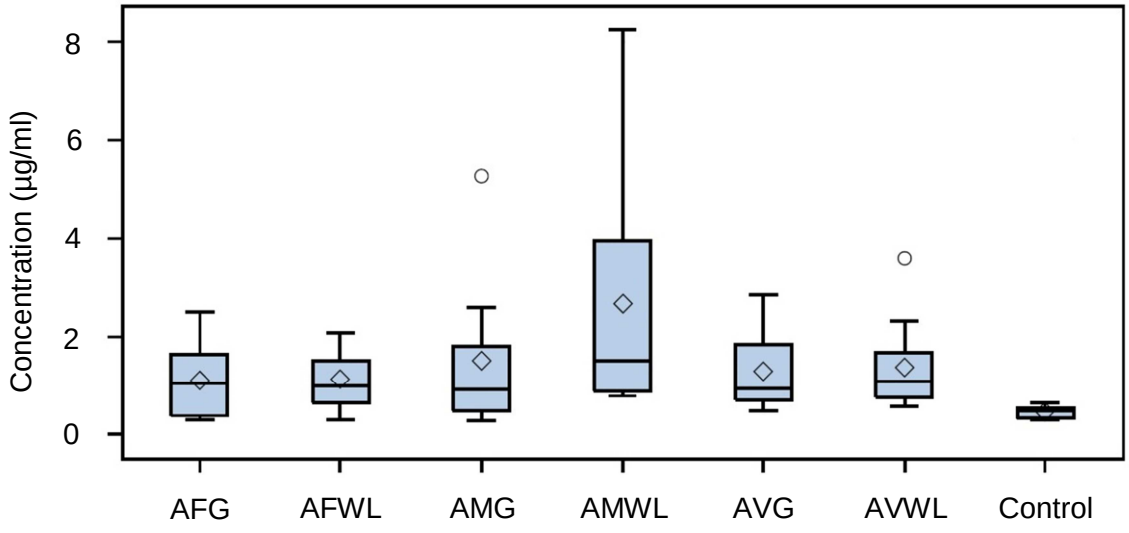


Figure E.21: Box-plots depicting the concentration (µg/ml) ketoprofen present in the ED for the different aloe leaf material solutions after tape stripping. The average and median concentration values are indicated by the diamond shapes and lines, respectively.

The box-plots in Figure E.21 are used to graphically compare the average and median ED concentrations for the various test solutions. Comparison of the average ED concentrations showed that AMWL (2.646 µg/ml) solution achieved the highest average concentration ketoprofen in the ED, followed by AMG (1.505 µg/ml), AVWL (1.382 µg/ml), AVG (1.295 µg/ml), AFWL (1.125 µg/ml), AFG (1.110 µg/ml) and finally the control group (0.467 µg/ml). These results show all the aloe leaf materials enhanced the permeation of ketoprofen to the dermal layer of the skin when compared to the control group.

Only small differences were noted between the average and median concentrations, except for AMWL. Therefore the median concentrations provide a more accurate representation of the true concentration as it was not affected by outliers in the data and takes all the data into consideration (Dawson & Trapp, 2004:30).

Overall, the average and median concentrations of ketoprofen in the ED were lower than for the SCE ketoprofen concentrations. This indicates that ketoprofen had a high tendency to leave the aqueous vehicle and migrate into the SC, but had some difficulty in penetrating the hydrophilic viable epidermis (Wiechers, 1989:189).

E.3.4 Inferential statistical data analysis

E.3.4.1 Membrane release studies

A three-way ANOVA was applied on the flux values and showed statistical significant differences existed between the aloe material concentrations ($p < 0.0001$), between the types of leaf material (i.e. gel vs. whole leaf) ($p = 0.001$) and between the aloe species ($p < 0.0001$). Statistical significant differences were found for the interactions between concentration and type ($p = 0.0063$), concentration and species ($p < 0.0001$), type and species ($p < 0.0001$), concentration and type and species ($p < 0.0001$).

Thereafter, the post-hoc Tukey's HSD test was performed. Statistical significant differences ($p < 0.0001$) were observed between the different concentrations (0.75%, 1.50% and 3.00% (w/v))) and the different types (gel and whole leaf). It was also found that AV was statistically significantly different ($p < 0.0001$) from AM and AF.

E.3.4.2 Skin diffusion studies

E.3.4.2.1 Flux

The parametric one-way ANOVA revealed a statistical significant difference ($p < 0001$) between the different treatment groups, i.e. aloe leaf materials and the control group. The seven test materials were compared with each other resulting in twenty-one different multiple group

comparisons. The Tukey post-hoc test revealed a larger number of statistical significant differences (fourteen out of twenty-one comparisons) when compared to the Kruskal-Wallis multiple comparisons test (six out of the twenty-one comparisons).

Although the Tukey post-hoc test revealed statistical significant differences between the control group and AMG and AFWL, it was not found to be the case with the Kruskal-Wallis multiple comparisons test. It is possible that more data points could have revealed more statistically significant differences. Therefore, the more conservative Kruskal-Wallis multiple comparisons table (Table E.6) was preferred to indicate some statistically significant differences (indicated in red coloured numbers).

Table E.6: Kruskal-Wallis multiple comparison test p-values for the flux obtained with the skin diffusion studies (red numbers indicate statistical significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.0003	1.0000	0.2096	1.0000	1.0000	0.2670	**Control**
	0.0004	1.0000	0.0002	0.0000	1.0000	**AVG**
		0.2710	1.0000	1.0000	0.3450	**AVWL**
			0.1970	0.0080	1.0000	**AMG**
				1	0.2530	**AMWL**
					0.0110	**AFG**

From Table E.6 it can be seen that AVG was statistically significantly different from the control group, AVWL, AMWL and AFG.

E.3.4.2.2 Alpha value

Tukey post-hoc tests were performed to investigate which groups differed statistically significantly in terms of the α-values. Note the Tukey post-hoc test revealed a larger number of statistical significant differences than the preferred Kruskal-Wallis multiple comparisons test, with eleven and six statistical significant differences revealed, respectively. It is possible that more data points could have revealed more statistically significant differences by the Kruskal-Wallis multiple comparisons test. Table E.7 contains the p-values obtained with the preferred Kruskal-Wallis multiple comparisons test (statistically significant differences indicated in red coloured numbers). This table shows that the α-value for AVG was statistically significantly higher than the control group. Although the α-value for AMG was higher than the control group, the difference was not statistically significant.

Table E.7: Kruskal-Wallis multiple comparisons test for α-values (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.005	1.000	0.847	1.000	0.514	1.0000	**Control**
	0.117	1.000	0.0001	0.000	0.004	**AVG**
		1.000	1.000	0.0199	1.000	**AVWL**
			0.085	0.0003	0.730	**AMG**
				1.000	1.000	**AMWL**
					0.603	**AFG**

E.3.4.2.3 Beta value

The Kruskal-Wallis multiple comparisons test was also preferred for statistical evaluation of the β-values and the obtained p-values are given in Table E.8 (statistically significant differences indicated in red coloured numbers).

The Kruskal-Wallis test revealed less statistical significant differences (i.e. five out of the twenty-one comparisons) than the Tukey test (i.e. nine out of the twenty-one comparisons). It is possible that more data points could have revealed more statistical significant differences. The obtained β-value for AFWL was statistically significant different from the β-value of the control group.

Table E.8: Kruskal-Wallis multiple comparisons test for β-values (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.7490	1.0000	0.5820	1.0000	1.0000	0.0002	**Control**
	0.0100	1.0000	1.0000	1.0000	0.3460	**AVG**
		0.0080	0.0900	0.0360	0.0000	**AVWL**
			1.0000	1.0000	0.6090	**AMG**
				1.0000	0.0550	**AMWL**
					0.1290	**AFG**

E.3.4.2.4 Partition coefficient (k_p)

The p-values obtained with the preferred Kruskal-Wallis multiple comparisons test are summarised in Table E.9. The red values indicate statistically significant differences in some cases when comparing test materials with one another. The Tukey test, once again, showed more statistically significant differences (i.e. fourteen out of twenty-one comparisons) than the

Kruskal-Wallis multiple comparisons test (i.e. six out of twenty-one comparisons). Potentially, the presence of more data points could have revealed more statistical significant differences. Important to notice is that only AVG statistically significantly differed from the control group in terms of the partition coefficient.

Table E.9: Kruskal-Wallis multiple comparisons test for the partition coefficient (k_p) (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.0002	1.0000	0.1690	1.0000	1.0000	0.2290	**Control**
	0.0004	1.0000	0.0003	0.0000	1.0000	**AVG**
		0.2800	1.0000	1.0000	0.3740	**AVWL**
			0.2170	0.0090	1.0000	**AMG**
				1.0000	0.2930	**AMWL**
					0.0140	**AFG**

E.3.4.2.5 Lag time

Of the twenty-one comparisons, the Tukey test revealed eleven statistically significant differences between the test solutions; whereas the preferred Kruskal-Wallis multiple comparisons test revealed only six statistically significant differences (Table E.10). As mentioned before, it is suspected that more statistical significant differences could have been observed if more data points were available. Statistically significant differences (as indicated by the red numbers in Table E.10) were observed between the control group and AFG and AFWL, indicating that this aloe species statistically significantly shortened the lag time compared to the control group.

Table E.10: Kruskal-Wallis multiple comparisons test for the lag times (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
1.0000	1.0000	0.3490	1.0000	0.0190	0.0000	**Control**
	0.0800	1.0000	1.0000	1.0000	0.0450	**AVG**
		0.0100	0.0530	0.0002	0.0000	**AVWL**
			1.0000	1.0000	0.4110	**AMG**
				1.0000	0.0680	**AMWL**
					1.0000	**AFG**

E.3.4.3 Tape stripping

A one-way ANOVA was used to determine whether there existed statistically significant differences amongst the SCE and ED data. The ANOVA revealed statistically significant differences with p-values of 0.008 and 0.023 for the SCE and ED experiments, respectively. The post-hoc Tukey and Dunnett's tests revealed statistically significant differences; however, due to the skewness of the data (as seen with the normal probability plot) the preferred Kruskal-Wallis multiple comparisons test was performed.

E.3.4.3.1 Epidermis (SCE)

Table E.11 shows the p-values obtained with the preferred Kruskal-Wallis multiple comparisons test for the SCE data. The results show that none of the solutions statistically significantly differed from each other. Conversely, two statistically significant differences were observed with the Tukey test.

Table E.11: Kruskal-Wallis multiple comparisons test for ketoprofen concentration in the SCE (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.8520	1.0000	1.0000	1.0000	1.0000	1.0000	**Control**
	1.0000	1.0000	1.0000	0.1520	0.1230	**AVG**
		1.0000	1.0000	1.0000	1.0000	**AVWL**
			1.0000	1.0000	1.0000	**AMG**
				0.3250	0.2620	**AMWL**
					1.0000	**AFG**

E.3.4.3.2 Dermis (ED)

The p-values obtained with the preferred Kruskal-Wallis multiple comparisons test as shown in Table E.12 for the ED data revealed two statistically significant differences (as indicated by the red numbers), i.e. between the control group and AVWL as well as between the control group and AMWL. However, the Tukey test revealed only one statistical significant difference out of twenty-one comparisons.

Table E.12: Kruskal-Wallis multiple comparisons test for ketoprofen concentration in the ED (red numbers indicate statistically significant differences)

AVG	AVWL	AMG	AMWL	AFG	AFWL	
0.0963	0.0360	0.3730	0.0007	0.3150	0.2580	**Control**
	1.0000	1.0000	1.0000	1.0000	1.0000	**AVG**
		1.0000	1.0000	1.0000	1.0000	**AVWL**
			1.0000	1.0000	1.0000	**AMG**
				1.0000	1.0000	**AMWL**
					1.0000	**AFG**

E.4 Conclusion

Penetration enhancers play a vital role in overcoming the barrier function of the skin in order to deliver the drug into and through the layers of the skin. The use of natural products as safe and effective drug permeation enhancers has been receiving an increasing amount of attention (Fang *et al.*, 2003:253). Therefore the use of aloe materials is an attractive alternative (Meadows, 1980:56; Cole & Heard, 2007:10) and their potential as penetration enhancers was investigated during the present study.

From the membrane release studies, it was determined that the majority of aloe leaf materials released the highest percentage ketoprofen from their gel-like structures at the lowest concentration (i.e. 0.75% (w/v)) investigated. Subsequently, it was decided to investigate the various aloe leaf materials' permeation enhancing abilities at this concentration.

Franz cell skin diffusion studies showed that AVG had the highest permeation enhancing effect (flux values) on ketoprofen when compared to the control group, which was statistically significant. This was followed by AMG and AFWL, although their effects were not statistically significantly different from the control group. Previous studies showed that AV does not exhibit penetration enhancing effects when used as a pre-treatment for the model drug ketoprofen and it was therefore suggested that the ketoprofen could not interact with the AV enhancing factor the same way as when used 'within-vehicle' (Ballam & Heard, 2010:116). The present study confirmed the need for the incorporation of the drug into the vehicle, as AV facilitated the transport of ketoprofen across the skin when included in the solution.

The high α-values of AVG and AMG, is a reflection that these two aloe leaf materials increased the skin partitioning of the ketoprofen when compared to the control group. Conversely, AFWL modified the diffusion characteristics of the skin toward ketoprofen as reflected by high β-value (Hadgraft *et al.,* 2003:141).

When comparing all the tested solutions, in terms of the SCE and ED concentrations of ketoprofen, AMWL displayed the highest values with average concentrations of 3.285 µg/ml and 2.646 µg/ml, respectively. In general, the ED concentrations of ketoprofen was found to be lower than for the SCE concentrations, indicating that the ketoprofen had a high tendency to leave the aqueous vehicle and migrate into the SC, but had some difficulty in penetrating the hydrophilic viable epidermis (Wiechers, 1989:189).

These differences in the penetration enhancing abilities of the different aloe leaf materials relate to differences in their chemical compositions as verified with the ^{1}H-NMR spectra (Appendix A). The AV proved to be a more effective penetration enhancer compared to the two indigenous species, AM and AF. Further research is needed to possibly determine the enhancing factor present in the aloe materials.

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Appendix F

Molecules: Guide for Authors

F.1 Submission of Manuscripts

F.1.1 Submission

Manuscripts should be submitted online at www.mdpi.com by registering and logging in to this website. Once you are registered, click here to go to the submission form.

F.1.2 Accepted File Formats

MS Word: Manuscript prepared in MS Word must be converted into a single file before submission. When preparing manuscripts in MS Word, the *Molecules* Microsoft Word template file must be used. Please do not insert any graphics (schemes, figures, *etc*.) into a movable frame which can superimpose the text and make the layout very difficult.

LaTeX: ensure to send a copy of your manuscript as a PDF file also, if you decided to use LaTeX. When preparing manuscripts in LaTeX, please use the MDPI LaTeX template files.

F.1.3 Coverletter

Check in your cover letter whether you supplied at least 5 referees. Check if the English corrections are done before submission.

F.2 Manuscript Preparation

Manuscripts should be prepared in English using a word processor. The MS Word template file should be used. MS Word for Macintosh or for Windows .doc or .rtf files are preferred. Manuscripts may be prepared with other software, provided that the full document (with figures, schemes and tables inserted into the text) is exported to a MS Word format for submission. Times or Times New Roman font is required. The font size is 12 pt and the line spacing "at least" 17 pt. Although our final output is in .pdf format, authors are asked to not send manuscripts in this format as editing them is much more difficult.

Special Notes regarding MS Word files:

- Please do not insert any graphics (schemes, figures, etc.) into a movable frame which can superimpose the text and make the layout very difficult.

- Most formatting codes will be removed or replaced on processing your article so there is no need to use excessive layout styling. In addition, options such as automatic word breaking, double columns, footnotes or automatic numbering should not be used. However, bold face, italic, subscripts, superscripts, etc. may be used for emphasis as needed. Authors from countries where right-to-left writing is used should ensure their manuscripts have Western style (left-to-right) formatting.

The standard style of *Molecules* should be followed. Prospective authors should consult any current issue of *Molecules* for examples of this style.

Authors' full mailing addresses, homepage addresses, phone and fax numbers, e-mail addresses and homepages can be included in the title page and these will be published in the manuscripts and the Table of Contents. The corresponding author should be clearly identified. It is the corresponding author's responsibility to ensure that all co-authors are aware of and approve of the contents of a submitted manuscript.

All authors who contributed significantly to the manuscript (including writing a section) should be listed on the first page of the manuscript, below the title of the article. Other parties, who provided only minor contributions, should be listed under Acknowledgments only. A minor contribution might be a discussion with the author, reading through the draft of the manuscript, or performing English corrections.

A brief (about 200 words) Abstract should be provided. The use in the Abstract of numbers to identify compounds should be avoided, unless these compounds are also identified by name.

A list of three to five keywords must be given, and placed after the Abstract. Keywords may be single words or very short sentences.

Although variations in accord with a manuscript's contents are permissible, in general all papers should have the following sections: Introduction, Results and Discussion, Conclusions, Acknowledgments (if applicable), Experimental and References (or References and Notes, if applicable).

Authors are encouraged to prepare figures and schemes in color. Full color graphics will be published free of charge. Conference slides, video sequences, software, etc., can also be included and will be published as supplementary material.

Tables should be inserted into the main text, and numbers and titles supplied for all tables. All table columns should have an explanatory heading. To facilitate layout of large tables, smaller fonts may be used, but in no case should these be less than 10 pt. in size. Authors should use

the Table option of MS Word to create tables, rather than tabs, as tab delimited columns are often difficult to format in .pdf for final output.

Figures and schemes should also be placed in numerical order in the appropriate place within the main text. Numbers, titles and legends should be provided for all schemes and figures. These should be prepared as a separate paragraph of the main text and placed in the main text before the figure or scheme.

Chemical structures and reaction schemes should be drawn using an appropriate software package designed for this purpose. As a guideline, these should be drawn to a scale such that all the details and text are clearly legible when placed in the manuscript (i.e. text should be no smaller that 8 – 9 pt.). To facilitate editing we recommend the use of any of the software packages widely available for this purpose: MDL® Isis/Draw, ACD/ChemSketch®, CS ChemDraw®, ChemWindow®, etc. Free versions of some of these products are available for personal or academic use from the respective publishers. If another less common structure drawing software is used, authors should ensure the figures are saved in a file format compatible with of one of these products.

X-ray crystallographic data. To avoid publication of extensive compilations of crystallographic data and facilitate refereeing of manuscripts, *Molecules* has entered a data deposition scheme with the Cambridge Crystallographic Data Centre (http://www.ccdc.cam.ac.uk/). Authors planning to include such results in their papers should contact the CCDC to deposit the crystallographic data and obtain the corresponding CCDC deposition number(s) before submitting their manuscripts to *Molecules* (for instructions on doing this, see: http://www.ccdc.cam.ac.uk/conts/depositing.html). The deposition number(s) (usually provided by the CCDC within 3 working days) should be included in the manuscript, along with the following text: "CCDC ..... contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk)". This text may be included in the General subsection of the Experimental or as a suitably referenced endnote.

Experimental data. Molecules is first and foremost a repository of synthetic procedures and data. The Experimental section should have an initial subsection labeled *General*, containing information on materials, methods, analytical instrumentation used, etc. To allow for correct abstracting of the manuscripts all compounds should be mentioned by correct chemical name, followed by any numerals used to refer to them in the paper. The use of the IUPAC nomenclature conventions is preferred, although alternate naming systems (for example CAS rules) may be used provided that a single consistent naming system is used throughout a

manuscript. For authors perhaps unfamiliar with chemical nomenclature in English we recommend the use of compound naming software such as AutoNom. Full experimental details must be provided, or, in the case of many compounds prepared by a similar method, a representative typical procedure should be given. The general style used in the *Journal of Organic Chemistry* is preferred. Complete characterization data must be given for all new compounds. For papers mentioning large numbers of compounds a tabular format is acceptable. In case of compound libraries containing more than 100 compounds, preferably 20% of the whole library. For known compounds appropriate literature references must be given.

References. See the Reference Preparation Guide. References should be numbered according to the order in which they appear in the text.

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Electronic Supplementary Information (ESI): Conference slides, video sequences, software, etc., can be included with the submission and published as supplementary material. Please read the information about Supplementary Material Deposit beneath.

F.3 Potential Conflicts of Interest

It is the authors' responsibility to identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of clinical research. If there is no conflict, please state here "The authors declare no conflict of interest". This should be conveyed in a separate "Conflict of Interest" statement preceding the "Acknowledgments" and "References" sections at the end of the manuscript. Financial support for the study must be fully disclosed under "Acknowledgments" section. It is the authors' responsibility to identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of clinical research. If there is no conflict, please state here "The authors declare no conflict of interest." This should

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F.4 Review / Referees

Authors should suggest at least 5 potential referees with the appropriate technical expertise, although the Editor will not necessarily approach them. Please provide as detailed contact information as possible about the proposed referees (address, homepage address, phone and fax numbers and e-mail address). At least two of the suggested referees must be from a different country than the authors. Additionally, at least two suggested referees must be from a western country (USA, Canada, Japan, Australia or western European country). To identify potential referees, you may check the Editorial Board of our journal and suggest board members with appropriate research interests. In addition, we suggest searching for related papers in Google Scholar and proposing their authors as possible reviewers. Another possibility is to propose authors that you frequently cited in your paper.

F.5 English Corrections

This journal is published in English, so it is essential that for proper refereeing and quick publication all manuscripts are submitted in grammatically correct English. For this purpose we ask that non-native English speakers ensure their manuscripts are checked before submitting them for consideration. We suggest that for this purpose your manuscript be revised by an English speaking colleague before submission. Authors can also use the services of American Journal Experts (AJE) for this purpose. Authors of articles submitted to MDPI journals benefit of a one-time 10% discount on AJE's charges. Simply follow the above link to make use of the referral discount.

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F.9 Correct identification of components of natural products

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* **2006**, *21*, 185. These recommendations may be summarized as follows:

Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully

potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.

F.10 Supplementary Material Deposit

- We wish to encourage the submission of supplementary data in electronic formats, so that important chemical, structural or scientific information is retained in full. Spectral data (NMR, IR, Raman, ESR, etc) can be submitted in JCAMP (.jdx) format.
- 3D coordinate structures (in pdb, mol, xyz or other common formats), if available, should also be submitted.

Appendix G

Pharmacognosy: Guide for Authors

G.1 About Phcog.Net

Natural products are the most consistent and successful source of drugs. In India, Ayurveda remains one of the most ancient and living traditions, which is practiced for the treatment of various diseases and disorders. India has many number of plant species and medicinal properties have been assigned to several thousands. Many major institutes and research centres are currently involved in exploring this opportunity to investigate newer drugs from ancient principles of ayurveda. Further researchers believe in combining the strengths of ayurveda with modern scientific techniques such as NMR, MS and chromatographic techniques to provide new functional leads with high therapeutic value in a short span of time. But Natural products research often comes across many hurdles, which dims the drug development goals. We believe that natural products research information can potentially benefit many researchers involved in this area. Also we believe that the research on natural products is often staggered due to lack of required information available for medicinal plants. In order to combat these problems, Pharmacognosy Network Worldwide (Phcog.net) - A Platform for Natural Product Researchers was started, which makes innovative use of best tools for information dissemination and solve the hurdles in Natural Product Research. Pharmacognosy Network Worldwide is a non-profit network dedicated to Natural Products Research in order to develop promising drugs.

G.2 About Journal

Pharmacognosy Magazine (Pharmacogn Mag.) [http://www.phcog.com] [ISSN: Print -0973-1296, Online - 0976-4062] is published quarterly and serves the need of different scientists and others involved in medicinal plant research and development. Each issue covers different topics in natural product drug discovery, and also publishes manuscripts that describe investigations, clinical reports, methods, techniques and applications of all forms of medicinal plant research and that are of broad readership interest to users in industry, academia, and government.

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G.3 Scope of the journal

The journal will cover research studies/reviews related to Natural products including some of the allied subjects. Articles with timely interest and newer research concepts will be given more preference.

G.4 The Editorial Process

A manuscript will be reviewed for possible publication with the understanding that it is being submitted to Phcog Mag. alone at that point in time and has not been published anywhere, simultaneously submitted, or already accepted for publication elsewhere. The journal expects that authors would authorize one of them to correspond with the Journal for all matters related to the manuscript. All manuscripts received are duly acknowledged. On submission, editors review all submitted manuscripts initially for suitability for formal review. Manuscripts with insufficient originality, serious scientific or technical flaws, or lack of a significant message are rejected before proceeding for formal peer-review. Manuscripts that are unlikely to be of interest to the Phcog Mag. readers are also liable to be rejected at this stage itself.

Manuscripts that are found suitable for publication in Phcog Mag. are sent to two or more expert reviewers. During submission, the contributor is requested to provide names of two or three qualified reviewers who have had experience in the subject of the submitted manuscript, but this is not mandatory. The reviewers should not be affiliated with the same institutes as the contributor/s. However, the selection of these reviewers is at the sole discretion of the editor. The journal follows a double-blind review process, wherein the reviewers and authors are unaware of each other's identity. Every manuscript is also assigned to a member of the editorial team, who based on the comments from the reviewers takes a final decision on the manuscript. The comments and suggestions (acceptance/ rejection/ amendments in manuscript) received from reviewers are conveyed to the corresponding author. If required, the author is requested to provide a point by point response to reviewers' comments and submit a revised version of the manuscript. This process is repeated till reviewers and editors are satisfied with the manuscript. Manuscripts accepted for publication are copy edited for grammar, punctuation, print style, and format. Page proofs are sent to the corresponding author. The corresponding author is expected to return the corrected proofs within three days. It may not be possible to incorporate corrections received after that period. The whole process of submission of the manuscript to final decision and sending and receiving proofs is completed online. To achieve faster and greater dissemination of knowledge and information, the journal publishes articles online as 'Ahead of Print' immediately on acceptance.

G.5 Editorial Policy

Authors should prepare their manuscripts submitted to the journal exactly according to the instructions given here. Manuscripts which do not follow the format and style of the journal may be returned to the authors for revision or rejected. The journal reserves the right to make any further formal changes and language corrections necessary in a manuscript accepted for publication so that it conforms to the formatting requirements of the journal. Manuscripts and figures are not returned to the authors, not even upon rejection of the paper.

G.6 Submission of Manuscripts

Manuscripts must conform to the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" http://www.icmje.org/. Contributions and Manuscripts must be written in English and submitted exclusively to Pharmacogn Mag. Manuscripts must be typewritten (double-spaced) with liberal margins and space at the top and bottom of the page.

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G.7 Publication / processing fee

The journal does not charge for processing fee of articles at the time of submission. Upon acceptance, authors are required to make necessary charges in support of Journal.

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Contributors should provide a description of contributions made by each of them towards the manuscript. Description should be divided in following categories, as applicable: concept, design, definition of intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review. Authors' contributions will be printed along with the article. One or more author should take responsibility for the integrity of the work as a whole from inception to published article and should be designated as 'guarantor'.

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A research paper typically should include in the following order

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- Introduction
- Materials and Methods
- Results
- Discussion
- Conclusion
- Acknowledgements (If any)
- References
- Tables and/or Figures
- Appendixes (if necessary)
- Abbreviations (if necessary)

G.14.1 Abstract – Limit of 250 Words

A brief summary of the research. The abstract should be in structured format include a brief introduction, a description of the hypothesis tested, the approach used to test the hypothesis, the results seen and the conclusions of the work.

Example

Background: Sophora flavescens Aiton is an important medicinal plant in China. Early *in vitro* researches of S. flavescens were focused on callus induction and cell suspension culture, only a few were concerned with *in vitro* multiplication. Objective: To establish and optimize the rapid propagation technology of S. flavescens and to generate and characterize polyploid plants of S. flavescens. Materials and Methods: The different concentrations of 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA) and kinetin (KT) were used to establish and screen the optimal rapid propagation technology of S. flavescens by orthogonal test; 0.2% colchicine solution was used to induce polyploid plants and the induced buds were identified by root-tip chromosome determination and stomatal apparatus observation. Results: A large number of buds could be induced directly from epicotyl and hypocotyl explants on the Murashige and Skoog medium (MS; 1962) supplemented with 1.4 – 1.6 mg/l 6-benzylaminopurine (BAP) and 0.3 mg/l indole-3-acetic acid (IAA). More than 50 lines of autotetraploid plants were obtained. The

chromosome number of the autotetraploid plantlet was 2n = 4x = 36. All tetraploid plants showed typical polyploid characteristics. Conclusion: Obtained autotetraploid lines will be of important genetic and breeding value and can be used for further selection and plant breeding.

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G.14.3 Introduction

Description of the research area, pertinent background information, and the hypotheses tested in the study should be included under this section. The introduction should provide sufficient background information such that a scientifically literate reader can understand and appreciate the experiments to be described. The introduction MUST include in-text citations including references to pertinent reviews and primary scientific literature. The specific aims of the project should be identified along with a rationale for the specific experiments and other work performed.

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This section should relate the results section to current understanding of the scientific problems being investigated in the field. Description of relevant references to other work/s in the field should be included here. This section also allows you to discuss the significance of your results i.e. does the data support the hypotheses you set out to test? This section should end with new answers/questions that arise as a result of your work.

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Natural products have proven to be a great source of new biologically active compounds. Thus, in an effort to discover new lead anti-malarial compounds, several research group screen plant extracts to detect secondary metabolites with relevant biological activities that could serve as templates for the development of new drugs. Flavonoids have been isolated and characterized from many medicinal plants used in malaria endemic areas.[10] However, controversial data have been obtained regarding their antiplasmodial activity, probably because of their structural diversity.[11-13] More recently, several flavonoids have been isolated from Artemisia afra[14] and Artemisia indica,[15] two plants related to Artemisia annua, the famous traditional Chinese medicinal plant from which artemisinin is isolated.

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1. Single/Multiple Authors

Halpern SD, Ubel PA, Caplan AL. Solid-organ transplantation in HIV-infected patients. N Engl J Med. 2002 Jul 25;347(4): 284-7.

2. More than six authors

Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res. 2002; 935(1-2): 40-6.

3. Organization as Author

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension. 2002; 40(5): 679-86.

4. Unknown Author

21st century heart solution may have a sting in the tail. BMJ. 2002; 325(7357): 184-5.

5. Journal article on the Internet

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

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6. Personal author(s)

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

7. Editor(s), compiler(s) as author

Gilstrap LC 3rd, Cunningham FG, VanDorsten JP, editors. Operative obstetrics. 2nd ed. New York: McGraw-Hill; 2002.

8. Author(s) and editor(s)

Breedlove GK, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services; 2001.

9. Organization(s) as author

Royal Adelaide Hospital; University of Adelaide, Department of Clinical Nursing. Compendium of nursing research and practice development, 1999-2000. Adelaide (Australia): Adelaide University; 2001.

10. Chapter in a book

Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93-113.

11. Conference proceedings

Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.

12. Thesis

N. Khoshakhlagh. The compositions of volatile fractions of Peganum harmala seeds and its smoke. Pharm. D. Thesis, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. (2002).

13. Websites

Website information

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Acknowledgements

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Appendix H

European Journal of Pharmaceutics and Biopharmaceutics: Guide for Authors

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H.4 Preparation

H.4.1 New submissions

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process. As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

H.4.1.1 References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

H.4.1.2 Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example: Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions. If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes. Divide the article into clearly defined sections.

H.4.1.3 Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

H.4.2 Revised submissions

Use of word processing software. Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

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H.4.4 Research papers

Should contain the following sections: Introduction, Theoretical Development (if applicable), Materials and Methods, Results, Discussion (or Results and Discussion) and References. For purely theoretical papers, appropriate sections may be selected. It should define the purpose of the research and reveal its connections with the principal work of other authors in the field.

H.4.4.1 Materials and methods

It should include materials, standard techniques and procedures relevant to the study. Published procedures and techniques should be cited unless significant modifications are involved. Exact specification of relevant materials and equipment must be given. Chemical terms must conform with IUPAC rules. Trademarks of commercial products must be labelled using a superscripted '(r)'. Names of products and equipment mentioned in the Materials and Methods section must be accompanied by the name of the manufacturer or distributor, location and state or country. This information must be stated in parentheses in the text, and not as a footnote. Any potential hazards connected with materials and procedures must be mentioned. A precise and detailed description should be given of those steps which are of vital importance in carrying out any repetition of the work. The Declarations of Helsinki and Tokyo for humans, and the European Community guidelines as accepted principles for the use of experimental animals, must be adhered to. Therefore, EJPB will only consider manuscripts that describe experiments that have been carried out under approval of an institutional or local ethics committee. Authors must state in the manuscript that the protocol complies with the particular recommendation and that approval of their protocols was obtained.

Equations must be part of the text and consecutively numbered on the right hand side using numbers in parentheses. References to equations in the text are also to be made with parentheses, e.g. using Eq. (3), etc.

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SI units must be used throughout.

Theory/calculation: a theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

H.4.4.2 Results

Results may be presented in tables, figures or schemes which must be referred to in the accompanying text, using appropriate numbering, e.g. Fig. 1, Table 2.

H.4.4.3 Discussion

It should focus on the interpretation of the results. It might be appropriate to combine RESULTS AND DISCUSSION in one section. If necessary at all, use CONCLUSIONS only to illustrate the general implication of the results and do not summarize the previous text.

H.4.4.4 Conclusions

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

Appendices: if there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

H.4.4.5 Essential title page information

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It should contain a brief and clear description of the aim of the paper, its principal results and major conclusions (100 – 200 words). The abstract should include all keywords pertinent to the subject.

H.4.4.6 Graphical abstract

A Graphical abstract is mandatory for this journal. I t should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system.v Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples. Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

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Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

H.4.4.9 Acknowledgements

Acknowledgements of financial support, gifts, technical help or other assistance may be given in an unnumbered paragraph under this heading preceding the references.

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Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

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References must be arranged as follows:

[1] A.– L. Cornaz, P. Buri, Nasal mucosa as an absorption barrier, Eur. J. Pharm. Biopharm. 40 (1994) 261– 270.

[5] C. Lanczos, Applied Analysis, Prentice-Hall, Englewood Cliffs, NJ, 1967, pp. 272– 280.

[10] D.M. Barends, Stability of active ingredients, in: H. Mü ller, W.H. Oeser (Eds.), Drug Master Files, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany, 1992, pp. 121– 128.

[14] E.A. Balazs, Ultrapure hyaluronic acid and the use thereof, U.S. Patent 4,141,973 (1979).

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List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text. Examples:

Reference to a journal publication: [1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.

Reference to a book: [2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book: [3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

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- All references mentioned in the Reference list are cited in the text, and vice versa
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The digital object identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document particularly "Articles in press" because they have not yet received their full bibliographic information.

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Notes

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