In vitro investigation of wound dynamics using Absorbatox® containing organic acid(s)

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"I know the Lord is always with me. I will not be shaken, for He is right beside me".

Psalm 16:8

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ABSTRACT

The skin provides an essential protective barrier between the internal tissues and the environment. A wound is defined as a defect or a break in the skin barrier as a result of physical or thermal damage. Wounds may also result due to an underlying medical or physiological condition, which compromises the tissue integrity. Spontaneous wound healing generally occurs due to a defined wound healing process.

Modern wound healing treatments are aimed at enhancing the understanding of the various interactions between the cells and mediators, such as cytokines, growth factors and lipid derivatives. Disruption of the normal wound healing cascade results in altered healing ability; creating abnormal, difficult to treat wounds. Normal wound treatment usually involves a specialised wound dressing, which is designed with the aim of improving the ability to maintain a sterile (free of bacterial contamination) environment, as well as eliminating the excess exudate.

The development of unique, new treatments for wounds such as burns and abrasions, acne and exuding wounds (typically lower leg ulcers) is in high demand, simply because current available treatments do not address all the issues in wound healing and often a “healed wound” is compromised with poor tissue quality or scarring. The wound healing process involves several different cell types, as well as different soluble mediators where the pH value on and within the wound both directly and indirectly influences the healing process. The pH on the surface of the wound plays an important role in infection control, anti-microbial action, oxygen release, angiogenesis, protease activity, as well as bacterial toxicity. Decreasing wound surface pH with topical applications containing organic acids will provide an environment unfavourable for microbial infestation and growth. Hence, the aim of this study was to formulate three different types of safe and effective wound dressings, which all include a unique combination of Absorbatox® bound to bound to an organic acid (fulvic acid, malic acid and citric acid) to promote an optimal wound healing environment, which optimises moisture control and ensures protection from the risks of maceration, as well as microbial contamination were formulated.

Initially, pre-formulation studies were performed determining the compatibility of the Absorbatox® with the different organic acids (fulvic acid, malic acid and citric acid separately) by means of differential scanning calorimetry (DSC), thermal activity monitoring (TAM) and Fourier-transform infrared spectroscopy (FTIR). Thereafter the optimised formula was utilised to formulate a silicone-based gel, hydrogel-based patch and a dry/sachet dressing. Assessment of the cytotoxicity of the active ingredients (separately) and the combination thereof used in the different formulations was performed using *in vitro* cell cultures, specifically human immortalised keratinocyte (HaCaT) cells. The enhanced fibroblast activity and architecture was also assessed when wound dressings were applied to human skin fibroblast (84BR) cells. The potential cell

cytotoxicity was determined by means of methylthiazol tetrazolium (MTT) assay to determine if the active ingredients could be considered safe for the application on human skin and essentially on wounds. Assessment of the wound healing potential of the active ingredients (separately) and the combination thereof used in the different formulations was performed using a cell migration assay, as well as a scratch wound healing assay. Evaluation of the prepared wound dressings with regards to API identification, pH, viscosity, mass loss, particle size, visual appearance and free swelling capacity was performed to determine the stability of the formulations.

Keywords: Absorbatox®; wound; organic acids; silicone-based gel; hydrogel-based patch; dry/sachet dressing

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ABBREVIATIONS

ABTX	Absorbatox®
A-HAs	Alpha-hydroxy Acids
API	Active Pharmaceutical Ingredient
ATR	Attenuated total reflection
CA	Citric acid
CEC	Cation Exchange Capacity
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSC	Differential Scanning Calorimetry
ENGORD	Endoscopically Negative Gastro-oesophageal Reflux Disease
EGF	Epidermal growth factor
FA	Fulvic acid
FBS	Foetal bovine serum
FBF-β	Fibroblast Growth Factor
FTIR	Fourier-Transform Infrared Spectroscopy
HaCaT	Human immortalised keratinocyte cells
ICH	International Conference on Harmonisation
IL-1	Interleukin-1
IL-6	Interleukin-6
IR	Infrared
KGF-2	Keratinocyte Growth Factor-2

MA	Malic acid
MCC	Medicines Control Council
MIC	Minimum Inhibitory Concentration
MTT	Methylthiazol tetrazolium
NEAA	Non-Essential Amino Acid
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory Drugs
OFA	Oxidised fulvic acid
PBS	Phosphate buffer solution
PDGF	platelet derived growth factor
PF-4	platelet factor-4
RH	Relative humidity
R_f	Retention factor
SAHPRA	South African Health Products Regulatory Authority
SD	Standard deviation
TAM	Thermal Activity Monitor
TLC	Thin Layer Chromatography
TGF-β	Transforming Growth Factor- β
TGT-β	Tumour Growth Factor-β
TNF-α	Tumour Necrosis Factor- α
84BR	Human skin fibroblast cells

CHAPTER 1

INTRODUCTION AND AIM OF THE STUDY

1.1 Introduction

Wounds and wound healing have been a challenge to man over the centuries and although the skin provides an essential protective barrier between the internal tissues and the environment (Kooistra-Smid *et al.,* 2009:57), it is the injury to this barrier, exposing deep tissue to the environment, which poses a risk not only to the underlying tissue, but to individuals' survival. A wound is defined as a defect or injury to the skin barrier as a result of physical or thermal damage. Wounds may also result due to an underlying medical or physiological condition, which compromises the tissue integrity (Boateng *et al.,* 2008:97). Spontaneous wound healing generally occurs due to a defined wound healing process. The problem is that many factors influence, delay or prevent spontaneous wound healing (Broughton *et al.,* 2006a:1e-S-32e-S).

Modern wound healing treatments stem from a better understanding of the various interactions between the cells and mediators, such as cytokines, growth factors and lipid derivatives/mediators. Disruption of the normal wound healing cascade results in altered healing ability; creating abnormal, difficult to treat wounds (Keller *et al.,* 2002:28). Modern wound treatment usually involves a specialised wound dressing, which is designed with the aim of improving the ability to maintain a sterile (free of bacterial contamination) environment, as well as eliminating the excess exudate produced by the exposed tissue (Von Cramon *et al*., 2017:46). The wound exudate often disrupts the healing process and acts as a medium to allow bacterial growth and even protects the micro-organisms from antibacterial agents applied locally or systemically (Von Cramon *et al*., 2017:46).

The development of unique, new treatments for wounds such as burns and abrasions, acne and exuding wounds is a high priority, simply because current available treatments do not address all the issues in wound healing and often a "healed wound" is compromised with poor tissue quality or scarring (Rutter, 2017:36). Producing a variety of unique dressings aimed at specifically addressing some of the unique challenges in wound healing, i.e. exudate, inflammation, infection and scarring forms an important aspect of this study. In this study three different types of safe and effective wound dressings will be developed, which all include a unique combination of Absorbatox® together with an organic acid to promote an optimal wound healing environment, which optimises moisture control and ensures protection from the risks of maceration, as well as microbial contamination (Boateng *et al.,* 2008:2892; Cuzzel, 1997:260).

In previous studies, it was confirmed that Absorbatox® has great epithelial protective properties, although the exact mechanism of the protective effect was not elucidated, it could be as a result

of its binding capability to biologically active amines and nitrates (Potgieter *et al.,* 2014:215). Absorbatox® is able to create an optimal wound healing environment through its unique ability to bind with specific harmful biologically active substances, which may be present in the wound exudate (Potgieter *et al.,* 2007). Absorbatox® is also able to generate capillary suction forces through its porous crystal structure; this makes topical wound dressing a possibility along with excellent exudate management (Potgieter *et al.,* 2007).

The acidity of the wound and wound environment directly and indirectly influences the healing process. The pH on the surface of the wound plays an important role in infection control, increases anti-microbial action, oxygen release, angiogenesis, protease activity, as well as bacterial toxicity. Decreasing wound surface pH with topical applications containing organic acids will provide an environment unfavourable for microbial infestation and growth (Prabhu *et al.,* 2014:38). Organic acids not only modulate the pH, but specific acids may have other properties such as direct antimicrobial activity and/or anti-inflammatory effects, which may even enhance fibroblast proliferation, all beneficial for optimal wound healing (Prabhu *et al.,* 2014:38; Van Rensburg *et al.,* 2001:53).

1.2 Problem statement

Wound treatment and healing are complex processes and are often poorly understood. Many patients experience severe pain and discomfort, while wound healing is frequently delayed with significant scar formation and loss of long-term function due to a myriad of reasons such as poor blood supply, infection, growth factor over or under stimulation (excessive or inadequate fibroblast growth), and wound bed exudate, to mention but a few aspects influencing clinical outcomes. It is therefore critically important that the optimal wound healing environment is established as quickly as possible considering the various types of wounds, the anatomical place and cause of wound (Keller *et al.,* 2002:28).

There are currently very few of the modern wound healing formulations and or dressings which address all or just some of the issues in wound healing as most treatments focus either on exudate control and or infection with little consideration to the growth factor environment and promotion of healing (Keller *et al.,* 2002:28). Thus, the formulation of Absorbatox® bound to organic acids into new unique wound healing products is worthy of further investigation; since it has the potential to address all the pillars of wound healing, i.e. optimisation of growth factors, control of exudate, protection against infection and reducing scar formation. In future, these newly formulated wound healing products could then be compared to more conventional wound healing products to establish their clinical benefit in addressing the various aspects of wound healing.

1.3 Aims and objectives

The aim of this study is to determine if the selected organic acids (fulvic acid, malic acid and citric acid separately) bound to Absorbatox® are effective wound healing agents when applied as "wound dressings" in an *in vitro* study using various pharmaceutical application products, i.e. silicone-based gel, hydrogel-based patch and dry powder sachets, based on different anticipated wound types graded on their level of exudation (low to high "exudating" wounds). Wounds will be imitated in a controlled cell culture lab to ensure replicability.

The study objectives are as follows:

- To perform the anti-bacterial characterisation with regards to *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* (outsourced).
- To assess the cytotoxicity of the active ingredients (separately) and the combination thereof used in the different formulations using *in vitro* cell cultures, specifically human immortalised keratinocyte (HaCaT) cells.
- To demonstrate enhanced fibroblast activity and wound architecture in cell culture when the formulated wound dressings are applied to human skin fibroblast (84BR) cells.
- To assess wound healing potential of the active ingredients (separately) and the combination thereof used in the different formulations using a cell migration assay, as well as a scratch wound healing assay.
- Determine the compatibility of the Absorbatox® with the different organic acids by means of differential scanning calorimetry (DSC), thermal activity monitoring (TAM) and Fourier-transform infrared spectroscopy (FTIR).
- To formulate a silicone-based wound dressing, a hydrogel-based patch and a sachet/powder dressing (dry form) containing the most suitable active pharmaceutical ingredient (API) based on the outcomes of the cell culture studies.
- Conducting stability tests on the different formulations stored at 25 °C/60% relative humidity (RH), 30 °C/65% RH and 40°C/75% RH.
- Evaluation of the prepared silicone-based gel with regards to pH, visual examination, viscosity, mass loss, as well as API identification during accelerated stability testing.
- Evaluation of the prepared sachet/powder dressing with regards to visual examination, mass loss, free swell capacity, as well as API identification during accelerated stability testing.
- Evaluation of the prepared hydrogel-based patch dressing with regards to visual examination, mass loss and API identification during accelerated stability testing.

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CHAPTER 2

ABSORBATOX® AND SELECTED ORGANIC ACIDS, ALONE AND BOUND, AS WOUND HEALING AGENTS

2.1 Introduction

The wound healing process has been extensively researched. It is an intricate, interactive process which involves cells and soluble mediators (Broughton *et al.,* 2006a:1e-S-32e-S). The wound healing cascade can be sequentially divided into three simple phases, namely (i) haemostasis and inflammation, (ii) proliferation (re-epithelialisation) and (iii) remodelling (neovascularisation) (Broughton *et al.,* 2006b:12S-34S; Diegelmann & Evans, 2004:9).

Conventional wound treatments have been designed based on the understanding of the interactions between these cells and mediators, which include various cytokines and growth factors. An alteration in the healing cascade is often due to an underlying pathological condition such as diabetes, Chron's disease, hypothyroidism, burns, etc. These may result in abnormal, difficult to treat wounds, e.g. chronic diabetic ulcers, chronic pressure ulcers, fibrosis, keloid scars etc. (Broughton *et al.,* 2006b:12S-34S; Diegelmann & Evans, 2004:9). These complicated wounds especially those in patients with immune disorders are often associated with significant morbidity and mortality (Keller *et al.,* 2002:28). The need for a safe, clinically effective, but also cost effective treatment is thus obvious.

2.2 Wound healing cascade

The wound healing cascade is divided into three distinct phases, namely: (i) haemostasis and inflammation, (ii) proliferation period and (iii) remodelling/ maturation phase as seen in Figure 2.1.

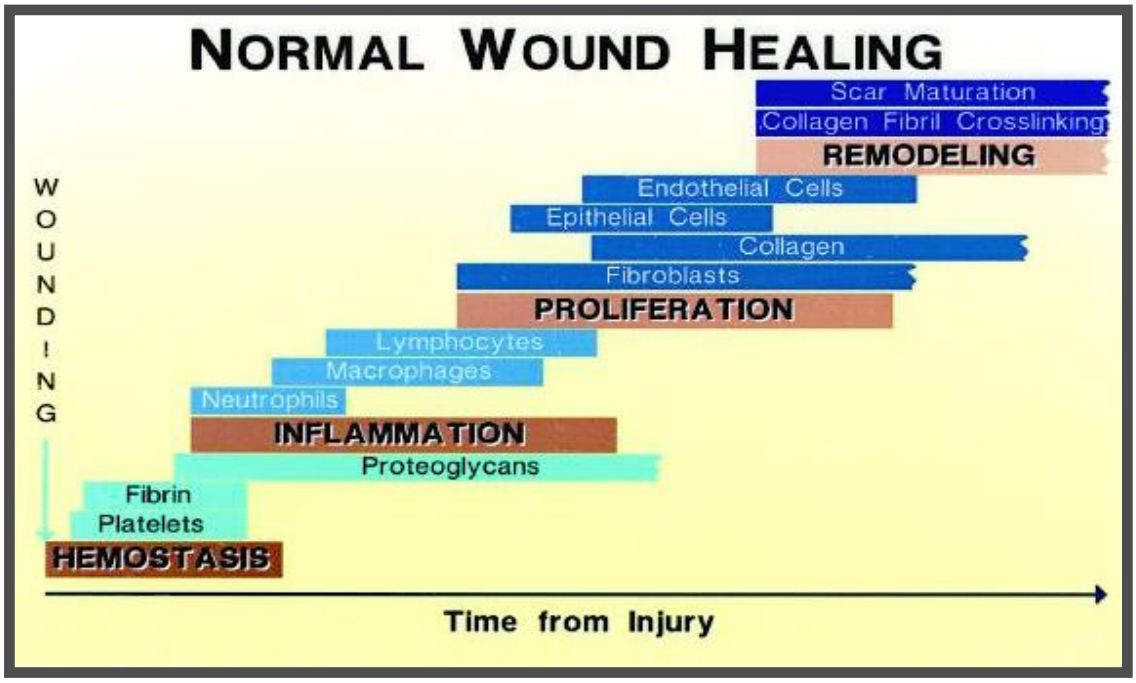

Figure 2.1: Normal sequence of wound healing (Diegelmann & Evans, 2004:9; Mast *et al.,* 1992).

2.2.1 Haemostasis and inflammation

Immediately after a traumatic insult to tissue integrity where blood vessels/capillaries are severed, the body aims to achieve haemostasis. This first step involves the aggregation of blood platelets, which adhere to the newly exposed collagen. Clotting can now take place; clots are made up of a combination of fibrin, collagen, blood cells, as well as platelets. These factors release both growth factors, which include, platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) and chemotactic cytokines (Diegelmann & Evans, 2004:9). The movement of immune cells into the wound area is the next step towards wound healing and is mediated by chemotaxis. Various cytokines and growth factors, such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) are involved. TGF-β and platelet factor-4 (PF-4) attracts neutrophils to the site of injury immediately after the clot has formed (Bevilacqua *et al.,* 1985:76; Pohlman *et al.,* 1986:136). The accumulated neutrophils act to clear the wound area of any necrotic cells and bacteria, as well as other contaminants (foreign material), which may be present due to the nature of the injury. These neutrophils are able to secrete additional cytokines which again attract the necessary monocytes which are in turn activated to become macrophages. These macrophages function as very important pro-inflammatory cells in the wound area. They are phagocytic and release PDGF and TGF-β. The proliferation phase is initialised by these factors resulting in the recruitment of fibroblasts to the wound site (Diegelmann & Evans, 2004:9).

The wound healing cascade involves many intrinsic pro-inflammatory processes which all are equally important for the achievement of successful wound regeneration. An alteration in these

inflammatory processes may disrupt the normal wound healing process which may decrease subsequent tissue quality (Davidson & Breyer, 2003). The pro-inflammatory process is essential for successful wound healing; however, it is also able to delay and reduce the progression in the course of normal wound healing (Blomme *et al.,* 2003: 148; Futagami *et al.,* 2002: 82; Witte *et al.,* 2002:51;). The enzyme cyclooxygenase and its products, i.e. pro-inflammatory eicosanoids, have an essential role in normal wound healing. For instance, nitric oxide (NO) is released by nitric oxide synthase (iNOS = inducible nitric oxide synthase) enzymes and arginine metabolism which are stimulated by produced prostaglandins (Salvemini *et al.,* 1993:90). This process further stimulates the production of proline which is an important collagen precursor. A decrease in collagen synthesis with a resultant reduced concentration found in wound fluid, is seen with the declining action of iNOS enzymes and ultimately NO production which delays the wound healing process (Witte & Barbul, 2002:183; Schäffer, *et al.,* 1999:165). It is therefore common knowledge that excessive inhibition of these pathways by e.g. non-steroidal anti-inflammatory substances (Cyclooxygenase (COX) inhibitors) may impair wound healing (Krischak, *et al.,* 2007:76).

2.2.2 Proliferation

The proliferation phase consists of a number of essential steps, namely epithelialisation, angiogenesis, granulation tissue formation and collagen deposition. The first step, epithelialisation, initiates once IL-1 and keratinocyte growth factor-2 (KGF-2) stimulate fibroblasts to produce KGF-2 and interleukin-6 (IL-6) (Broughton *et al.,* 2006a:117), these cytokines allow the proliferation and migration of keratinocytes (Broughton *et al.,* 2006b:117). A protective barrier layer is re-established by epithelial layers and endothelial cell migration and angiogenesis (capillary growth) proceeds to the new tissue growth (Broughton *et al.,* 2006a:117). The angiogenesis that has taken place allows for essential nutrient delivery which is critical for proper granulation and collagen formation alongside the deposition of extracellular matrix. Synthesis of proteoglycans and fibronectin is stimulated by an increase in fibroblasts, the latter stimulated by PDGD and epidermal growth factor (EGF) that originate from macrophages and blood platelets (Broughton *et al.,* 2006b:117). Fibroblast synthesis and proliferation is further stimulated by TGF-β; this also prevents collagen degradation and acts as a mediator for cellular adhesion to extracellular matrix (Broughton *et al.,* 2006a:117; Diegelmann & Evans, 2004:9).

2.2.3 Remodelling

The final phase of the wound healing cascade aims to establish equilibrium in collagen deposition by continuous synthesis and degradation (Diegelmann & Evans, 2004:9). A thicker, stronger extracellular matrix is formed in this way by continuous collagen deposition as fibronectin and proteoglycans are replaced by type-I collagen in this process (Broughton *et al.,* 2006b:117). Specialised collagenase enzymes found in fibroblasts, neutrophils and macrophages, keep the

collagen synthesis within the required range for optimal healing (Broughton *et al.,* 2006a:117; Broughton *et al.,* 2006b:117; Diegelmann & Evans, 2004:9).

2.3 Impaired wound healing

Wounds often do not heal successfully in the expected time if an alteration in the normal wound healing cascade is at play; this may lead to chronic wounds. Excessive amounts of wound exudate are usually associated with chronic wounds as a result of oedema caused by inflammation, decreased mobility and venous or lymphatic insufficiency (Boateng *et al.,* 2008:97). Exudate levels may also be increased by autolytic debridement, a process which causes necrotic tissue to develop into a wet sloughy, mass (Boateng *et al.,* 2008:97). It is thus evident that wound moisture must be effectively balanced, where cellular mediators can function optimally and cell growth can take place, allowing for matrix deposition (Alvarez *et al.,* 1983:35; Winter, 1962:193).

2.4 Description of wound conditions

2.4.1 Burn wounds

Burn wounds compromise the skin's protective barrier properties thus increasing the risk of bacterial contamination and growth (Alexander, 1990:30). Excessive heat causes the affected skin cells to die which leads to severe damage to the skin creating a typical burn wound. During the burn insult, free radicals and superoxide's are released adding to collateral skin damage. Dying cells also release cytokines which trigger the recruitment of immune competent cells and putative inflammation. Absorbatox® plays an important role in sorption of these components (Snyman *et al,.*2002:57). Most burn wounds are manageable on an outpatient basis. Burns occur as a result of exposure to extreme heat, such as fire or steam, radiation, friction, contact with heated objects or chemicals and electricity (Der Sarkissian, 2017). Burn wounds are classified into three different degrees depending on severity. First degree burns result in erythema of the epidermis of the skin which is considered to be a mild burn wound. Second degree burn wounds result in the erythema and swelling of the affected epidermis and upper layers of the papillary dermis of the skin, these types of burns constitute the majority of burn injuries. Second degree burns are classified as superficial partial thickness burns which cause pain, erythema, swelling and blistering of the affected area (Benson *et al.* 2006:332; International, 2014; Loyd & Rodgers, 2012:85). Third degree burns result in white or blackened, charring and numbness of the affected skin. Third degree burns are classified as full thickness burns (Der Sarkissian, 2017). A critical factor in burn wound healing is prevention of microbial contamination, especially in deeper, partial and full-thickness burn wounds (Benson *et al.* 2006:332; International, 2014; Loyd & Rodgers, 2012:85).

Burn wounds, thermal destruction of the skin barrier, cause a decreased sensitisation in local and systemic host cell and humoral immune response. These factors play an important role in combatting infection. The surface of the burn wound is initially sterile, directly after thermal insult, but soon gives way to a growth medium for micro-organisms. Micro-organisms thrive and colonise on the protein rich wound surface which consists of avascular necrotic tissue which provides a favourable growth environment. The extent of micro-organism colonisation determines the risk for future invasive wound infection. Micro-organisms such as staphylococci, gram-positive bacteria, often survive thermal damage to the skin, or colonise from adjacent skin, allowing for heavy colonisation of the wound surface unless anti-microbial substances are used immediately after burn injury. (Church *et al.,* 2006: 19).

2.4.2 Abrasions

A superficial abrasion of the skin is described as a superficial or shallow wound to the epidermal layer of the skin. It often occurs as a result of an excoriation or circumscribed removal of the superficial layers of skin or mucus membrane by rubbing or scraping against a rough surface (Chandler, 2017). Abrasions are not by nature sterile and are usually contaminated from the onset and may even include environmental spores such as *Aspergilus* over and above normal skin flora (Stevens *et al.,* 2005:41). An abrasion differs from burn wounds only in the mechanism of wound creation. Both the treatment of burn wounds and superficial skin abrasions remains similar in the sense that using a wet wound dressing results in faster healing and a faster onset of pain relief as well as a decreased incidence of scar formation (Beam, 2007: 42).

2.4.3 Acne

Acne is described as an intense perifollicular inflammatory skin condition in which skin pores are engorged with an excess of sebum production, dead skin cells and may include the proliferation of bacteria, usually as a result of increased sebaceous gland activity during puberty. The swelling of the skin pores causes a break in the follicle wall, follicular hyperkeratinisation, which is caused by a cascade of pro-inflammatory cytokines. This follicular rupture may cause perifollicular abscess as well as deeper lesions. Often these abscesses are sterile as the inflammation is elicited by fatty acid breakdown products which elicits the immune response. If infected, bacterial material is allowed to spill into surrounding skin tissue. This series of events leads to the abnormal healing cascade which results in an imbalance of matrix degradation, as well as the biosynthesis of collagen (Fabbrocini, 2010:893080; Urioste *et al.,*1999:18). Acne usually presents as spots, whiteheads, blackheads, cysts, nodules and pimples on the surface of the skin, giving it a rough appearance. The areas mostly affected are the face, shoulders, back, neck, chest and upper arms (Nordqvist, 2017).

Acne scars are a result of a deviation in the order of normal wound healing. Acne scars are classified as either atrophic or hypertrophic. Atrophic scars are caused by inflammatory processes causing the destruction of collagen which appear as dermal depressions due to dermal atrophy. Atrophic scars appear erythematous initially followed by an increasingly hypopigmented and fibrotic phase (Jacob *et al.* 2001: 45). Hypertrophic acne scars appear as erythematous, elevated, firm nodular lesions and are limited to the site of original tissue injury. Keloids are also seen as a common appearance of acne. Keloids typically present as deeper reddish-purple papules and nodules, often appearing on the anterior chest, shoulders and upper back. Keloids are recognised by thickened bundles of hyalinised acellular collagen arranged in whorls and nodules (Alster & Tanzi, 2003: 4).

Modern acne treatment ranges from dermabrasion, chemical peels to laser therapy i.e. superficial abrasions or burns. One of the main goals in the effective prevention and treatment of acne is the continuous use of a topical treatment that contains a topical anti-microbial, the latter often used for its anti-metabolic or anti-inflammatory activity rather than its anti-microbial activity as resistance is usually seen within a week of treatment (e.g. tetracyclines and macrolides). Successful treatment of acne usually calls for the use of topical benzoyl peroxide together with a topical or oral anti-microbial, as well as the option of using a retinoid, in order to decrease the incidence of bacterial resistance and reduce sebum production (Krader, 2014). Studies have shown that occluding the affected area of skin after the application of the active formulation, promotes skin hydration of the underlying stratum corneum. This allows for an increase in absorption of the topically applied active ingredient, thus allowing for faster healing and decreased incidence of scarring (Martin *et al.,* 2000:26). Development of a thick, flexible hydrogel dressing is ideal to protect the affected area from trauma, i.e. scratching, as well as absorb excess fluid and microbial material from the skin, allowing for an anti-inflammatory effect to manifest if ingredients are selected well (Ladenheim *et al.,* 1996:48; Martin *et al.,* 2000:26).

2.4.4 Exuding wounds

Exuding wounds such as lower leg ulcers are associated with delayed healing and are prone to infection, making them a problem, especially among the elderly. Chronic exuding wounds express a disrupted healing process, halted at one or more of the different stages of normal wound healing. This develops into a wound which does not progress to healing with the conventional wound healing treatments available. The exuding wound is characterised by a loss of skin and/or the underlying soft tissue which fails to heal (Boynton *et al.,* 1999:34; Frantz & Gardner, 1994:20; Schultz *et al.,* 2003:11). Lower leg ulcers are a result of increased hydrostatic pressures in the venous system thus causing the veins to distend (e.g. varicose veins or blood flow obstruction). This causes widening of the cell junctions and allows for fluid to pass into the tissue and or wound (Majno & Joris, 1996). Maceration results around the wound due to

excessive exudate, this establishes an environment for bacterial colonisation with organisms such as staphylococci, enterococci and *Pseudomonas aeruginosa*, and delayed healing (Cutting & White, 2002:18; Fiers, 1996: 42; Majno & Joris, 1996; Schmidtchen *et al.,* 2003:34).

For the optimal treatment of wounds, an adequate, effective exudate, ad- and absorbing, wound dressing which maintains a moist environment at the surface of the wound which is not frequently removed. Too frequent changes of wound dressings could cause damage to the wound bed (new epithelium stripped off) and increase the risk of bacterial contamination. The optimal wound healing environment is critical for successful healing (Hampton, 2004). Leg ulcers more often than not produce large amounts of exudate due to their larger surface areas, which may decrease growth factor availability for cell proliferation and increased proteolytic activity (Muldoon, 2013; WUWHS, 2007).

The aim of wound treatment is essentially to promote re-epithelialisation as well as preventing both infection and desiccation, while providing effective pain relief. One conventional approach to effective wound care is to regularly change the absorptive wound dressings along with the topical application of an anti-microbial (Hajská *et al*., 2014: 40).

2.5 Wound dressings

The main goal of treating any type of wound is to create an optimal environment for healing, by providing a well vascularised, stable wound bed that is conductive to normal and timely healing (Schultz *et al.,* 2003:1-28). Modern wound dressings are free of the disadvantages of traditional dressings as they have improved textile materials, flexible designs and possess combined properties such as wound healing and antimicrobial activity. This expands the function of newer dressings having more advantages of being atraumatic in character, possess effective curative action as well as reduced therapy duration (Yudanova & Reshetov, 2006:24).

2.5.1 Silicone-based gels

Silicone-based devices have proven to improve the occlusion and hydration of wound beds as well as increase the hydration of the stratum corneum and elevating the skin's surface temperature (Bleasdale et al., 2015:4). Silicone as a form of topical gel or gel sheeting is often used in the treatment of cutaneous wounds to promote healing and to prevent the risk of scar formation, as well as to reduce the skin's acute inflammatory response (Kim *et al.,* 2014:29; Kwon *et al.,* 2014:28; Parry *et al.,* 2013:34).

During this study the development of an organic acid, Absorbatox® combination silicone-based gel for the treatment of a superficial skin abrasions and burn wounds will be created, with the following qualities: creating the optimal wound healing environment as it optimises pH, scavenges free radicals, promotes cell proliferation and protects the wound surface from environment will.

2.5.2 Hydrogel-based patches

Hydrogel-based patches allow for an increase in application time; thus, resulting in prolonged delivery of active substance and or creating an ad- and absorbing environment for bacteria and unwanted free radicals. These patches are able to provide an occlusive covering which promotes hydration of the underlying stratum corneum which accelerates the healing process and reduces pain and inflammation (Kamoun *et al.,* 2017). Hydrogel-based patches are classified as macromolecular networks which prove them excellent for wound healing applications (Kamoun *et al.,* 2017). Hydrogels possess unique properties such as high-sensitivity to physiological environments, hydrophilic in nature, soft tissue-like water content, as well as adequate flexibility which all add to their excellent wound healing capability. Hydrogels have the ability to both swell and de-swell wound exudate in a reversible direction which shows specific environmental stimuli-responsive e.g. temperature and pH (Kamoun *et al.,* 2017). Hydrogel wound patches can absorb as well as retain wound exudate which promotes fibroblast proliferation and keratinocyte migration, both of which are necessary for complete epithelialisation and wound healing. The tight mesh size of the hydrogel structure allows for wound protection against infection and microorganism and bacterial wound penetration. The hydrogel structure allows transport of API, by the entrapment of the API into the hydrogel network during the gelling process, while the molecules are then exchanged with absorbing wound exudate during adequate contact with the wound surface (Kamoun *et al.,* 2017). Hydrogel patches are simply a hydrophilic polymeric network cross-linked in a specific way to produce an elastic structure usually formulated by the addition of powders such as pectin and/or sodium carboxymethylcellulose to a heated premix, comprised of hydrophobic synthetic and semisynthetic elastomers, trackifying resins and mineral oils, to produce a homogeneous dispersion (Ahmed, 2015). An adhesive sheet of uniform thickness is produced and laminated between a sheet of polyethylene film and silicone release paper (Martin *et al.,* 2000:26). Owing to its success as an occlusive wound cover is its unique property to absorb exudate from wound cavities as well as deliver topically applied drugs (Ladenheim *et al.,* 1996: 48). A hydrogel dressing will have very similar properties to the silicone dressing but with more hygroscopic activity with the potential to address superficial exudation and inflammation better in order to optimise the healing environment. Again, the cell proliferative and anti-inflammatory effects or organic acids will be combined with the anti-oxidant and scavenging properties of a specially formulated Absorbatox® in the hydrogel patch.

2.5.3 Powder dressing (dry form)

Dry powder dressings in sachet type wound dressings are able to absorb more wound exudate and associated bacteria and irreversibly bound to such materials. It may also create capillary suction forces, which aids in removing excessive moisture from the wound if the correct material is selected. A dry ceramic powder (e.g. Cerdak™) is contained in a non-woven material sachet

with these claims. The dry ceramic wound dressing is able to improve the healing process by providing effective infection control by preventing microbial colonisation as well as decrease the risk of scar formation (Available from Cerdak™ website). In this study a sachet based wound dressing much like Cerdak™ containing the activated clinoptilolite, Absorbatox® will be developed. The latter has known ad and absorption qualities as well as the ability to create capillary action to control moisture but also to have significant anti-bacterial and anti-oxidant qualities when applied to wounds (Muldoon, 2013; WUWHS, 2007). This make it an ideal ingredient for a dry powder dressing. Adding these latter properties to that of organic acids seems to be worthy of exploration.

2.6 Organic acids and Absorbatox®: experimental wound treatments

2.6.1 Zeolites

Zeolites are known as naturally occurring minerals which are mined in various parts across the world. Not all zeolites are equal, zeolites which are commonly used commercially are produced synthetically, and thus are used for different applications (Lenntech, 2019).

Nearly fifty different types of zeolites exist (clinoptilolite, chabazite, phillipsite, mordenite, etc.) which all have unique physical and chemical properties. The crystal structure and chemical composition of these minerals account for the largest differences. Whereas, particle density, cation selectivity, molecular pore size as well as strength (“hardness”) are some of the properties which differ to a lesser extent depending on the zeolite in question. Most naturally occurring and synthetically produced zeolites, each with a unique structure, have a pore size ranging from approximately 0.0003 – 0.0008 µm (Lenntech, 2019).

The biggest difference between naturally occurring and synthetic zeolites is the method in which these aluminosilicates are manufactured. Synthetic zeolites are artificially manufactured from energy consuming chemicals which have a silica to alumina ratio of 1 to 1, whereas natural zeolites are processed from natural ore bodies having a silica to alumina ratio of 5 to 1. Zeolites are formed in cavities in lava flows and in plutonic rocks. These are well-defined, microporous, three-dimensional, tetrahedral, crystalline structures which have an excessive negative charge as seen in Figure 2.2 and 2.3. This negative charge is compensated for by cations, such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^+), etc., which can be exchanged for others in a contact solution in a patented process (Seetharam & Saville, 2002: 31). Void spaces, as seen in Figure 2.4, within the crystalline structure can selectively exchange specific types of molecules and are thus capable of hosting cations, water and other organic molecules. The exchange process results in a product which is able to exchange specific molecules. Hydrated aluminosilicates have many uses both in the industry and agriculture, these uses are mainly attributed to the unique physicochemical properties which include a higher cation exchange capacity (CEC), adsorbent nature, size exclusion framework (through manipulating the

pore size and charge) as well as catalytic properties, all of which have proven to be safe in animals and humans (Potgieter *et al.,* 2014:7; Rodriguez-Fuentes *et al.,* 1997: 19). Different particle sizes and charges of Absorbatox® result in different CEC values, according to which it is classified. For this study Absorbatox® 2.4D is used (with a CEC equal to 7.2).

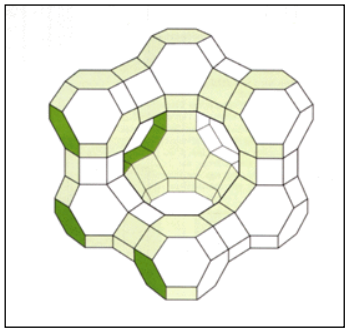

Figure 2.2: Three-dimensional tetrahedral structure of zeolite (clinoptilolite) (Absorbatox (Pty) Ltd, 2008)

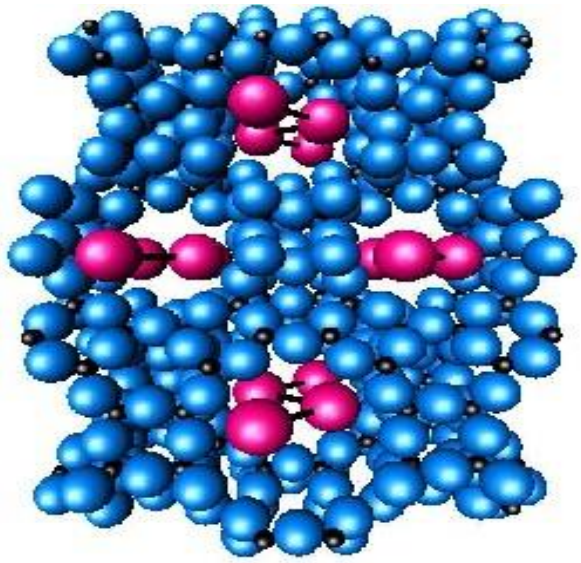

Figure 2.3: Binding position of cations within zeolite (clinoptilolite) structure (Absorbatox (Pty) Ltd, 2008)

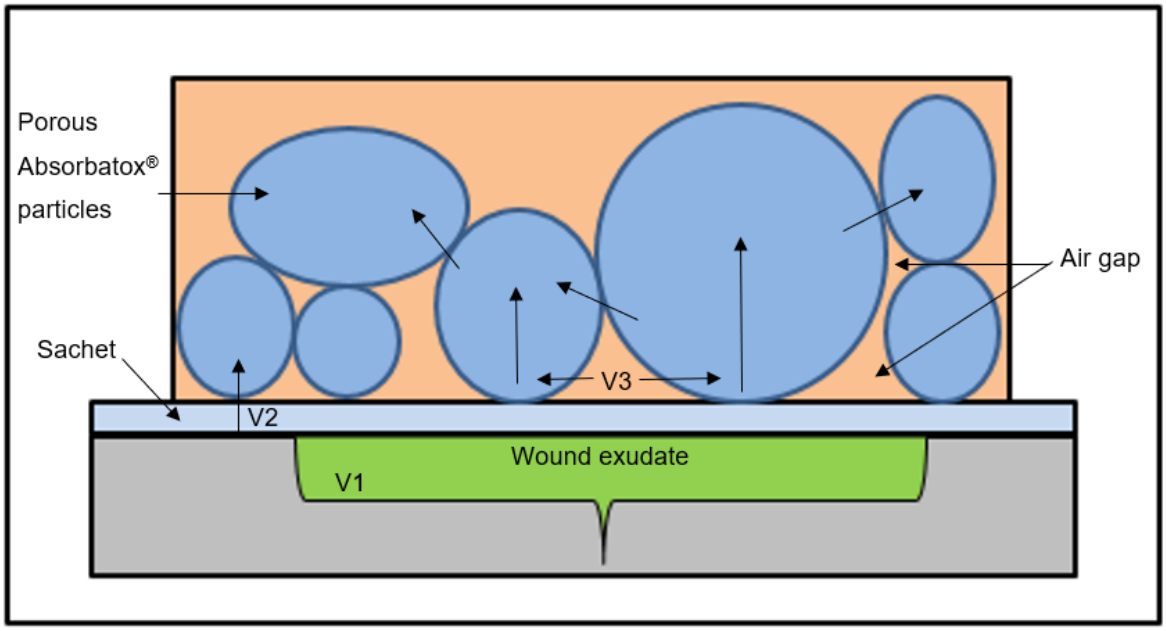

Figure 2.4: Schematic representation of the mechanism of action of Absorbatox® (Absorbatox (Pty) Ltd, 2008)

The wound produces exudate at a rate V_1. The exudate is absorbed by the sachet and passes through it at the same rate ($V_2 = V_1$). Upon reaching the particles, the exudate is absorbed at a rate V_3, which is much faster than the rate of supply. This creates an air gap at the point of contact with the sachet. When this happens, the absorption process stops until the wound supplies more moisture, and then it repeats itself (Absorbatox (Pty) Ltd, 2008).

2.6.2 Absorbatox®

Absorbatox® ($(Na,Ca,K)_6Si_{30}Al_6O_{72}{\cdot}nH_2O$) is a natural or synthetically enhanced zeolite, with a particle size between 30 – 400 µm, its unique properties making it like no other crystal hydrated aluminosilicate, which belongs to the clinoptilolite family. Absorbatox® is proven to have epithelial protective properties in patients with endoscopically negative gastro-oesophageal reflux disease (ENGORD) as well as patients taking non-steroidal anti-inflammatory drugs (NSAIDs) (Potgieter, *et al.,* 2014:7). The protective effect confirmed in studies is a result of binding to biologically active amines and nitrates (Potgieter *et al.,* 2007). Absorbatox® is able to create an optimal wound healing environment through its unique ability to bind with harmful biologically active substances which may be present in the wound exudate. Absorbatox® generates capillary suction forces through its porous crystal structure, this makes topical wound dressing a possibility along with excellent exudate management (Mncube, 2013; Potgieter *et al.,* 2007)

The successful use of Absorbatox® in a skin-mask to treat environmentally exposed skin, i.e. chlorine and sun exposure is attributed to its anti-oxidative and free-radical scavenging properties

(data on file dermaV pharmaceuticals (Pty) Ltd). The addition of Absorbatox® in a topical skin formulation aids in managing and preventing acute skin damage and enhance re-epithelialisation with evidently reduced scarring when used in wound healing applications.

2.7 Organic acids in wound healing

The pH value on the surface of an existing wound is both directly and indirectly affected by all biological reactions which occur during the wound healing process. The surface pH of a wound plays an important part in effective wound healing as it aids in infection control and increase of anti-microbial activity, oxygen release, angiogenesis, protease activity as well as bacterial toxicity. In this way, the pH affects regular cellular events in wound healing. Organic acids have the ability to successfully decrease wound surface pH when incorporated into modern wound healing products (Nagoba *et al.,* 2015:5).

Organic acids assist the wound healing process by providing infection control as most bacteria associated with infected wounds in humans require a pH above 6, thus a decrease in pH by the organic acid inhibits the growth and multiplication of bacteria by creating an unsuitable environment. Alteration of protease activity is also seen by the application of a pH decreasing acid to the wound surface. Protease are enzymes produced by the wound itself and function optimally at a specific pH allowing for more rapid ability to cleave proteins needed for wound healing. A decrease in pH value leads to the Bohr-effect (i.e. increase in the amount of available oxygen of cells). This increase in the level of oxygen delivery to damaged tissue increases resistance to infection as well as promote healing. A reduction in toxicity of bacterial end products such as ammonia is also a benefit of a decreased pH value on the surface of a wound. An acidic environment also promotes epithelisation by boosting fibroblastic growth and neovascularisation which increases microcirculation of wounds that in turn enables the formation of new healthy granulation tissue and ultimately faster wound healing (Nagoba *et al.,* 2015:5).

2.7.1 Fulvic acid

Humic acid is obtained by a unique conversion process of bituminous coal (Bergh *et al.,* 1997:76). Humic acid substances can be divided into three components, namely, humic acid, fulvic acid and humin based on their solubility in water as a function of pH (Van Rensburg *et al.,* 2001:53). The humic substances are naturally formed substances, formed during the decay of plant and animal residues (Snyman *et al.,* 2002:57).

Fulvic acid is a unique derivative which is soluble in water under all pH conditions, it is lower in molecular size and weight and it also appears with a lower colour intensity than the other humic acids (Van Rensburg *et al.,* 2001:53). Its anti-microbial and anti-inflammatory effects were observed in the topical application of oxidised fulvic acid (OFA) to laboratory mice, which acted

to suppress superoxide production by neutrophils (Van Rensburg *et al.,* 2001:53). Fulvic acid has a wide anti-microbial spectrum, as well as the anti-inflammatory properties when used in topical formulations.

2.7.2 Malic acid

Malic acid is a unique colourless to white crystalline solid, which is present naturally in many plants such as flowers, vegetables, spices and wine grapes. Malic acid is part of a group of organic acids, called alpha-hydroxy acids (A-HAs), which possess anti-oxidant, anti-inflammatory and anti-platelet aggregating properties (Dharmappa *et al.,* 2009:75; Takada *et al.,* 2010:17). Malic acid is able to successfully balance the pH levels of the skin, as well as encourage a high cell turnover rate; as it is able to penetrate into the deeper layers of skin to promote collagen formation, which is ideal for wound healing. Malic acid is also a humectant which helps with moisture retention necessary for optimal wound healing and is highly soluble in water. The acid has unique exfoliation properties making it an ideal substance in acne treatment (Marusinec & Ashpari, 2014).

Malic acid has proven efficacy as a topical anti-microbial agent; seeing that it is able to cause significant damage to the cytoplasm of micro-organism cells allowing for their anti-microbial activity (Eswaranandam *et al.,* 2004:69).

2.7.3 Citric acid

Citric acid is a colourless, odourless crystalline structure which has hygroscopic properties as well as highly water soluble (Ciriminna *et al.,* 2017:11). Citric acid has proven antiseptic, as well as anti-oxidant properties. Citric acid is used as an effective topical anti-microbial agent against various bacteria, including *Pseudomonas aeruginosa* from burn wounds. Its anti-microbial activity is attributed to its pH lowering ability (Watts & Frehner, 2016:24). Citric acid exhibits excellent metal ion chelating properties, which add to its suitability as a pharmaceutical and is commonly used as an effervescent in powders (Ciriminna *et al.,* 2017:11). Citric acid has hydrophobic properties that aid in its anti-microbial activity as microbial cell wall consists of lipid material, thus the acid is able to interact with this lipid layer and disrupt microbial activity (Bartek ingredients, 2008).

2.8 Conclusion

In previous studies it is confirmed that Absorbatox® has great epithelial protective properties, although the exact mechanism of the protective effect was not elucidated, it could be as a result of its binding capability to biologically active amines and nitrates (Potgieter *et al.,* 2007). As aforesaid, Absorbatox® is able to create an optimal wound healing environment through its unique

ability to bind with these harmful biologically active substances which may be present in the wound exudate (Potgieter *et al.,* 2007).

As previously stated, Absorbatox® is able to generate capillary suction forces through its porous crystal structure, this makes topical wound dressing a possibility along with excellent exudate management (Mncube, 2013; Potgieter *et al.,* 2007).

The pH value on and within the wound both directly and indirectly influences the healing process. The pH on the surface of the wound plays an important role in infection control, increases anti-microbial action, oxygen release, angiogenesis, protease activity as well as bacterial toxicity. Decreasing wound surface pH with topical applications containing organic acids will provide an environment unfavourable for microbial infestation and growth (Prabhu *et al.* 2014:2).

The properties of Absorbatox® combined with specifically selected organic acids i.e. fulvic, malic and or citric acid have the potential for the formulation of unique new wound dressings, able to addresses many unmet needs in wound care. These combinations will be explored for uniqueness and the appropriate combination selected for specifically targeting each unique wound challenge such as exudate handling, anti-infective and anti-inflammatory properties as well as the ability to promote collagen and epithelial function.

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CHAPTER 3

ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

This chapter is written in article format for publication in The International Journal of Pharmaceutics. For the ease of reading, the paragraphs of this chapter have been justified. The author's guide states that the article can be written in either UK or US English, and the authors decided to write in UK English. The complete author's guide is listed in Appendix F.

Wound healing effects of Absorbatox® bound to selected organic acids: *in vitro* investigations using the HaCaT and 84BR cell culture models

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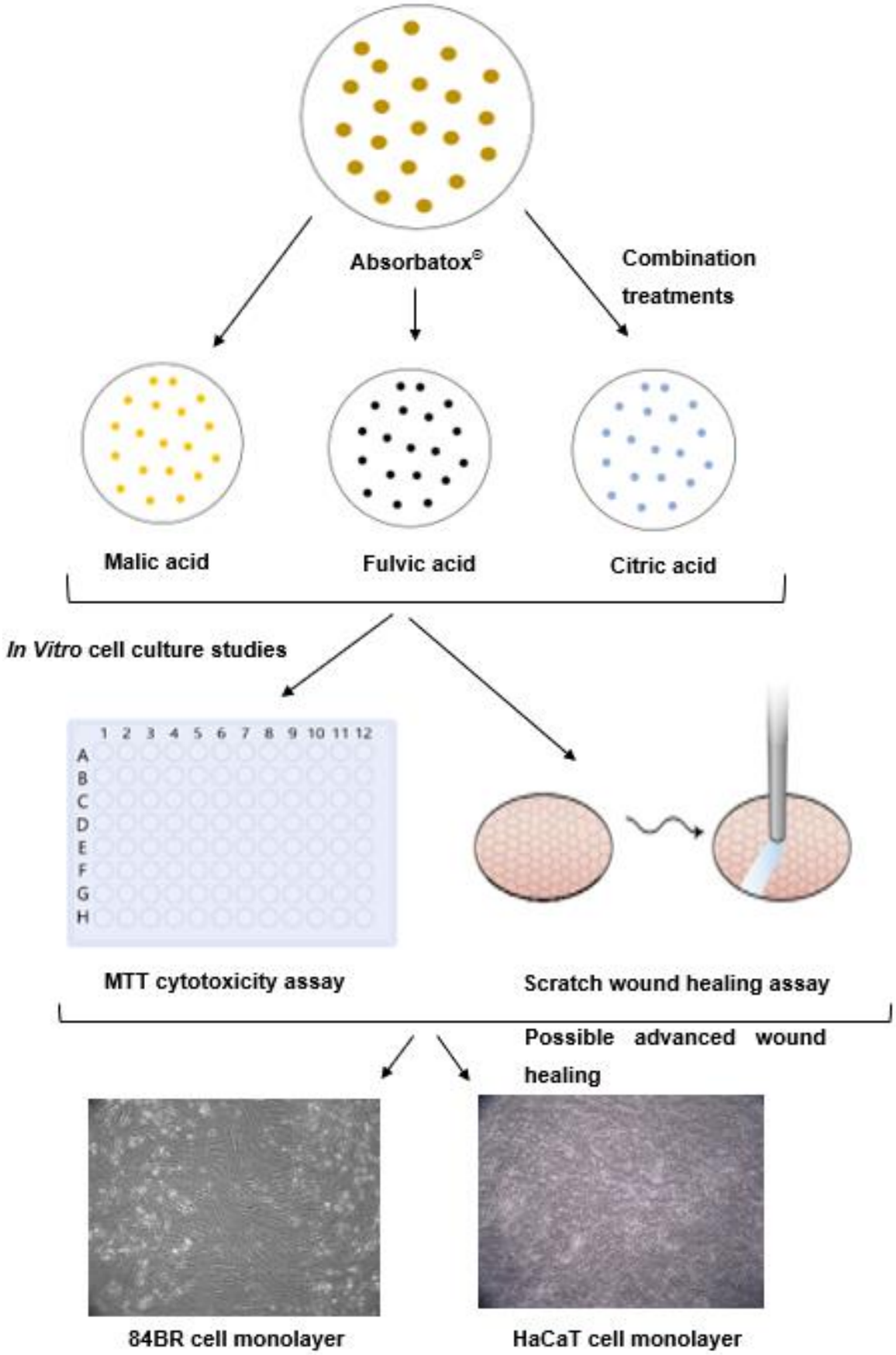
Abstract

The development of new treatments for wounds is in high demand, as current available treatments do not address all the issues in wound healing and often a “healed wound” is compromised. The wound pH plays an important role and therefore organic acids with known antimicrobial activity was tested bound to Absorbatox® a potentiated clinoptilolite with known free radical scavenging and wound enhancement properties. The purpose of this study was to investigate the effects of the active ingredient, which consisted of a patented combination of products containing Absorbatox® with a selected organic acid (citric, malic and fulvic acid) *on in vitro* cell cultures, specifically human immortalised keratinocyte (HaCaT) cells and human dermal fibroblast (84BR) cells. The cell cytotoxicity was determined by means of methylthiazol tetrazolium (MTT) assay to determine the safety for the application on human skin and essentially on wounds. *In vitro* wound assays were used mimicking wound healing, i.e. scratch assay, as well as a cell migration assay. The Absorbatox® bound to fulvic acid demonstrated the most significant growth promotion when compared to no treatment at all and increased cell proliferation and healing by 30%.

Keywords: Absorbatox®, Organic acids, Wound assay, Cytotoxicity, MTT-assay

Graphical abstract



1 Introduction

A wound is defined as break in the continuity of the skin as a result of physical or thermal damage. Although the skin provides the essential barrier between the internal tissues and the external environment, it is injury to this barrier, exposing deep tissue which poses the largest risk to the underlying tissue as well as, in severe cases, survival (Boateng *et al.,* 2008; Kooistra-Smid *et al.,* 2009). Wounds as well as wound healing have proven to be a challenge over many centuries. The healing process has been extensively researched and proves to be both intricate and interactive, involving both soluble mediators and cells. Wound healing is considered to occur spontaneously when the natural process of healing is not interrupted or prevented (Broughton *et al.,* 2006). The normal wound healing cascade can be sequentially divided into three distinct phases, namely haemostasis and inflammation, proliferation (re-epithelialisation) and lastly, remodelling (neovascularisation) (Broughton *et al*., 2006; Diegelmann & Evans, 2004). Alteration in this normal wound healing cascade is often as a result of underlying pathological conditions such as diabetes mellitus, hypothyroidism, Crohn's disease and burns, etc. These conditions may result in abnormal and difficult to treat wounds, i.e. chronic pressure ulcers, keloid scars, fibrosis, etc. Patients suffering from immune disorders are of particular concern and when associated with these complicated wounds may result in significant morbidity and mortality (Keller *et al.,* 2002). Wound treatment and healing are complex processes, which are generally poorly understood. Patients may experience severe pain and discomfort, while wound healing is frequently delayed with significant scarring and loss of long-term function due to a myriad of reasons such as poor blood supply, infection, growth factor over or under stimulation (excessive or inadequate fibroblast growth), and wound bed exudate, to mention but a few aspects influencing clinical outcomes. It is therefore important that the optimal wound healing environment is established as quickly as possible considering the various types of wounds, the anatomical place and cause of wound (Keller *et al.,* 2002).

Modern wound healing treatments stem from a better understanding of the various interactions between the cells and mediators, such as cytokines, growth factors and lipid

derivatives/mediators (such as prostaglandins, etc.). These wound treatments usually involve a specialised wound dressing, designed with the aim of improving the ability to maintain a sterile (free of bacterial contamination) environment, as well as eliminating the excess exudate produced by the exposed tissue (Von Cramon *et al*., 2017). The wound exudate often disrupts the healing process and acts as a medium to allow bacterial growth and even protects the micro-organisms from antibacterial agents applied locally or systemically (Von Cramon *et al*., 2017).

In previous studies, it was confirmed that Absorbatox® has great epithelial protective properties, although the exact mechanism of the protective effect was not elucidated, it could be as a result of its binding capability to biologically active amines and nitrates (Potgieter *et al.,* 2014). Absorbatox® is able to create an optimal wound healing environment through its unique ability to bind with specific harmful biologically active substances, which may be present in the wound exudate (Potgieter *et al.,* 2007). Absorbatox® is also able to generate capillary suction forces through its porous crystal structure; this makes topical wound dressing a possibility along with excellent exudate management (Potgieter *et al.,* 2007).

The acidity of the wound and wound environment directly and indirectly influences the healing process. The pH on the surface of the wound plays an important role in infection control, increases anti-microbial action, oxygen release, angiogenesis, protease activity, as well as bacterial toxicity. Decreasing wound surface pH with topical applications containing organic acids will provide an environment unfavourable for microbial infestation and growth (Prabhu *et al.,* 2014). Organic acids not only modulate the pH, but specific acids may have other properties such as direct antimicrobial activity and/or anti-inflammatory effects, which may even enhance fibroblast proliferation; all beneficial for optimal wound healing (Prabhu *et al.,* 2014; Van Rensburg *et al.,* 2001).

The purpose of this study was to investigate the effects of Absorbatox® alone and when bound with a selected organic acid, as well as selected organic acids alone on both human immortalised keratinocyte (HaCaT) cells and human skin fibroblast (84BR) cell lines. A methyl thiazolyl tetrazolium (MTT) assay was performed to assess if the active ingredient presented

with cytotoxic effects, followed by a scratch wound healing assay to determine the wound healing potential of the Absorbatox® in bound to a selected organic acid.

The skin consists of three types of cells namely keratinocytes, fibroblasts, as well as melanocytes. Previous studies on wound healing and cell cultures proved that HaCaT cells may be used as a successful *in vitro* model. The spontaneously immortalised cell line has characteristics of easy propagation and near normal phenotype (Lópes-Garcia *et al*., 2014:44). 84BR cells are known as one of the most ubiquitous cells in complex organisms, such as humans. 84BR cells are essentially involved in the repair and healing of damaged tissue. They are the main cells of stomal tissue, indicating their role during toxicity studies in an *in vitro* model (Rieske *et al*., 2005).

Cytotoxicity testing is one of the most important methods of biological assessment and is a key biological evaluation that makes use of tissue cells *in vitro* to observe cell growth, reproduction, as well as morphological effects resulting from the application of pharmaceutical active ingredients (Li *et al*., 2015). Cytotoxicity studies were generally only performed later in the development process of new chemical entity discovery, causing an increase in the rate of drug attrition as a result of toxicology-related problems (Peternel *et al*., 2009). In more modern research, it has become one of the preferred indications for toxicity evaluation as it is simple, fast and exhibits high sensitivity (Li *et al*., 2015). Although cytotoxicity studies may present as an expensive process, it is an important tool used to eliminate unsuitable formulations or chemical entities before the commencement of pre-clinical trials, aiding in the initial screening of new pharmaceutical agents (Van Tonder *et al*., 2015).

Although *in vitro* and *in vivo* conditions are very different, it can be said that toxicological assessments performed during the development of new drug delivery systems determine if any of the excipients of the formulation are toxic to human tissue. This adds to the importance of *in vitro* cell culture studies, providing the building blocks on which future *in vivo* studies are based (Wang *et al*., 2010, Yoon *et al*., 2012).

2 Materials and methods

2.1 Materials

Absorbatox® as well as Absorbatox® bound to selected organic acids was obtained from Absorbatox (Pty) Ltd (SA). Citric acid, fulvic acid, as well as malic acid were obtained from ASAP Pharma Care (PTY) Ltd (SA). Phosphate buffered saline (PBS) and high glucose Dulbecco's Modified Eagle's Medium (DMEM) were obtained from HyClone™ (Hyclone, USA). Trypsin-ethylenediaminetetraacetic acid (EDTA), non-essential amino acids (NEAA) and L-glutamine were obtained from Lonza™ (Lonza, Switzerland). Foetal bovine serum (FBS) was obtained from Gibco™ (Gibco, SA). Trypan blue, methylthiazol tetrazolium (MTT), dimethyl sulfoxide (DMSO), Triton™ X-100 and penicillin/streptomycin (pen/strep) were all obtained from Sigma-Aldrich® (Johannesburg, RSA).

2.2 Methods

2.2.1 Selection of an appropriate cell line and cell culture preparation

Both HaCaT and 84BR cells were used as the models on which the *in vitro* cell culture studies were performed. The HaCaT cells are suitable for wound healing studies, because they are human epidermal cells. The HaCaT cell line was maintained in 75 cm^2 cell culture flasks, each containing DMEM supplemented with 1% NEAA, 10% FBS, 2 mM L-glutamine and 1% pen/strep. The 84BR cell line was maintained in 75 cm^2 cell culture flasks, each containing DMEM supplemented with 1% NEAA, 15% FBS, 1% pen/strep and 2 mM L-glutamine. Standard incubation conditions were set at 37°C, 5% CO_2 and 95% humidity. For sub-culturing, the adherent cells were washed with PBS, detached with 5% trypsin-EDTA, re-suspended in growth medium and centrifuged at 140 *x g* for 5 min. Thereafter, the supernatant was discarded and the cell pellet re-suspended in the appropriate medium for either sub-culturing into new flasks or seeding into multi-well plates for assays. To maintain the cells, the culture medium was refreshed every two to three days (Mazumder *et al.,* 2016). The cells were checked regularly for bacterial contamination and the confluence estimated using an inverted light microscope.

2.2.2 Concentrations used for exposure

The cell lines were treated with the following formulations: a control group (Absorbatox®), as well as vehicle groups containing both Absorbatox® in bound to a specific organic acid (malic acid, citric acid or fulvic acid). Treatments for the MTT-assay were prepared in two concentrations of each combination of Absorbatox® alone, as well as Absorbatox® bound to an organic acid (0.15 mg/ml and 0.30 mg/ml), i.e. Absorbatox® 0.15 mg/ml bound to 2% malic acid, Absorbatox® 0.30 mg/ml bound to 2% malic acid, Absorbatox® 0.15 mg/ml bound to 4% malic acid and Absorbatox® 0.30 mg/ml bound to 4% malic acid. The treatments for the acids alone were prepared in concentrations of 0.5, 1.0, 2.0 and 4.0%.

2.2.3 *In vitro* cytotoxicity assays

Cultured HaCaT and 84BR cells were evaluated based on cell viability, when exposed to the Absorbatox® and organic acid formulations, as well as exposure to selected organic acids alone (citric, malic and fulvic acid). The cell lines were cultured to 80% confluence, trypsinised, the cell count determined, cell suspensions with specific concentrations prepared and seeded in 96-well plates.

The HaCaT and 84BR cells were seeded into clear 96-well plates at 50 000 cells/ml (10 000 cells/well) and 75 000 cells/ml (15 000 cells/well) for HaCaT and 84BR cells respectively, and incubated for 24 h to form a monolayer. The cells were then treated with various concentrations between 0.5 and 4.0%, of the organic acids separately, Absorbatox® alone and Absorbatox® bound to an organic acid. The *in vitro* cytotoxicity of the selected organic acids and Absorbatox® on the HaCaT and 84BR cells was determined by means of a colorimetric method such as the MTT-assay. The cultured cells were seeded at different concentrations and the plates were incubated for 24 h at 37°C, 5% CO_2 and 95% humidity to ensure cells were properly adhered to the plate surface.

2.2.3.1 Methylthiazol tetrazolium assay

The purpose of the MTT-assay was to measure the cell viability of the HaCaT and 84BR cell lines, after an exposure period of 24 and 48h to the various treatment groups. Three different combinations of Absorbatox® bound to an organic acid concentration were tested, in addition

a negative control (dead cells obtained with Triton™ X-100) and a positive control group (untreated cells) were also included. All the treatments were conducted in six replicates. Blanks with DMSO or media only were also included. For the organic acids, the highest tested concentration was also loaded separately to check for any possible interference with MTT.

The absorbance of the wells was recorded at 560 nm, as well as at 630 nm for background interference, using a microplate spectrophotometer. This absorbance was used as an indication of the viable cells capable of reducing the yellow colour of the MTT to purple formazan crystals. Such a colour change is a result of dehydrogenase enzymes present in the mitochondria of the metabolically active cells (Fotakis & Timbrell, 2006). The calculation of the percentage cell viability was performed using Equation 1.

$$\%\text{Cell viability} = \frac{\Delta \text{ Absorbance of sample} - \Delta \text{ Absorbance of blank}}{\Delta \text{ Absorbance of untreated} - \Delta \text{ Absorbance of blank}} \times 100 \quad \textbf{(Eq. 1)}$$

Δ Absorbance = Absorbance of treated cells$_{560}$ – Absorbance of treated cells$_{630}$

Δ Blank = Mean absorbance of blank$_{560}$ – Mean absorbance of blank$_{630}$

Δ Control = Absorbance of control$_{560}$ – Absorbance of control$_{630}$

2.2.4 Scratch wound healing assay

The purpose of the scratch assay was to measure the *in vitro* wound healing efficacy of Absorbatox® formulations on the HaCaT and 84BR cell lines, after inducing a scratch area. The scratch assay was performed using 12-well plates with the cultured HaCaT and 84BR cells seeded at 400 000 cells/ml and 200 000 cells/ml respectively, and incubated for 24 h to form a monolayer. Three different Absorbatox® bound to an organic acid concentrations were tested, as well as an untreated group. Fresh growth medium containing the formulations were added to the scratched wells. The cells were then cultured for an additional 48 h during which photographs were taken of the designated wound area at 8, 16, 24, 32, 40 and 48 h intervals (Chen, 2012). The percentage wound closure of each wound was calculated according to Equation 2.

$$\text{Wound closure\%} = \frac{(\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{(\text{Pre-migration})_{\text{surface area}}} \times 100 \quad \textbf{(Eq. 2)}$$

The migration rate over time was calculated using Equation 3.

$$\text{Migration rate } (\mu m^2/h) = \frac{(\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{\text{Time (hour)}} \quad \textbf{(Eq. 3)}$$

2.2.5 *In vitro* cell migration assay

The CytoSelect™ cell migration assay was performed on the HaCaT cells (Mazumder *et al.,* 2016). The absorbance was measured with the microplate spectrophotometer at 560 nm. The percentage cell migration was calculated using the following equation.

$$\%\text{Cell migration} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \quad \textbf{(Eq. 4)}$$

2.2.6 Statistical analysis

The Brown-Forsythe test was performed on the results in order to determine if the data collected was normally distributed. The analysis of variance (ANOVA) test, followed by Tukey's HSD and Kruskal-Wallis tests, were performed on the results to determine if there were statistically significant differences among the various treatment groups. A p-value below 0.05 was considered as statistically significant.

3 Results and discussion

3.1 MTT-Assay

Cytotoxicity of a specific treatment can be divided into four groups namely, strong, moderate, weak and non-cytotoxic, depending on the degree of cytotoxicity to the cells, as seen in Table 1. The results are cell line and assay specific (López-Garcia *et al*., 2014). Visual observation of the 96-well plates after treatment indicated a clear increase in purple colour intensity, where the exposure to the Absorbatox® bound to an organic acid was favourable for cell proliferation, inducing a higher cell viability. Increased absorbance was a result of increased cell number and consequently, an increased amount of MTT formazan formed (Baluchamy *et al*., 2010). MTT results obtained after 24 and 48 h exposure periods of the HaCaT and 84BR cells to various treatments of Absorbatox® bound to different organic acids are shown in Fig. 1 and Fig. 2, with the results clearly indicating cell proliferation. The results obtained indicate a significant increase in cell viability when cells were exposed to Absorbatox® bound to citric, malic or fulvic acid, with Absorbatox® bound to both citric and fulvic acid attaining cell viability well over 100%; when applied to 84BR cells, a clear indication of cell growth. Treatments

consisting of Absorbatox® bound with either citric or fulvic acid, resulted in the highest cell viability. According to López-Garcia *et al*. (2014), a compound with a %cell viability between 60-80% is weakly cytotoxic and a compound with a %cell viability above 80% is considered non-cytotoxic. The results thus prove Absorbatox® bound to organic acids are not toxic to the selected keratinocyte and fibroblast cells and are therefore safe for use in the formulation of new wound healing treatments. Direct correlation can be observed between 24 and 48 h cell viability, except in treatment with Absorbatox® bound to 2% citric acid. The results obtained from both the HaCaT and 84BR cells indicated no significant different between treatment concentrations of 0.15 and 0.30 mg/ml. No significant difference was observed between the 2% and 4% concentrations of the organic acids bound to the Absorbatox®.

Tukey's HSD test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate a significant difference between the untreated control groups compared to all three organic acids namely, citric, malic and fulvic aids in concentrations of 0.5%. 1.0%, 2.0% and 4%. The Kruskal-Wallis test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate a significant difference between the untreated control groups compared to treatments containing citric acid 2% and 4% at a 48 h exposure period.

The results obtained from Tukey's HSD test on the results of the HaCaT cells indicated statistical significant differences after 24 h exposure between the untreated control group and Absorbatox® (0.30 mg/ml). There were statistical significant differences between Absorbatox® (0.15 mg/ml) and Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), as well as 4% citric acid (0.15 mg/ml). Lastly, the test indicated statistical significant differences between all the treatment groups consisting of Absorbatox® (0.30 mg/ml) bound to 0.15 mg/ml concentrations of malic acid (2 and 4%), citric acid (2 and 4%) and fulvic acid (2 and 4%), as well as bound to 0.30 mg/ml concentrations of malic acid (2 and 4%), citric acid (2 and 4%) and fulvic acid (2 and 4%).

The results obtained from Tukey's HSD test on the results of the HaCaT cells indicated statistical significant differences after 48 h exposure between the untreated control group and Absorbatox® bound to 4% citric acid (0.15 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.15 mg/ml and 0.30 mg/ml). Statistical significant differences were seen between Absorbatox® (0.15 mg/ml) and treatments consisting of Absorbatox® bound to groups containing 4% malic acid (0.30 mg/ml), 2% citric acid (0.15 mg/ml), 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant differences for Absorbatox® bound to groups containing 2% citric acid (0.15 mg/ml), 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml).

The results obtained from Tukey's HSD test on the results of the 84BR cells indicated statistical significant differences after 24 h exposure between Absorbatox® (0.30 mg/ml) and Absorbatox® bound to groups containing 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.30 mg/ml).

At 48 h, Tukey's test on the results of the 84BR cells indicated statistical significant differences between the untreated control group and Absorbatox® bound to 2% citric acid (0.15 mg/ml), as well as Absorbatox® bound to groups containing 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 2% citric acid (0.15 mg/ml), as well as 4% fulvic acid (0.30 mg/ml).

Post exposure results of the HaCaT and 84 BR cells to the various organic acid treatments is shown in Fig. 3 and Fig. 4. The results obtained show a clear indication of concentration dependent decrease in cell viability when both the HaCaT and 84BR cells were exposed to the organic acids individually, however, no indication of cell death was observed. When compared to the other organic acids, fulvic acid proved to be the least toxic to both cell lines even though it still generated, %cell viability values lower than 40%. This result is considered as an indication of strong cytotoxicity to cells (López-García *et al.,* 2014) and thus not suitable for use in wound healing dressings. The results obtained show direct correlation between the

HaCaT and 84BR cell lines when treated with organic acids alone. The organic acids caused a clear pH decrease when added to the media resulting in a distinct colour change, going from a pink colour to bright yellow, malic and citric acid, and a dark brown-black colour when fulvic acid was used alone. Although many organic acids alone have been used extensively in dermatological procedures as peeling agents causing effects such as swelling, burning and pruritus of the skin, which is yet another strong indication that acids alone cannot be used in wound healing products. Organic acids function as to promote cellular apoptosis (programmed cell death) (Tang & Yang, 2018).

Death receptors as well as BH3-interacting domain death agonist proteins are activated by citric acid which in turn increases apoptosis-inducing factor. Malic acid has the ability to activate antiproliferative effect on HaCaT cells by inhibiting the cell cycle progression, thus inducing apoptosis through molecular pathways which includes endoplasmic reticulum stress and mitochondria-dependent signalling pathways. Although citric acid and malic acid have different structures, they activate the same apoptotic pathways. Treatment of HaCaT cells with either citric or malic acid have strong apoptotic features are which include DNA damage, as well as apoptotic bodies (Tang & Yang, 2018).

From the cytotoxicity results, it was decided to not include the organic acids alone for wound healing, seeing as a strong cytotoxic compound would not benefit the wound healing process. Tukey's HSD test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate statistical significant differences between the untreated control groups compared to all three organic acids (citric, malic and fulvic aids) in concentrations of 0.5, 1.0, 2.0 and 4.0%. The Kruskal-Wallis test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate statistical significant differences between the untreated control groups compared to treatments containing citric acid (2 and 4%) at a 48 h exposure period.

3.2 Scratch wound healing assay results and discussion

The results obtained from the %wound closure obtained at 24 and 48 h exposures of the HaCaT and 84BR cells, respectively is indicated in Fig. 5 and Fig. 6. Both cell lines indicated increase in improved wound closure when exposed to Absorbatox® bound to an organic acid when compared to the untreated control, as well as Absorbatox® alone. HaCaT cells exposed to Absorbatox® bound to 2% citric acid and 4% fulvic acid, respectively, showed the highest percentage wound closure, with wounds indicating a percentage of over 90% closure after a 48h exposure period. 84BR cells exposed to Absorbatox® bound to 2% citric acid and 4% fulvic acid respectively, showed the highest percentage wound closure, with wounds indicating a 100% closure after a 48 h exposure period. Therefore, the Absorbatox® combinations with high acid ratios improved the wound healing potential of Absorbatox® alone.

Previous *in vivo* wound treatment studies have proved that wound treatment with Absorbatox® resulted in a rapid decrease of wound depth in comparison to a control group with a conventional treatment. The results were clear from day 7 of treatment, with the largest difference on day 10 where micronised and granular Absorbatox® resulted in 96 and 92% decrease in depth, respectively vs. 88% for the control group (Data on file Absorbatox (Pty) Ltd, 2008).

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated group and Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml) and 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.15 mg/ml) indicated statistical significant differences for the treatments containing Absorbatox® bound to 2% malic acid (0.30 mg/ml) and Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) displayed statistical significant differences for the treatments containing Absorbatox® bound to 2% malic acid (0.30 mg/ml) and Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated group

and Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml) 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented statistical significant differences for the treatments containing Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant differences for the treatments containing Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated control group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 2 and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) displayed statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated control group and Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 2 and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% citric acid (0.15 and 0.30 mg/ml, 4% citric acid (0.15 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant differences for

Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml) and also 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).

The HaCaT and 84BR cell migration rate when exposed to Absorbatox® bound to each organic acid over a 24 and 48 h exposure period is indicated in Fig. 7 and Fig. 8. The results clearly indicate increased migration rate when exposed to various treatments containing Absorbatox® bound to various organic acids compared to the untreated control, as well as Absorbatox® alone. Both cell lines treated with Absorbatox® bound to malic acid indicated a significant increase in migration rate compared to the untreated control group. Both cell lines treated with Absorbatox® bound to both citric and fulvic acid indicate the highest significant difference in migration rate compared to the untreated control group. The highest wound close rate is observed within the first 24 h for both the HaCaT and 84BR cell lines, the closure rate slows after 24 to 48 h. This may be attributed to cellular contact inhibition; this occurs as a result of increased wound healing. As the cells grow closer to one another, they exert an inhibitory contact force, which slows migration as the space becomes smaller. This is evident in both Fig. 11 and Fig. 12, as the plateau is reached. The results obtained indicate an average faster wound migration rate when treated with concentrations of 0.15 mg/ml of the Absorbatox® bound to organic acid treatments. The graphs indicate a faster migration rate observed with Absorbatox® bound to an acid in a concentration of 2% compared to a concentration of 4%.

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% fulvic acid (0.30 mg/ml) , as well as Absorbatox® bound to 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.15 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.30 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic

357 acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.30 mg/ml), as well as 4% fulvic acid (0.15 and
358 0.30 mg/ml).
359 Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments
360 indicated statistical significant differences after 48 h exposure between the untreated group
361 and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound
362 to 4% malic acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.15
363 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences
364 for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15
365 and 0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml)
366 displayed statistical significant differences for Absorbatox® bound to groups containing 2%
367 malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), and also 4% fulvic acid (0.15
368 and 0.30 mg/ml).
369 Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments
370 indicated statistical significant differences after 24 h exposure between the untreated group
371 and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound
372 to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and
373 0.30 mg/ml), as well as Absorbatox® bound to 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).
374 Absorbatox® (0.15 mg/ml) showed statistical significant differences for Absorbatox® bound to
375 groups containing 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30
376 mg/ml), as well as 2% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) presented
377 with statistical significant differences for Absorbatox® bound to groups containing 4% malic
378 acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4%
379 fulvic acid (0.15 and 0.30 mg/ml).
380 Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments
381 indicated statistical significant differences after 48 h exposure between the untreated group
382 and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound
383 to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and
384 0.30 mg/ml), as well as Absorbatox® bound to 2 and4% fulvic acid (0.15 and 0.30 mg/ml).

Absorbatox® (0.15 mg/ml) displayed statistical significant differences for Absorbatox® bound to groups containing 4% malic (0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml) and 2% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 4% malic acid (0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).

3.3 *In vitro* cell migration assay results and discussion

The results obtained after performing the migration assay with HaCaT cells exposed to various concentrations of the Absorbatox® bound to organic acids for 24 h is illustrated in Figure 9. The results indicate the largest increase in cell migration with the HaCaT cells treated with Absorbatox® bound to 2% fulvic acid at a concentration of 0.30 mg/ml. This result is a clear indication that cell migration plays an important role in wound healing especially at longer exposure time (i.e. 48 h), as seen during the scratch assay results in Section 3.2.

4 Conclusion

Results from both the cytotoxicity and scratch assays give a clear indication that Absorbatox® bound to specifically selected organic acid(s), prove to be beneficial as new wound healing agents. The treatments tested prove enhanced wound closure at an increased rate as well as providing an environment for optimal wound healing. This may be attributed to the unique combination of Absorbatox® which is bound to a selected organic acid in a patented process as a single compound to provide a pH optimal for healing, which protects against microbial infection and has the ability to create a moist environment optimal for healing.

The cytotoxicity assay was performed with the aim of determining whether Absorbatox® bound to selected organic acid as well as the Absorbatox® and organic acids separately pose to have cytotoxic effects on the cellular viability of the HaCaT and 84BR cells. This MTT-assay was performed as a common application of cytotoxicity, which is based on cell viability by means of staining (Chiba *et al.,* 1998). The results obtained from the MTT-assay were comparable between the HaCaT and 84BR cell lines. When measuring the %cell viability of the HaCaT cells treated with the various combinations of Absorbatox® bound to an organic acid compared to the 84BR cells over exposure periods of both 24 and 48 h, the results indicated significant

similarities. Analysis of the results obtained from the organic acid treatments alone, indicated a low %cell viability proving to be strongly cytotoxic (<40% cell viability) to both cell lines. Cellular proliferation was observed when cell lines were treated with a combination of Absorbatox® bound to an organic acid, especially when bound to fulvic acid, which resulted in a very high cell viability (above 80% cell viability).

The results obtained from the scratch wound healing assay proved that Absorbatox® bound to a selected organic acid is advantageous for wound closure, as well as increasing the rate at which a wound will close. The results indicate the highest wound closure, as well as wound closure rate when cells were treated with Absorbatox® bound to fulvic acid.

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Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1:

Classification of treatment cytotoxicity according to %cell viability

%Cell viability	**Cytotoxicity**
<40%	Strong
40-60%	Moderate
60-80%	Weak
>80%	Non-cytotoxic

Figures

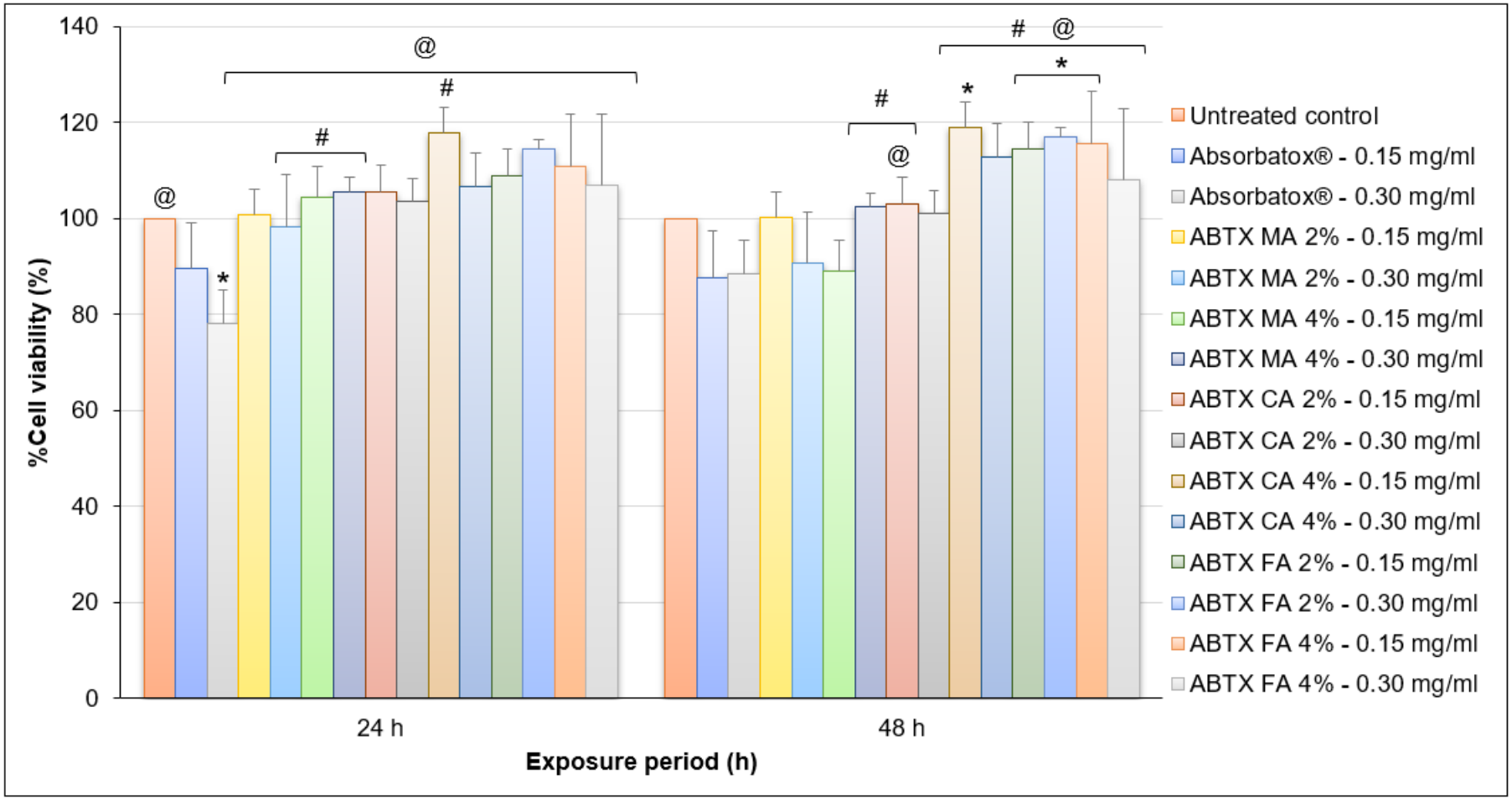

Fig. 1: %Cell viability of the HaCaT cell line treated with Absorbatox® bound to an organic acid for 24 and 48 h as determined by MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

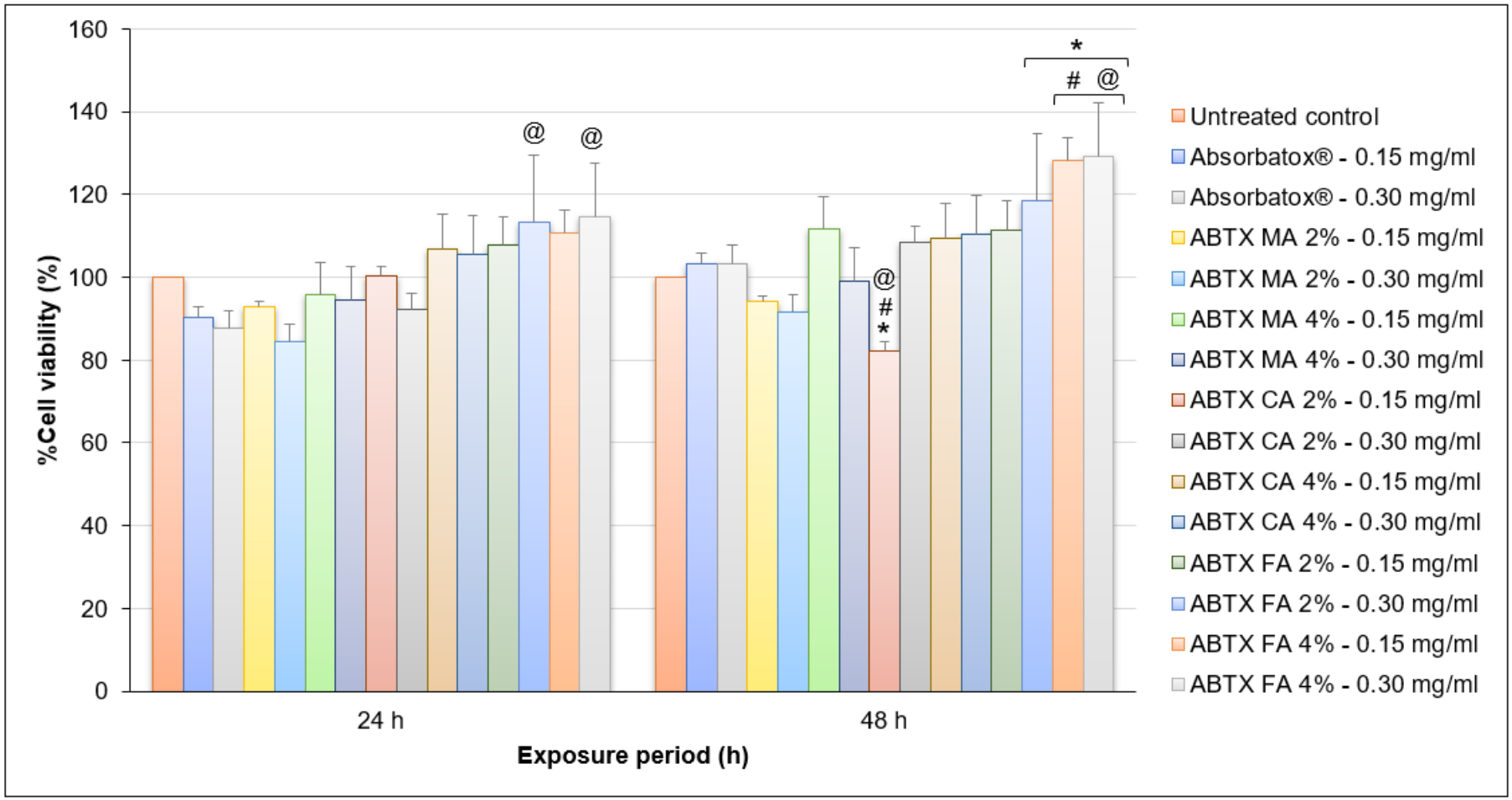

Fig. 2: %Cell viability of the 84BR cell line treated with Absorbatox® bound to an organic acid for 24 and48 h as determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

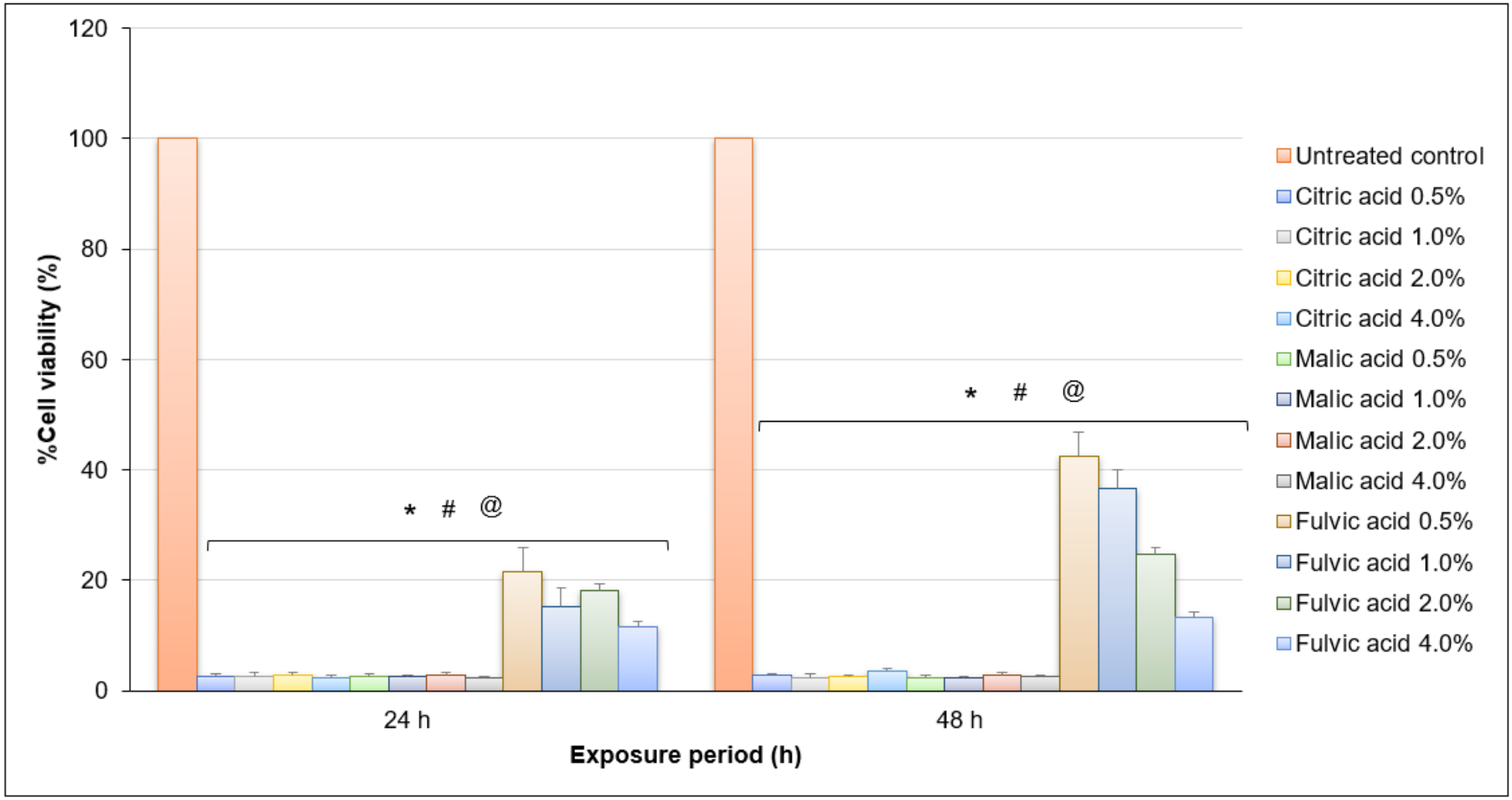

Fig. 3: %Cell viability of the HaCaT cell line treated with an organic acid for 24 and 48 h determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

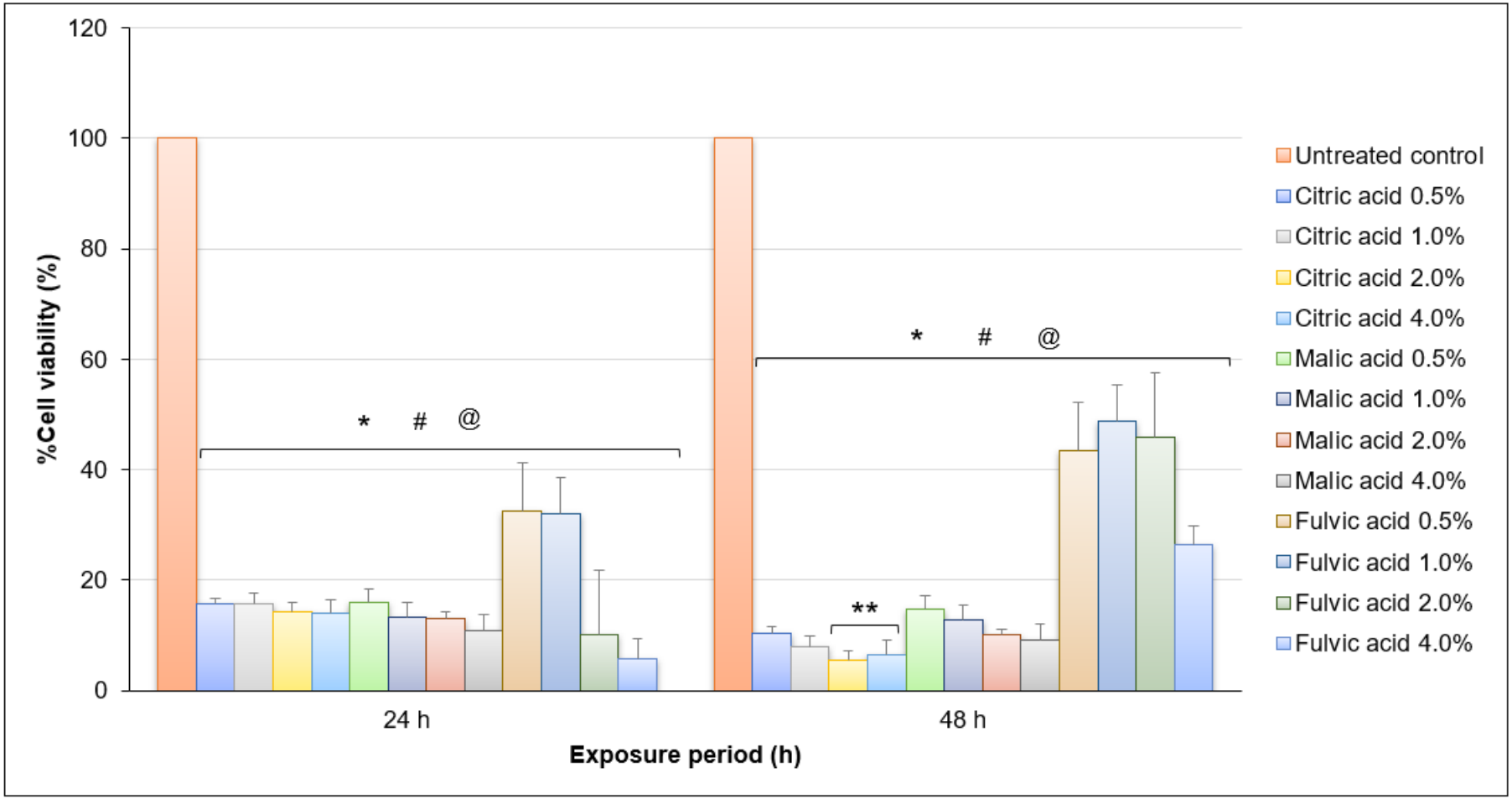

Fig. 4: %Cell viability of the 84BR cell line treated with an organic acid for 24 and 48 h determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid), Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml), Kruskal-Wallis test (** significantly different to the untreated)

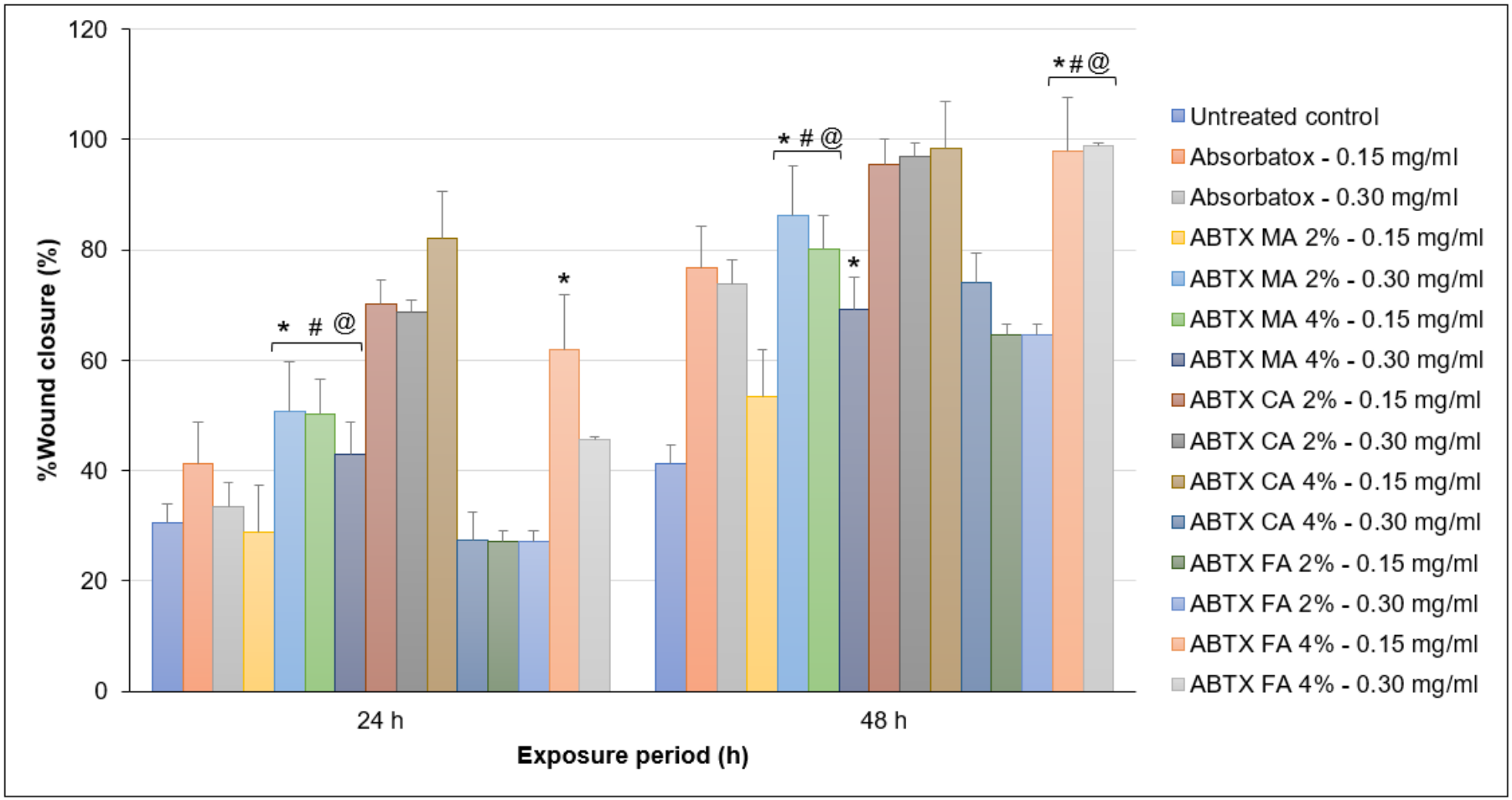

Fig. 5: HaCaT cell %wound closure results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

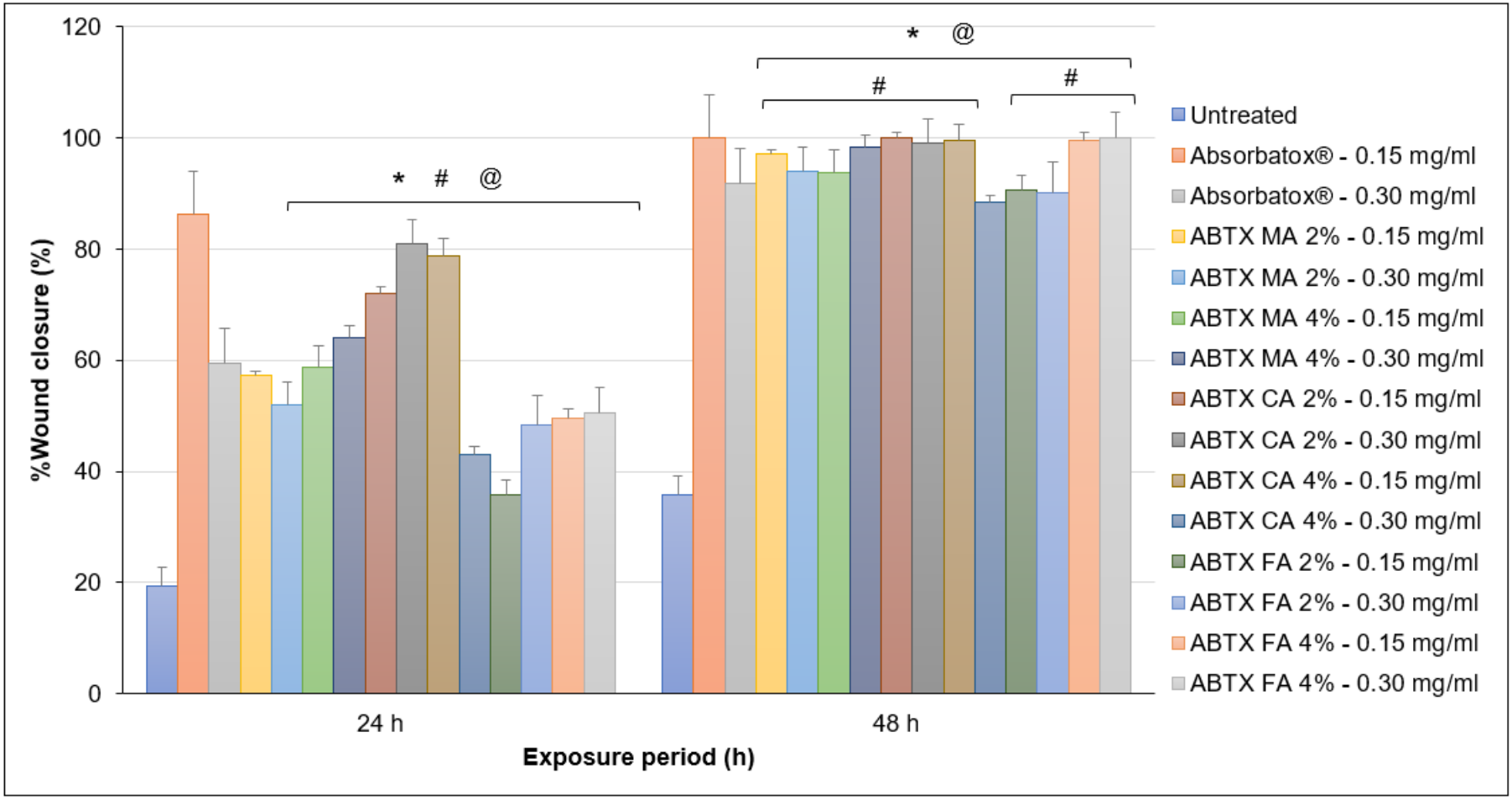

Fig. 6: 84BR cell %wound closure results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

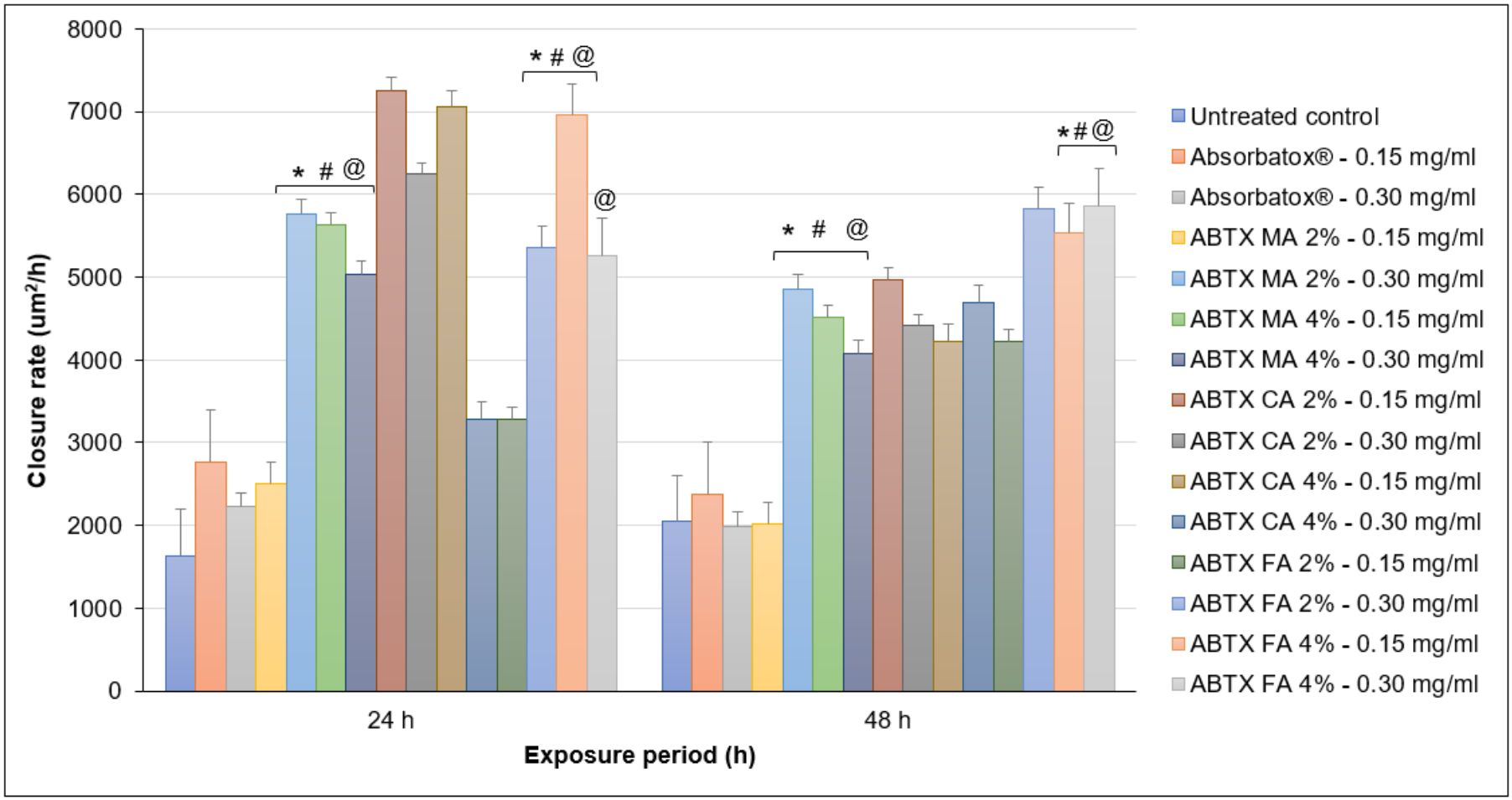

Fig. 7: HaCaT cell wound closure rate results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

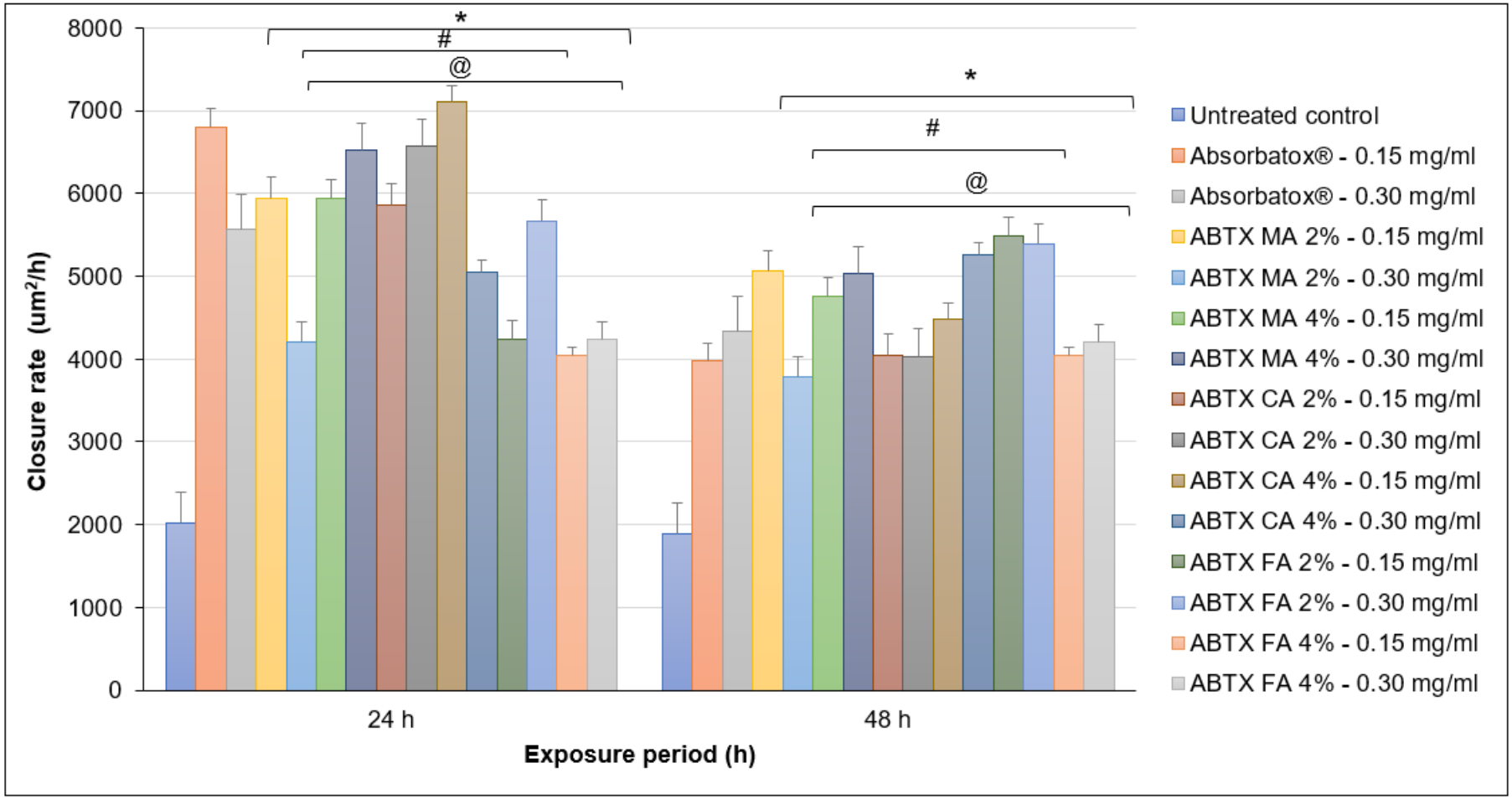

Fig. 8: 84BR cell wound closure rate results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

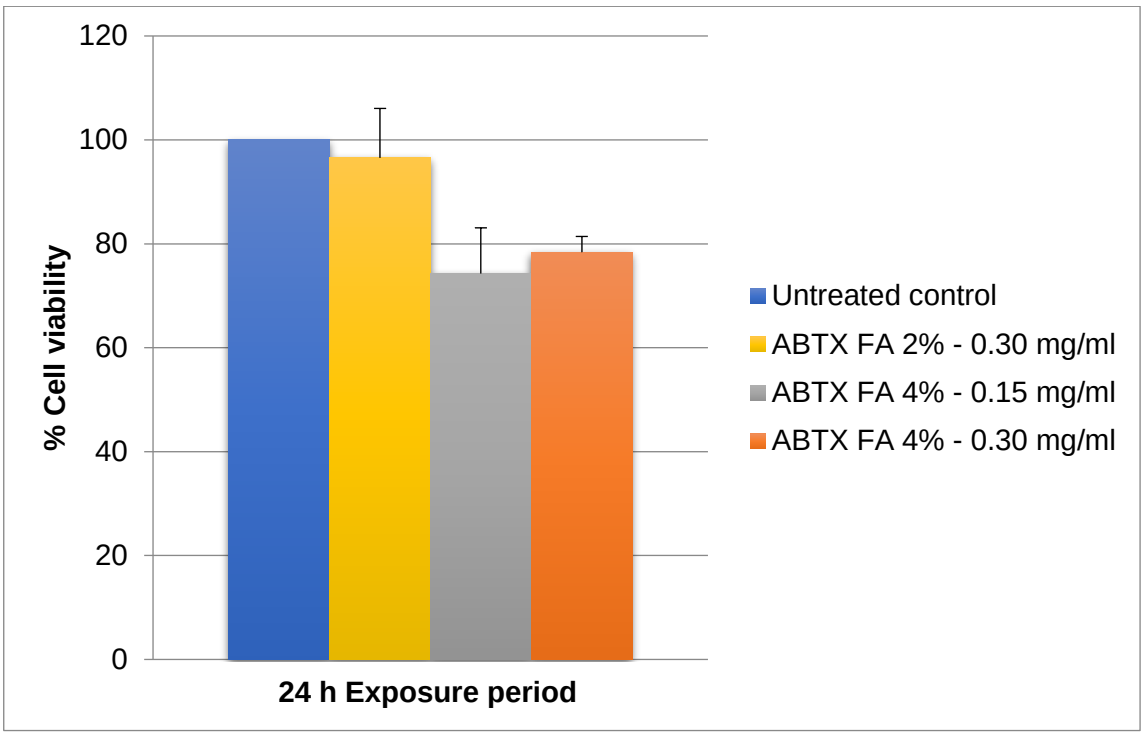

Fig. 9: Cell migration results of HaCaT cells treated with Absorbatox® bound to an organic acid (with ABTX (Absorbatox®) and FA (fulvic acid))

CHAPTER 4
FINAL CONCLUSION AND FUTURE PROSPECTS

Wound healing and tissue repair is the fundamental process that is required for homeostasis and regeneration following damage to the skin and underlying tissue (Razzel, 2014:141). It is known that every species has the ability to restore and regenerate the disruption of the normal continuity of tissue after physical injury (mechanical, chemical, radiation, disruption of tissue integrity, etc.) (Lee *et al.,* 2012:213). Today, large numbers of treatments and dressings are available for the management of different wounds, which represents a multi-billion-dollar industry across the globe. These wound dressings often include conventional dressings (simple gauze or cotton wool based), dressings which incorporate growth factors (that stimulate and facilitate the healing process), as well as skin substitutes incorporating patient derived cells (Murray *et al.,* 2019:1).

Wounds can be divided into two distinct groups depending on their healing time, acute and non-healing chronic wounds. A wound is referred to as a chronic, non-healing wound if it does not heal within 3 months, which is often associated with underlying pathologies that reduce the healing ability, i.e. patients with arterial or venous insufficiencies or patients who suffer from diabetes mellitus (often due to peripheral neuropathy and arterial occlusion). According to the type of wound, dressings are divided into different types, namely traditional and advanced wound care. Traditional wound care is considered applicable for the treatment of an acute wound, which aims to cover and protect the wound from the environment. When a wound fails to heal, advanced wound care (usually in the form of a specialised dressing) is used to ensure the wound environment remains moist (Öhnstedt *et al.,* 2019:485).

The newest dressings are made of materials including films and foam dressings, which serve to create a sealed wound environment to provide protection from infection while creating an ideally moist environment that has the ability to promote the healing process (Murray *et al.,* 2019:1). The main focus of this study was to create new, advanced wound dressings made from various materials that have known anti-microbial properties and agents capable of facilitating cell migration for optimal healing (Murray *et al.,* 2019:1). Lowering the pH is known to counteract the risk of infection and aids in the acceleration of healing, which was first mentioned by Hippocrates, who recommended vinegar to clean wounds before the application of a dressing (Öhnstedt *et al.,* 2019:485).

The aim of this study was to determine if the selected organic acids (fulvic acid, malic acid and citric acid separately) bound to Absorbatox® were effective wound healing agents when applied as “wound dressings” in an *in vitro* study using various pharmaceutical application products, i.e. silicone-based gel, hydrogel-based patch and dry powder sachets, based on different anticipated wound types graded on their level of exudation (low to high “exudating” wounds).

To achieve this goal, the following study objectives were set:

- To perform the anti-bacterial characterisation with regards to *S. aureus*, *E. coli* and *S. typhimurium* (outsourced).
- To assess the cytotoxicity of the active ingredients (separately) and the combination thereof used in the different formulations using *in vitro* cell cultures, specifically HaCaT cells.
- To demonstrate enhanced fibroblast activity and wound architecture in cell culture when the formulated wound dressings are applied to 84BR cells.
- To assess wound healing potential of the active ingredients (separately) and the combination thereof used in the different formulations using a cell migration assay, as well as a scratch wound healing assay.
- Determine the compatibility of the Absorbatox® with the different organic acids by means of DSC, TAM and FTIR.
- To formulate a silicone-based wound dressing, a hydrogel-based patch and a sachet/powder dressing (dry form) containing the most suitable API based on the outcomes of the cell culture studies.
- Conducting stability tests on the different formulations stored at 25 °C/60% RH, 30 °C/65% RH and 40°C/75% RH.
- Evaluation of the prepared silicone-based gel with regards to pH, visual examination, viscosity, mass loss, as well as API identification during accelerated stability testing.
- Evaluation of the prepared sachet/powder dressing with regards to visual examination, mass loss, free swell capacity, as well as API identification during accelerated stability testing.
- Evaluation of the prepared hydrogel-based patch dressing with regards to visual examination, mass loss and API identification during accelerated stability testing.

As seen from the anti-bacterial characterisation results, Absorbatox® proved to be effective against the cultured organisms used during testing. The analysis results provided important anti-microbial efficacy data required to formulate the wound dressings. Fulvic acid was the most effective against the organisms tested (Simpson, 2018b), although citric as well as malic acid also provided effective anti-microbial activity. Hence, the organic acids in combination with Absorbatox® may be effective when used as new wound healing formulations. Cytotoxicity studies were performed *in vitro* on both a keratinocyte (HaCaT) as well as a fibroblast (84BR) cell line, to determine the cell viability after exposure to the active ingredients, namely Absorbatox® (separately) as well as Absorbatox® bound to organic acids. Assessment wound healing potential

of the active ingredients used in the different formulations was determined using a cell migration assay, as well as a scratch wound healing assay.

The MTT-assay results on both the HaCaT and 84BR cell lines indicated increased cell viability when treatments were applied, with Absorbatox® bound to both citric and fulvic acid attaining cell viability well over 100%, a clear indication of cell growth. Formulations containing Absorbatox® bound to both citric and fulvic acid, indicate the highest cell viability. This is an indication that the Absorbatox® bound to organic acids are not toxic to keratinocyte and fibroblast cells and are thus safe and effective for use in the formulation of new wound healing treatments. Treatment with organic acids alone, resulted in a concentration dependent decrease in cell viability as measured with the MTT-assay, however, no indication of cell death is observed for the HaCaT and 84BR cells. Fulvic acid proves to be the least toxic to both cell lines. The results prove pure organic acids to be strongly cytotoxic to cells and are not suitable for use in wound healing dressings as single agents.

The HaCaT cells exposed to Absorbatox® bound to an organic acid at 24 h and 48 h treatment periods showed a concentration dependent increase in improved wound closure when compared to the untreated control. HaCaT cells exposed to Absorbatox® bound to 4% citric acid and 4% fulvic acid, respectively, showed the largest percentage wound closure; with wounds indicating a percentage of over 90% closure after a 48 h exposure period.

The 84BR cells exposed to Absorbatox® bound to an organic acid at 24 h and 48 h treatment periods showed a concentration dependent increase in improved wound closure when compared to the untreated control. 84BR cells exposed to Absorbatox® bound to 4% citric acid and 4% fulvic acid, respectively, showed the largest percentage wound closure; with wounds indicating a 100% closure after a 48 h exposure period.

The HaCaT cell migration rate when exposed to Absorbatox® bound to an organic acid over a 48 h exposure period indicates an increase. The results obtained clearly indicate the cells exposed to the various treatments show an increased migration compared to the control. Cells treated with Absorbatox® bound to malic acid indicate a notable increase in migration rate compared to the untreated control group. Cells treated with Absorbatox® bound to both citric and fulvic acid indicate the largest difference in migration rate compared to the untreated control group.

The 84BR cell migration rate, when exposed to Absorbatox® bound to an organic acid over a 48 h exposure period, indicates a clear increase. The results obtained clearly indicate the cells exposed to the various treatments show an increased migration compared to the control. Cells treated with Absorbatox® bound to malic acid indicate a notable increase in migration rate compared to the untreated control group. Cells treated with Absorbatox® bound to both citric and

fulvic acid indicate the largest difference in migration rate compared to the untreated control group.

The migration assay results of the HaCaT cells exposed to various concentrations of the Absorbatox® bound to an organic acid for 24 h is illustrated in Appendix B.. The results obtained indicate a very high cell migration of the treated cells compared untreated control group. The results obtained indicate a decrease in cell migration compared to the untreated control. The largest increase in cell migration is observed were the HaCaT cells are treated with Absorbatox® bound to 2% fulvic acid. This shows that cell migration may play an important role in wound healing at a longer exposure time, i.e. 48 h, as seen during the scratch assay results in Appendix B.

Physical characteristics and stability of both Absorbatox® and the selected organic acids were investigated by the interpretation of the DSC thermograms, TAM compatibility graphs, as well as FTIR spectra obtained. The results from the TAM showed complete compatibility of Absorbatox® bound to both the 2% and 4% fulvic acid, whereas the compatibility of Absorbatox® bound to 4% citric acid may be indicative of a possible incompatibility (Aucamp, 2019:2). The results obtained from the FTIR spectra indicated that Absorbatox® was dominant when measuring different samples (bound with and physical mixture) containing both Absorbatox® and fulvic acid. No large differences in absorption bands were observed on the IR graphs for Absorbatox® when Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid were compared; the acid concentration is too low to be presented on the IR spectra. From the data and results collected, it was evident that Absorbatox® and fulvic acid proved to be a favourable combination for wound dressing formulations developed during this study.

Three different wound dressing formulations were formulated namely a silicone-based gel, a hydrogel-based patch, as well as a sachet/dry dressing. The formulations contained an active ingredient of Absorbatox® bound to fulvic acid for the gel and sachet formulations and citric acid for the hydrogel patch formulation. Stability tests were performed on the different formulations over a 3-month period. The formulations were stored under three different conditions, 25 °C/60% RH, 30 °C/65% RH and 40°C/75% RH. The stability tests (discussed in Appendix D) were determined on months 0, 1, 2 and 3 on the different formulations. The formulations all proved to be stable throughout the 3-month stability testing period. The identification of the active ingredient in the formulations showed no or minimal indication of change and remained within the formulation as specified throughout the stability testing.

The identification of the active ingredient namely, Absorbatox® bound to 2% fulvic acid, in the silicone-based gel formulation was successful throughout the 3-month stability testing period under all three storage conditions. The silicone-based gel formulation was designed for the

treatment of burn wounds and abrasions, as discussed in Chapter 2, the optimal wound healing environment is considered at a pH value of below 6, since most bacteria associated with infected wounds in humans require a higher pH (Nagoba *et al.,* 2015:5). A decrease in pH, created by the presence of an organic acid, i.e. fulvic acid, thus aids in wound healing processes by providing infection control. A decrease in pH value leads to the Bohr-effect (i.e. increase in the amount of available oxygen of cells). This increase in the level of oxygen delivery to damaged tissue increases resistance to infection, as well as promote healing. An acidic environment also promotes epithelisation by boosting fibroblastic growth and neovascularisation, which increases microcirculation of wounds that in turn enables the formation of new healthy granulation tissue and ultimately faster wound healing (Nagoba *et al.,* 2015:5). Viscosity results obtained over the 3-month period did not change significantly. An increase in viscosity can be a result of the settling process of the gel, before the formulation reaches a state of equilibrium, as well as an average loss in moisture during stability testing over the 3-month period, which results in a higher viscosity value. The mass of the silicone-based gel formulation remained relatively stable over the 3-month storage period with no significant change in mass. The slight variation in results obtained may be a result of insufficient container sealing, which allowed for evaporation of moisture; escaping from the containers during the storage periods. This may be a result due to the conveying of moisture from a higher moisture content in the gel formulation to an atmosphere with a lower moisture content. The results obtained from the particle size measurements confirmed the particles within the gel formulation demonstrate adherence to one another to form aggregates of successively increasing in size. This phenomenon is referred to as flocculation and may occur as a result of the particles under the influence of gravity (Eccleston, 2013:460). As the Absorbatox® containing 2% fulvic acid particles are not intended to dissolve within the gel formulation, but rather remain suspended therein, the aggregation of particles does not render the product unstable. The particles may decreased in size when the gel is mechanically stirred or after rubbing it onto the patient's skin. The colour and visual appearance of the gel formulation showed no significant change over the 3-month period. The results obtained indicate that the silicone-based gel formulation met the stability criteria, proving to be a stable formulation.

The identification of the active ingredient namely, Absorbatox® bound to 4% citric acid, in the hydrogel-based patch formulation was successful throughout the 3-month stability testing period under all three storage conditions. The mass of the hydrogel-based patch showed a slight increase over the 3-month storage period with no significant change in mass. The results obtained may be the result of insufficient container sealing, which allowed for swelling and increase in moisture content of the formulations during storage periods. The patch formulation is designed to have the ability to both swell and de-swell wound exudate in a reversible direction, which shows specific environmental stimuli-responsivity e.g. temperature and pH (Kamoun *et al.,* 2017). Hydrogel wound patches can absorb, as well as retain wound exudate, which promotes

fibroblast proliferation and keratinocyte migration, both of which are necessary for complete epithelialisation and wound healing (Kamoun *et al.,* 2017). This indicates that the swelling/moisture increase of the patch observed during the 3-month testing period is not considered an instability, but rather an indication that better care must be taken in future to sufficiently seal containers to prevent premature swelling of the patches before they are to come in contact with the wound surface. No visual or colour changes where observed during the 3-month testing period. The visual appearance, as well as the API identification and mass results obtained during testing was a clear indication of the stability of the hydrogel-based patch formulation.

The identification of the active ingredient namely, Absorbatox® bound to 4% fulvic acid, within the dry (sachet) dressing formulation was successful throughout the 3-month stability testing period under all three storage conditions. The mass of the dry dressing formulation remained relatively stable over the 3-month storage period with no significant change in mass. The sachets were designed to absorb moisture (extremely hygroscopic), thus results obtained may be due to insufficient container sealing, which allowed for moisture to enter the containers during storage periods. The overall appearance of the dry (sachet) dressing formulation remained constant throughout the 3-month stability testing period. The colour and texture of all the sachets did not change over the testing period. No signs of moisture uptake were visible and sachets remained intact. The free swell capacity of the dry (sachet) dressing was measured and used as an indication to estimate the potential ability to absorb wound exudate when placed on an exuding wound such as a leg ulcer. The absorption capacity of the sachet dressings decreased over the 3-month period. This result may be attributed to insufficient sealing of containers while storing, sachets may have been exposed to moisture prior to the free swell capacity testing. The dressings were formulated with absorption of fluid as a key factor, thus indicating a successful result. The overall results obtained from the dry (sachet) dressing were a clear indication of formulation stability.

Future prospects for further investigation include the following:

- Use of more suitable storage containers such as sealable glass or plastic containers during stability testing and especially when packaged for wound healing in a commercial environment.
- Further testing on the potential use of different organic acids, as well as in different concentrations bound to Absorbatox® used as an active ingredient such as hyaluronic acid.
- Further *in vivo* and clinical testing for the elimination of potential adverse reactions and refinement to formulations.

This study clearly demonstrates the potential that Absorbatox® bound to organic acids as new wound healing agents have when used in different formulations. The use of both the keratinocyte and fibroblast cell lines for *in vitro* screening for cellular cytotoxicity, as well as the use of the scratch wound healing assay have enabled immeasurable advances in the development of modern wound healing agents and dressings. The results obtained during stability testing give a clear indication that the formulations were stable and suitable for further clinical trials on suitable wounds.

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APPENDIX A
CHARACTERISATION OF ABSORBATOX® AND SELECTED ORGANIC ACIDS

A.1 Purpose of characterisation

Characterisation studies during pre-formulation are important prior to the commencement of the formulation development. Logical development of a safe, stable and efficacious dosage form all form part of the pre-formulation process, which also involves the classification of physicochemical properties of the active ingredients. Pre-formulation studies are important for the optimal dosage form to be developed and researched (Walters & Brain, 2002:319).

A.2 Characterisation methods

A.2.1 Thermal analysis

This technique differential scanning calorimetry (DSC) is commonly used in the pharmaceutical industry to measure melting points, detect changes in slopes and used to detect different polymorphic forms and or hydrates and solvates.

Thermal analysis and more specific DSC is the technique where the temperature difference between the sample and reference is measured. Both sample and reference were held at the same temperature throughout the duration of the experiment. The accuracy and sensitivity are important features of this technique. The thermogram is usually a change in heat over time (dH/dt) vs temperature graph (Soni, 2017:2).

DSC analysis is used to establish the connection between temperature and specific physical properties of the active ingredients. The principle of DSC determines the thermal changes occurring as a result of a loss or gain in heat during a controlled heating and cooling of a specific sample when compared to an inert reference (Laye, 2002:220).

Thermal methods of analysis include DSC and thermal activity monitor (TAM); both methods of analysis were performed on the organic acid powders (citric acid, fulvic acid and malic acid).

A.2.1.1 Differential scanning calorimetry

The DSC thermograms were recorded by a Shimadzu (Kyoto, Japan) DSC-60 instrument. The recorded DSC thermograms indicate the difference in heat-flow (mW) against time (min). The different samples, weighing approximately 3 – 5 mg, were heated in a sealed aluminium crimp cell, from 25 – 200 °C at a rate of 10 °C/min under a nitrogen gas purge of 35 ml/min. Instrument

calibration was done using an ultra-pure indium standard, which has a melting point of 156.4 °C. The DSC results obtained were used to determine the melting points. The Merck Index (O'Neil; 2001) reports softening of citric acid at approximately 75 °C and a melting point of 100 °C; malic acid has a melting point between 131 – 132 °C.

As seen in the DSC thermograms (Figure A.1), the softening of citric acid at 69.68 °C and a melting point of 101.95 °C. In Figures A.2 and A.3, the melting points of fulvic acid and malic acid were 125.86 °C and 125.39 °C, respectively.

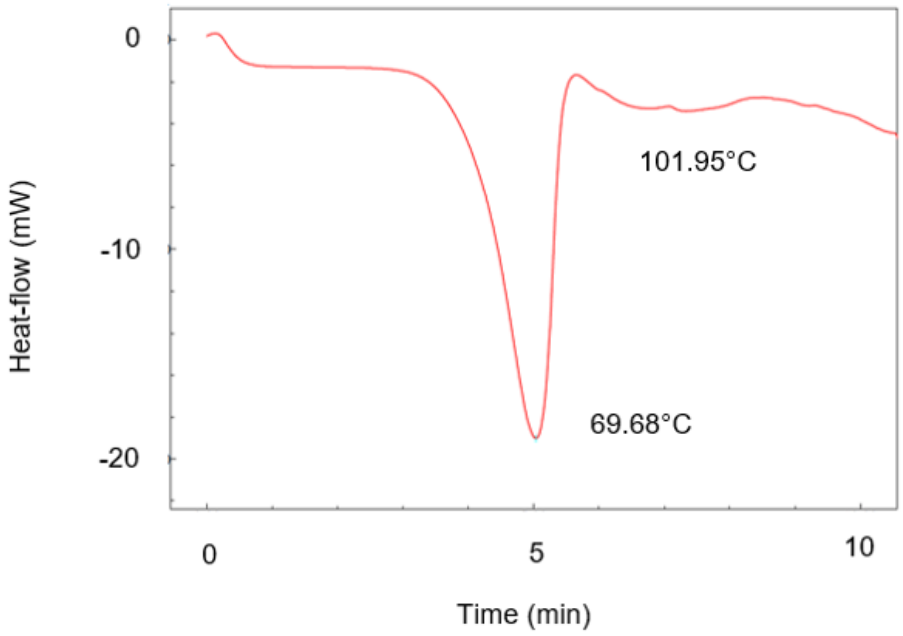

Figure A.1: DSC thermogram showing the softening of citric acid at 69.68 °C and a melting point of 101.95 °C

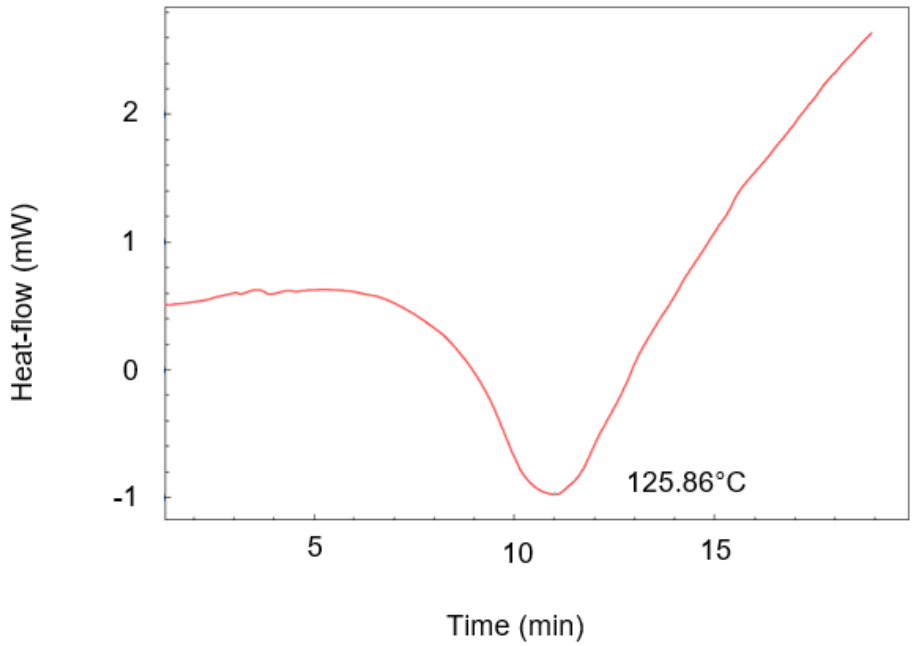

Figure A.2: DSC thermogram of fulvic acid showing a broad melting endotherm at125.86 °C

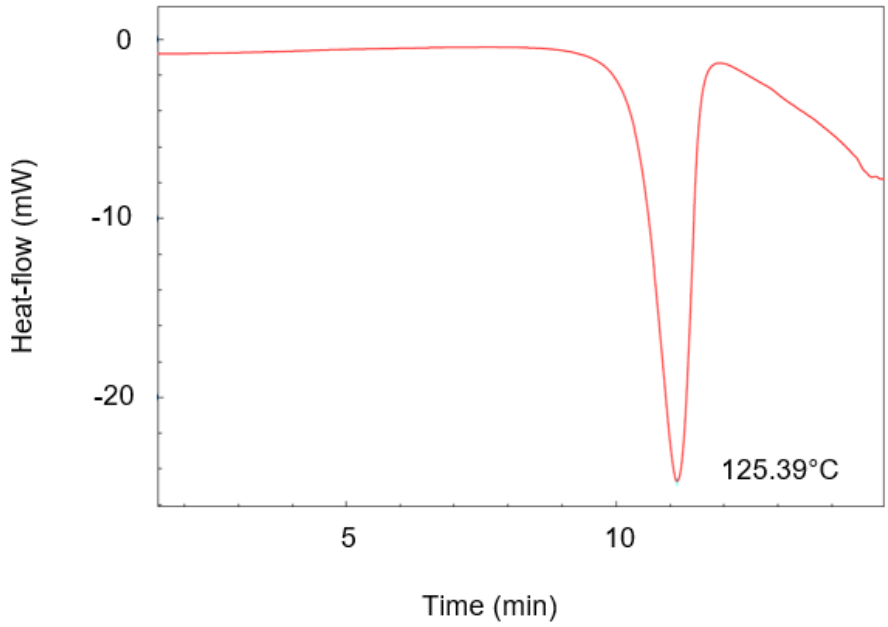

Figure A.3: DSC thermogram of malic acid showing the melting endotherm at 125.39 °C

A.2.1.2 Thermal Activity Monitor

TAM is a unique micro-calorimetric system which is able to monitor a wide range of chemical and biological reactions (Hou *et al.,* 2006:169). TAM was used to provide a more sensitive and direct measure of the rate of energy gain or -loss as a function of time at actual storage or stability temperatures (Sahni *et al.,* 2016:27). TAM is therefore a suitable method to measure any incompatibilities between different excipients and active ingredients. This process allows for a sample to be maintained in isothermal conditions without any destruction (Phipps & Mackin, 2000:9). A TAM (TAM-III) apparatus (TA Instruments, USA) was used during analysis of samples.

The compatibility of Absorbatox® bound to 2% fulvic acid was determined through isothermal microcalorimetry. The temperature of the microcalorimeters were maintained at 40 °C. Figure A.4 represents the heat-flow graph obtained with the mixture of Absorbatox® bound to 2% fulvic acid; an average heat-flow of 1.83 ± 4.01 µW/g was measured. From the heat-flow graph, it is apparent that no interaction occurred between the two compounds. This is evident from the sameness of the theoretical and measured heat-flow curves (Aucamp, 2019:1).

Figure A.5 depicts the heat-flow graph obtained with Absorbatox® bound to 4% fulvic acid; an average heat-flow of 5.45 ± 5.86 µW/g was measured. From the graph, it is also evident that the two compounds are compatible with one another due to a lack of a sloped interaction curve and no significant difference between the measured and theoretically calculated heat-flow curve (Aucamp, 2019:1).

The heat-flow curve obtained with Absorbatox® bound to 4% citric acid is shown in Figure A.6. With this combination, the possibility of an incompatibility exists with an average heat-flow of 24.96 ± 37.08 µW/g. However, it must be mentioned that the Absorbatox® heat-flow data was used for this analysis, since no run of only Absorbatox® was done as part of the compatibility testing experimental setup (Aucamp, 2019:2).

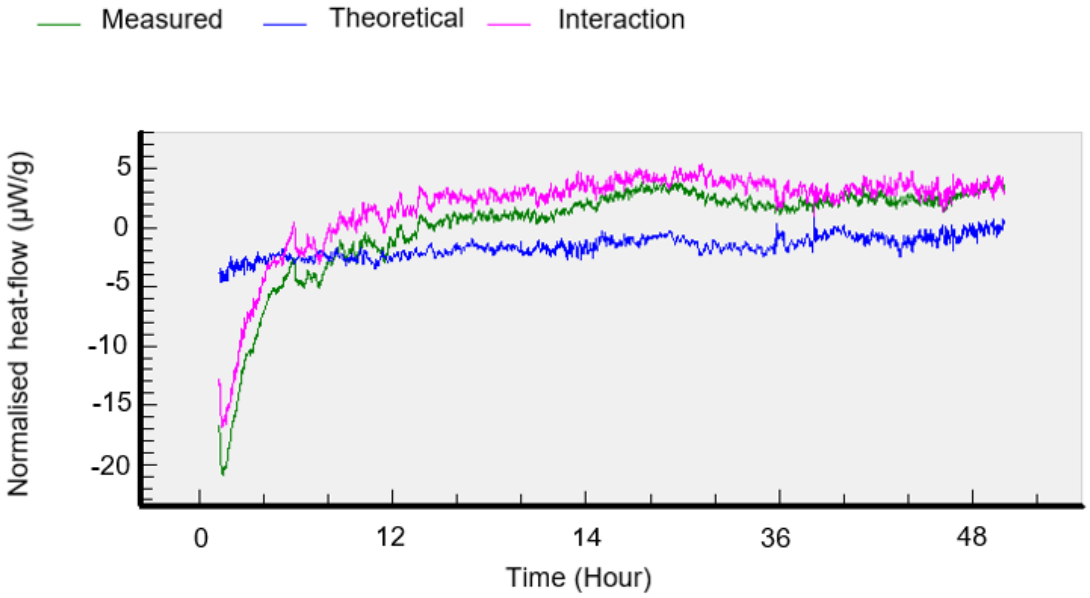

Figure A.4: Heat-flow graph obtained during the isothermal analysis of Absorbatox® bound to 2% fulvic acid

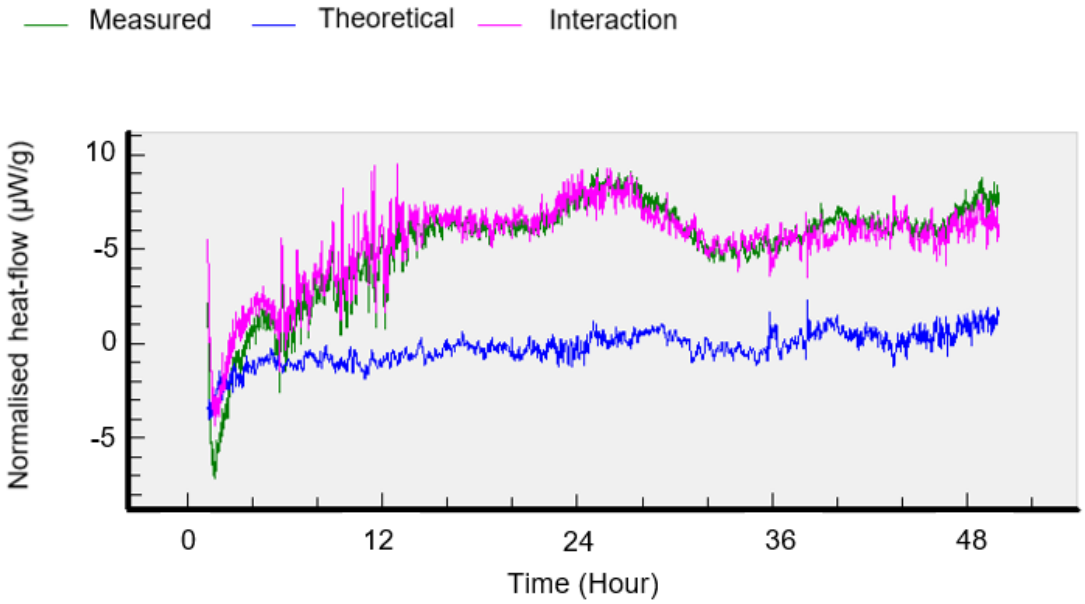

Figure A.5: Heat-flow graph obtained during the isothermal analysis of Absorbatox® bound to 4% fulvic acid

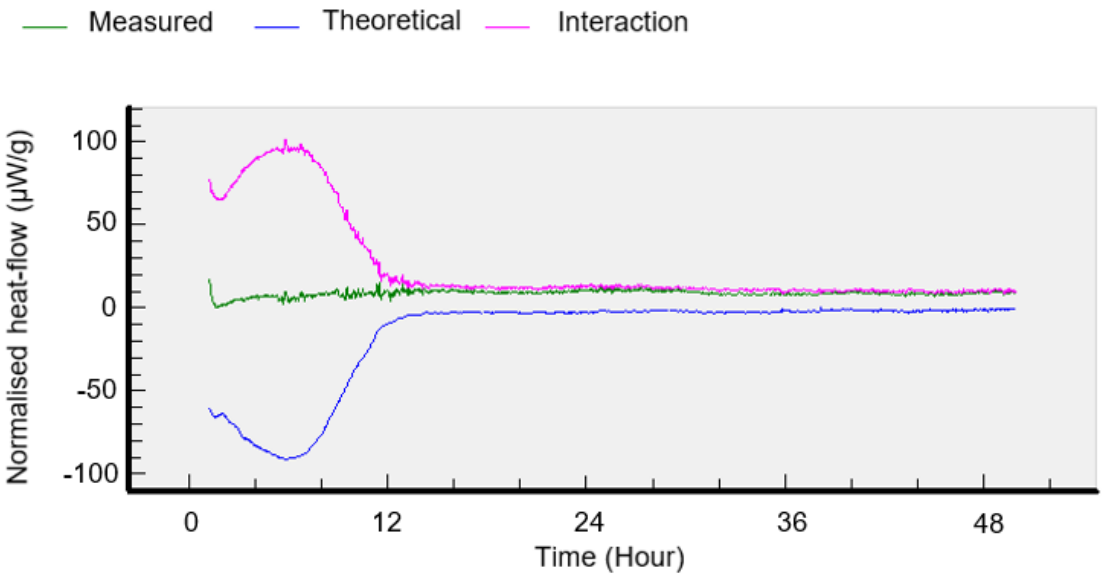

Figure A.6: Heat-flow graph obtained during the isothermal analysis of Absorbatox® bound to 4% citric acid

In conclusion, the single runs of citric acid and fulvic acid that was part of the experiment and Absorbatox® was compared with Absorbatox® bound to 2% fulvic acid, Absorbatox® bound to 4% fulvic acid and Absorbatox® bound to 4% citric acid. These results showed compatibility of Absorbatox® with fulvic acid, irrespective of the fulvic acid concentration, whilst the results of Absorbatox® bound to citric acid could indicate a possible incompatibility (Aucamp, 2019:2).

A.2.2 Spectroscopy

Spectroscopy is an identification method that allows for the identification of short-range arrangement of molecules in solids.

A.2.2.1 Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) spectroscopy is a technique used to obtain information on the structure and molecular conformation of a specific sample by measuring the vibration modes of bonded atoms. FTIR is a standard technique for the characterisation of compounds in the current context of solid materials (Bernstein, 2002:410; Rodriguez-Spong *et al.,* 2004:242). Infrared (IR)-spectra was recorded on a Bruker ALPHA Platinum spectrophotometer (Bruker, Billerica, USA) across a range of 400 – 4 000 cm^{-1}. The ALPHA Platinum Module has the attenuated total reflection (ATR) accessory designed for minimal operator induced variations, sampling with no preparation and excellent reproducibility. OPUS software was used to analyse the data.

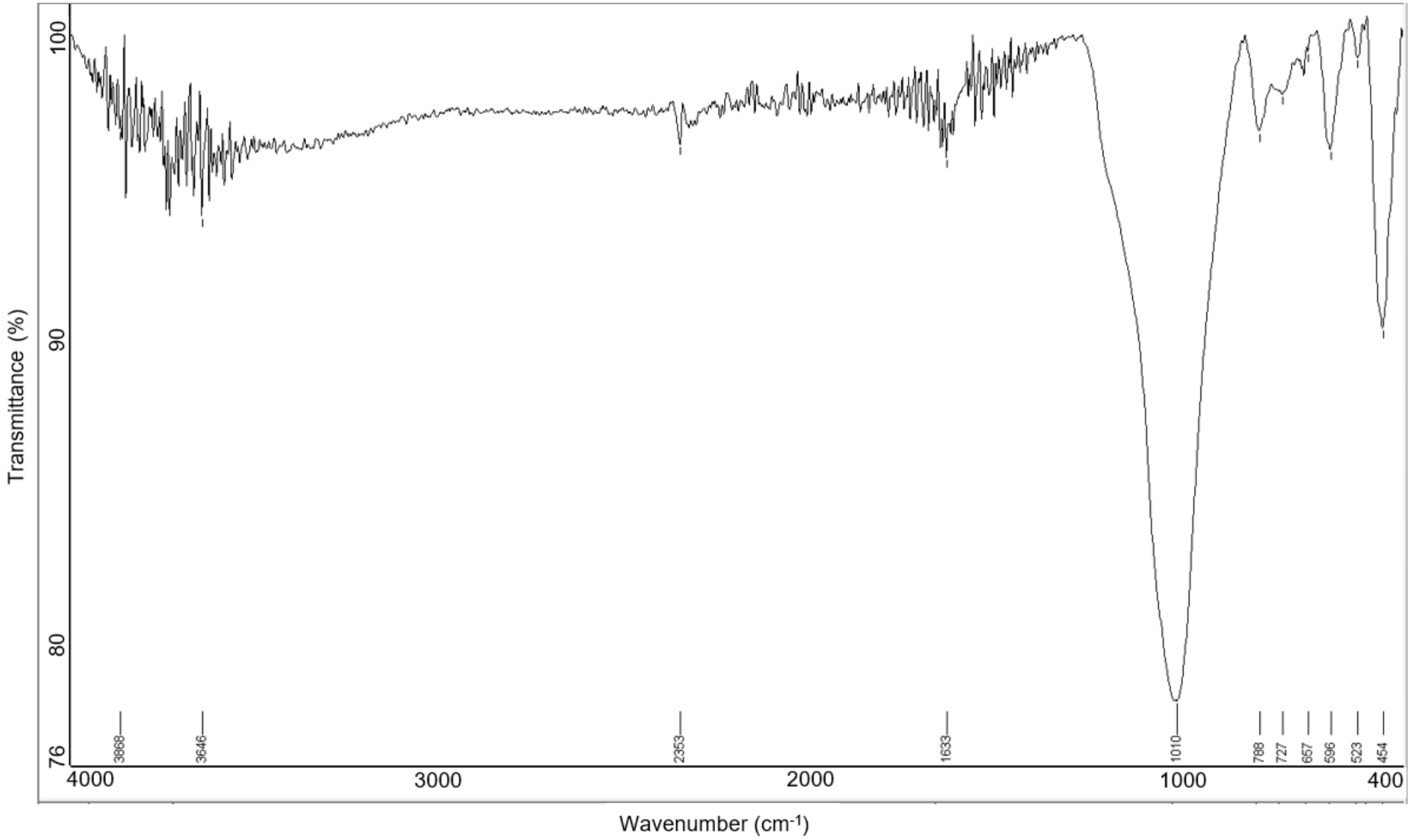

Figure A.7: FTIR spectrum of Absorbatox®

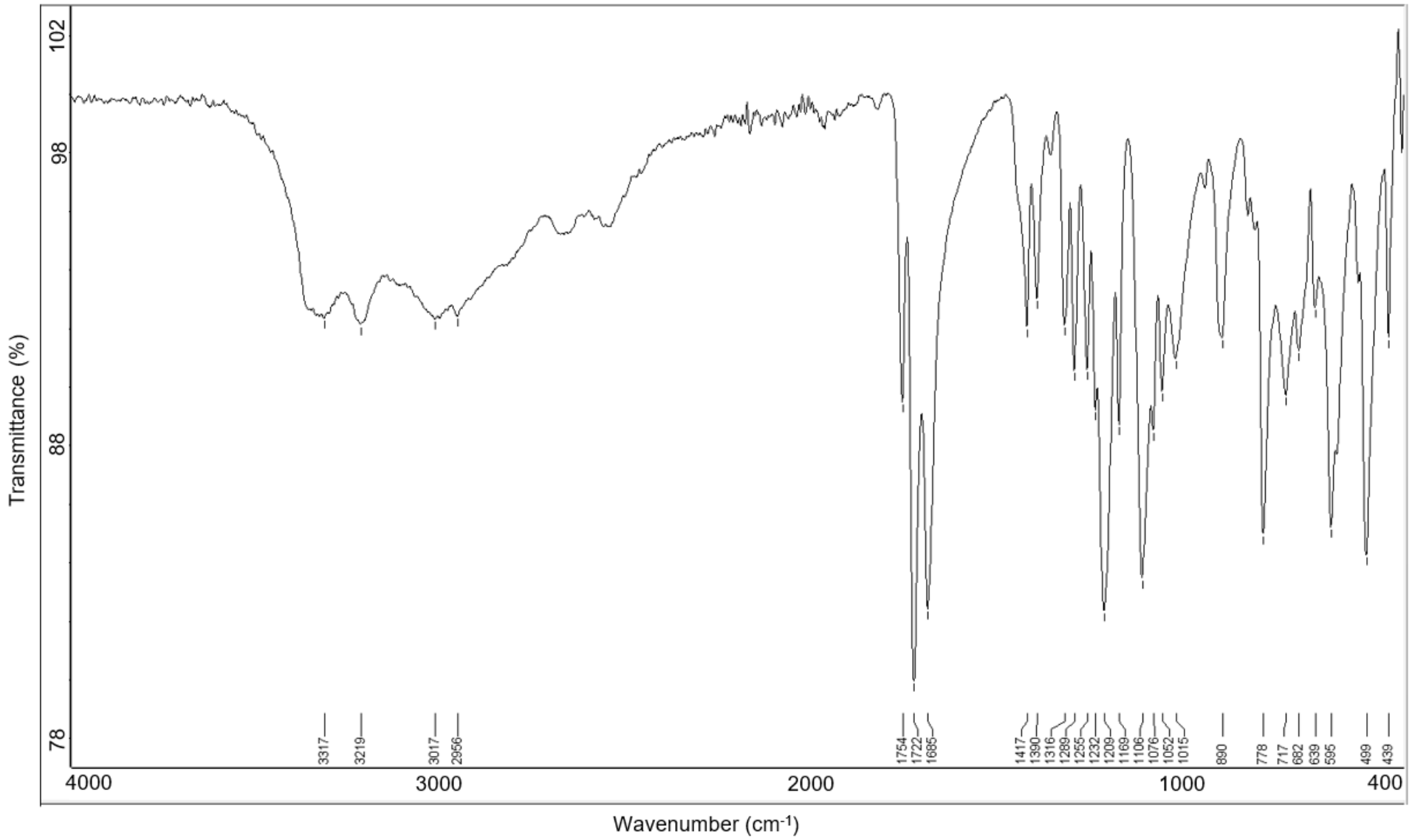

Figure A.8: FTIR spectrum of citric acid monohydrate

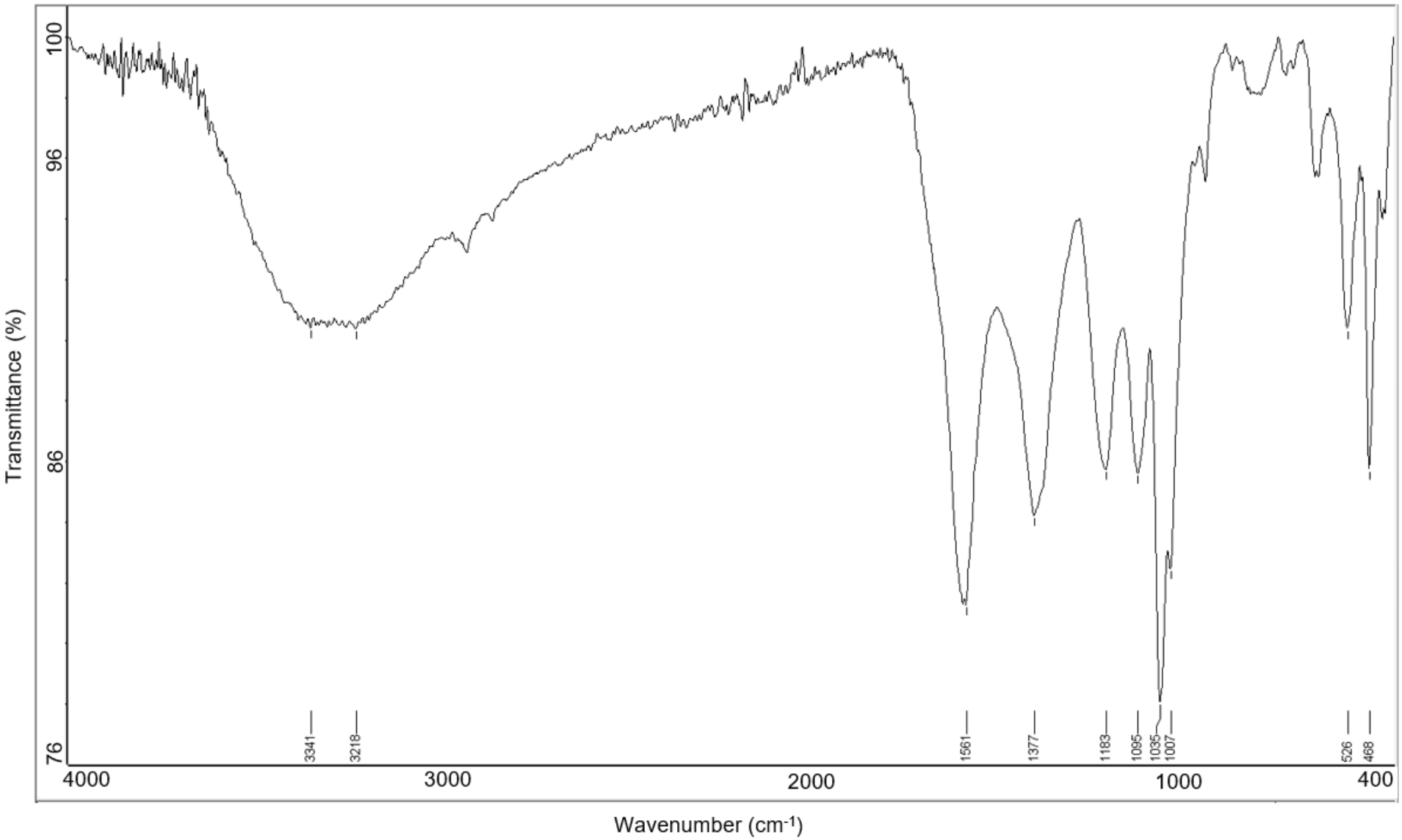

Figure A.9: FTIR spectrum of fulvic acid

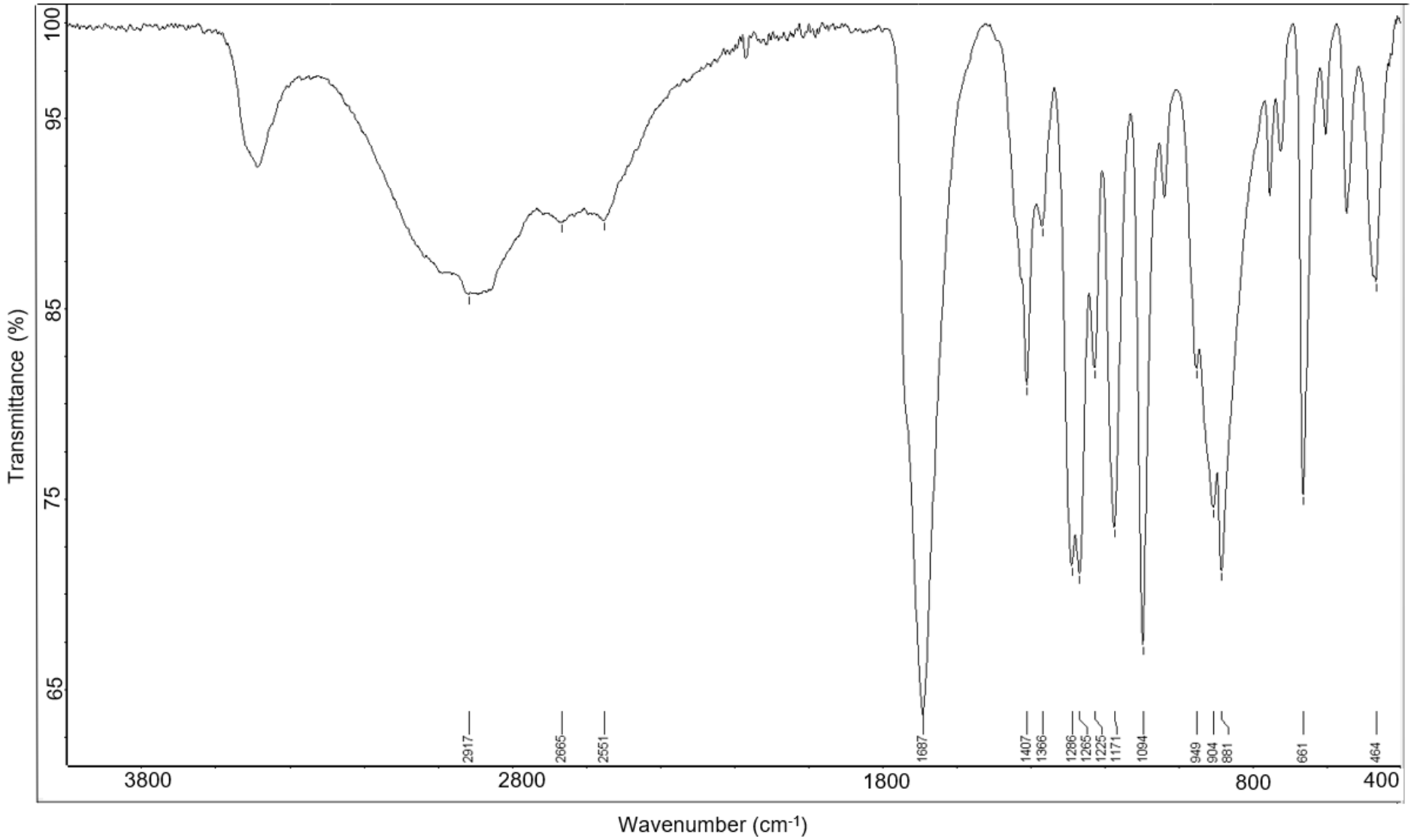

Figure A.10: FTIR spectrum of malic acid

During the FTIR analysis, Absorbatox® bound to 4% fulvic acid was tested as a single entity, bound together, whereas the physical mixture refers to the physical mixing of Absorbatox® with 4% fulvic acid in a beaker, which was also tested on FTIR. Figures A.7, A.8, A.9 and A.10 illustrate the IR spectra of Absorbatox®, citric acid, fulvic acid and malic acid, respectively; whilst the main absorptions are listed in Tables A.1 and A.2.

Table A.1: Main absorptions of the IR spectrum of Absorbatox® raw material, as well as selected organic acids

Wave number (cm^{-1})	Absorbatox®	Malic acid	Citric acid	Fulvic acid
	1010	1686	1753	1561
	454	1407	1722	1376
		1285	1685	1182
		1265	1209	1095
		1224	1168	1034
		903	1105	604
		721	778	525
		542	594	467
			499	
			439	

Table A.2 represents the main absorptions of the IR spectrum of Absorbatox® raw material in comparison with Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid, which will be used as reference point when comparing data from the stability testing as discussed in Appendix D.

Table A.2: Main absorptions of the IR spectrum of Absorbatox® raw material in comparison with Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid

Wave number (cm^{-1})	Absorbatox®	Absorbatox® bound to 4% fulvic acid	Physical mix of Absorbatox® and 4% fulvic acid
	1010	1011	1026
	454	459	453

Table A.2 indicates there are no large differences in the absorption bands for the Absorbatox® bound to 4% fulvic acid when compared to the physical mix of Absorbatox® and 4% fulvic acid. As seen in Figures A.11 and A.12, Absorbatox® is dominant in the spectra, as only 4% of the fulvic acid is present in each. The percentage acid is not enough to be observed on the IR.

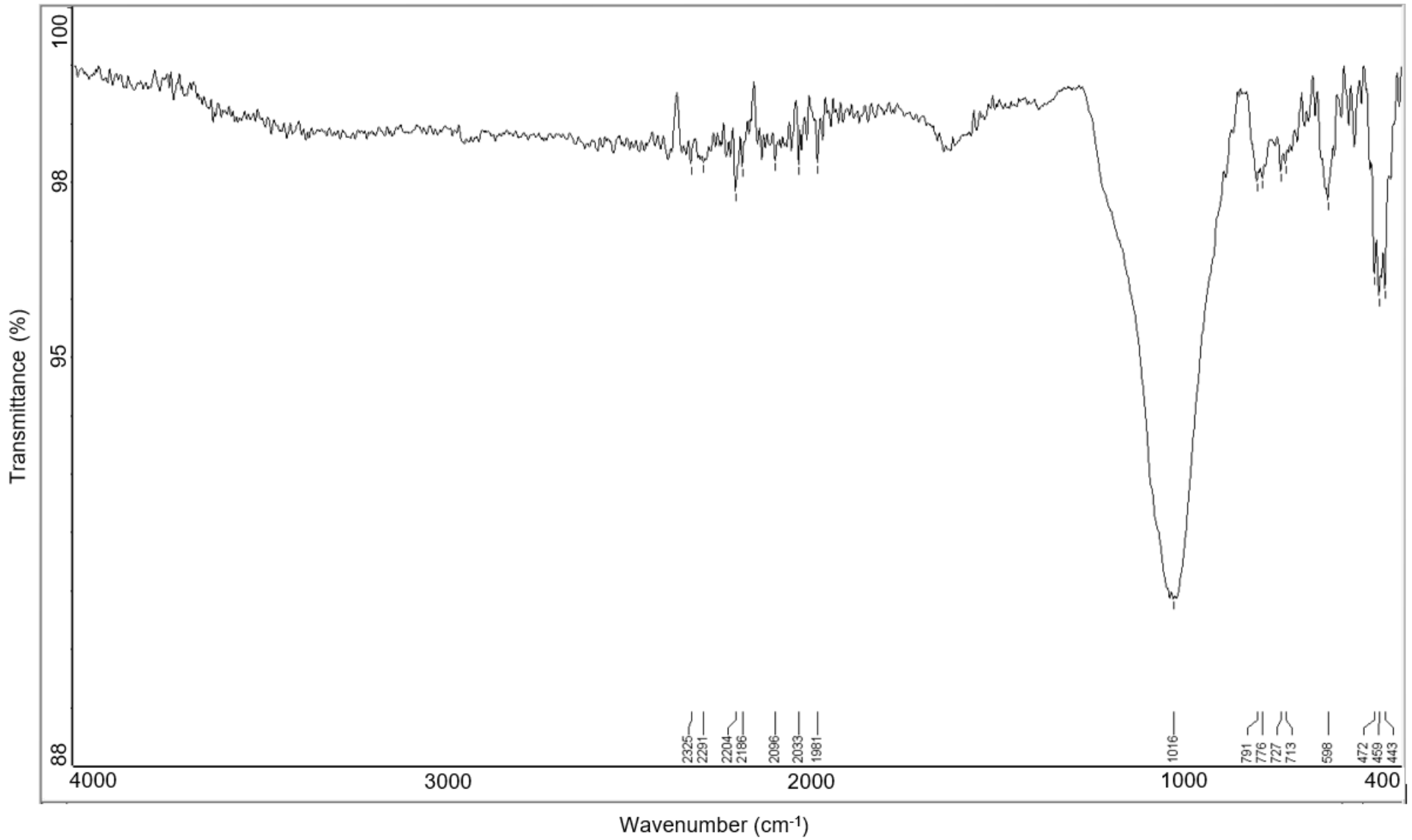

Figure A.11 FTIR spectrum of a physical mix of Absorbatox® and 4% fulvic acid

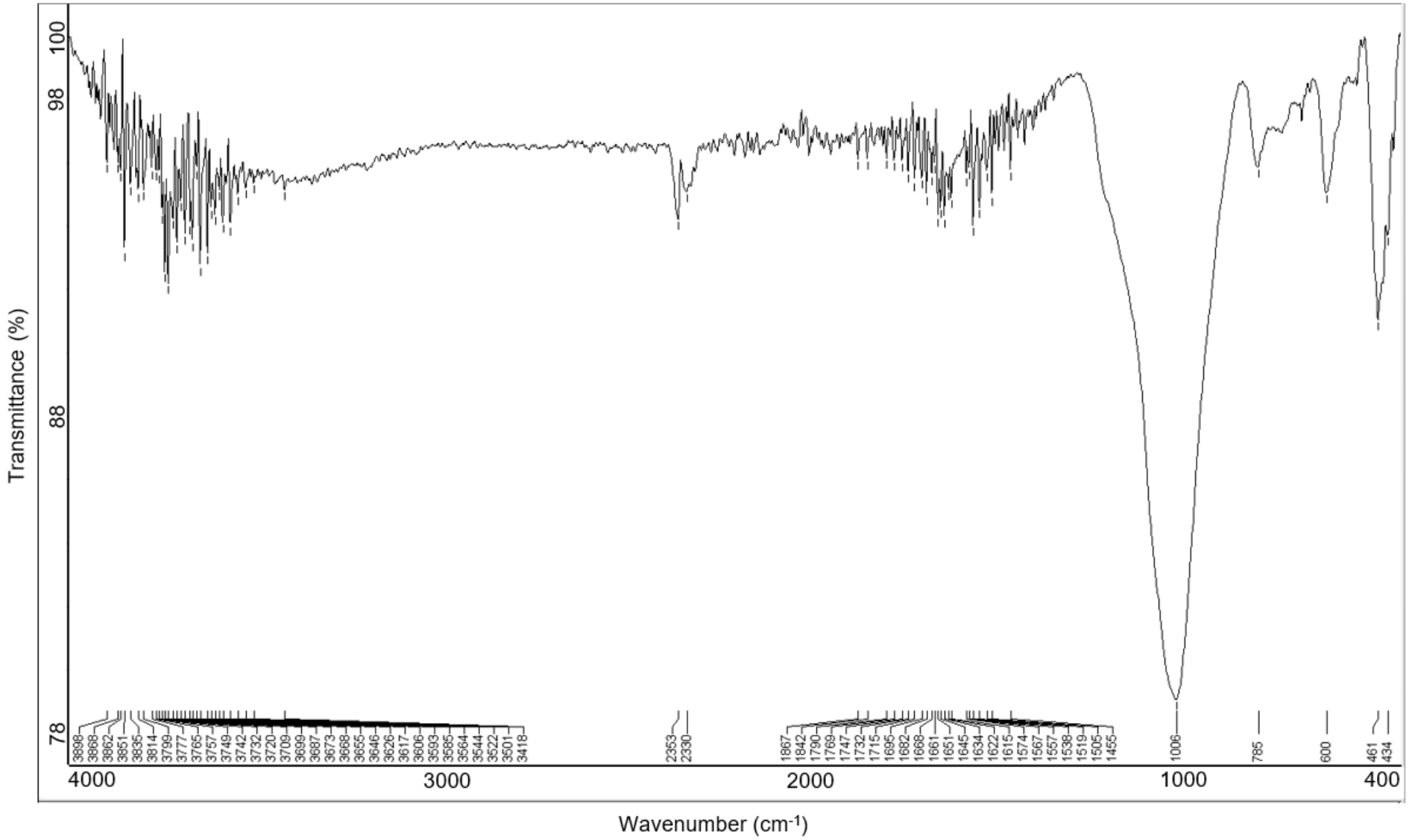

Figure A.12 FTIR spectrum of Absorbatox® bound to 4% fulvic acid

A.3 Conclusion

Physical characteristics and stability of both Absorbatox® and the selected organic acids were investigated by the interpretation of the DSC thermograms, TAM compatibility graphs, as well as FTIR spectra obtained. The results from the TAM showed complete compatibility of Absorbatox® bound to both the 2% and 4% fulvic acid, whereas the compatibility of Absorbatox® bound to 4% citric acid may be indicative of a possible incompatibility (Aucamp, 2019:2). The results obtained from the FTIR spectra indicated that Absorbatox® is dominant when measuring different samples (bound with and physical mixture) containing both Absorbatox® and fulvic acid. No large differences in absorption bands were observed on the IR graphs for Absorbatox® when Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid were compared; the acid concentration is too low to be presented on the IR spectra. From the data and results collected it is evident that Absorbatox® and fulvic acid proves to be a favourable combination for wound dressing formulations developed during this study.

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APPENDIX B

IN VITRO CYTOTOXICITY AND WOUND HEALING STUDIES USING ABSORBATOX® BOUND TO AN ORGANIC ACID

B.1 Introduction

Cytotoxicity testing is considered as one of the most important methods of biological assessment. Cytotoxicity testing is a key biological evaluation that makes use of tissue cells *in vitro* to observe cell growth, reproduction, as well as morphological effects resulting from the application of pharmaceutical active ingredients (Li *et al*., 2015:617). Cytotoxicity studies were previously only performed later in the process of new chemical entity discovery, causing an increase in the rate of drug attrition as a result of toxicology-related problems (Peternel *et al*., 2009:142). Today it has become one of the preferred indicators for toxicity evaluation, since it is simple, fast and exhibits high sensitivity (Li *et al*., 2015:617). Although cytotoxicity studies may present as an expensive process, it is a key tool to eliminate unsuitable formulations or chemical entities before the commencement of pre-clinical trials, aiding in the initial screening of new pharmaceutical agents (Van Tonder *et al*., 2015:2).

Although *in vitro* and *in vivo* conditions differ greatly, it can be said that toxicological assessments performed during the development of new drug delivery systems determine if any of the excipients of the formulation are toxic to human tissue. This adds to the importance of *in vitro* cell culture studies, providing the building blocks on which future *in vivo* studies are based (Wang *et al*., 2010:e10202, Yoon *et al*., 2012:634).

The skin consists of three types of cells namely keratinocytes, fibroblasts, as well as melanocytes. Previous studies on wound healing and cell cultures proved that human keratinocyte (HaCaT) cells may be used as a successful *in vitro* model. The spontaneously immortalised cell line has characteristics of easy propagation and near normal phenotype (López-Garcia *et al*., 2014:44). Human fibroblast (84BR) cells are known as one of the most ubiquitous cells in complex organisms, such as humans. 84BR cells are essentially involved in the repair and healing of damaged tissue. They are the main cells of stomal tissue, indicating their role during toxicity studies in an *in vitro* model (Rieske *et al*., 2005:474).

The purpose of this study was to investigate the effects of Absorbatox® bound to a selected organic acid on both HaCaT and 84BR cell lines. A methyl thiazolyl tetrazolium (MTT) assay was performed to assess if the API presented with cytotoxic effects, followed by a scratch wound healing assay to determine the wound healing potential of the API.

B.2 Materials and methods

The reagents used for the cytotoxicity studies, as well as the procedures prior to the commencement of the study (i.e. cell cultivation, treatment of cell lines, feeding and seeding of cells in 96- and 12-well plates) are given in Table B.1.

Table B.1: Reagents used during the *in vitro* cytotoxicity studies

Reagents	Supplier	Batch number
Phosphate buffered saline (PBS)	HyClone™	AB212873
Trypsin-EDTA (0.5%)	Lonza™	Not available
Trypan blue solution (0.4%)	Sigma-Aldrich®	RNBC9030
MTT	Sigma-Aldrich®	MKBX6716V
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich®	SHBH2447V
Dulbecco's Modified Eagle Medium (DMEM)	HyClone™	AC11223315
Triton™ X-100	Sigma-Aldrich®	SLFL3227V
Non-essential amino acids (NEAA)	Lonza™	5MB124
Foetal bovine serum (FBS)	Gibco™	42Q9352K
Penicillin/streptomycin (pen/strep) (1.0%)	Sigma-Aldrich®	SLBG0033v
200 mM L-glutamine	Lonza™	5MB180

B.2.1 Selection of an appropriate cell line and cell culture preparation

Both HaCaT and 84BR cells were used as the models on which the *in vitro* cell culture studies were performed. The HaCaT cells are suitable for wound healing studies, because they are human epidermal cells. The HaCaT cell line was maintained in 75 cm^2 cell culture flasks, each containing high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% non-essential amino acids (NEAA), 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (pen/strep). The 84BR cell line was maintained in 75 cm^2 cell culture flasks, each containing DMEM supplemented with 1% NEAA, 15% FBS, 1% pen/strep and 2 mM L-glutamine. Standard incubation conditions were set at 37 °C, 5% CO_2 and 95% humidity. For sub-culturing, the adherent cells were washed with phosphate buffered saline (PBS), detached with 5% trypsin-ethylenediaminetetraacetic acid (EDTA), re-suspended in growth medium and centrifuged at 140 *x g* for 5 min. Thereafter, the supernatant was discarded and the cell pellet re-suspended in the appropriate medium for either sub-culturing into new flasks or seeding into multi-well plates for assays. To maintain the cells, the culture medium was refreshed every two to three days (Mazumder *et al.,* 2016). The cells were checked regularly for bacterial contamination and the confluence estimated using an inverted light microscope.

B.2.2 Concentrations used for exposure

The cell lines were treated with the following formulations: a control group (Absorbatox®), as well as vehicle groups containing both Absorbatox® bound to a specific organic acid (malic acid, citric acid or fulvic acid).

The treatments for the MTT-assay were prepared according to Equation B.1.

$C_1V_1 = C_2V_2$ **Equation B.1**

Where:

C_1 = 1.00 mg/ml

C_2 = 0.15 mg/ml or 0.30 mg/ml

V_2 = 1.50 ml

V_1 = calculated as the volume of the stock solution per treatment to be added to make up 1.50 ml.

Example:

1 mg/ml x V_1 = 0.30 mg/ml x 1.50 ml

V_1 = 0.45 ml = 450 µl

1.50 ml - 0.45 ml = 1.05 ml media to be added to stock solution for treatment.

The treatments for the scratch wound healing assay were prepared according to Equation B.1. Where:

C_1 = 1.00 mg/ml

C_2 = 0.15 mg/ml or 0.30 mg/ml

V_2 = 15.0 ml

V_1 = calculated as the volume of the stock solution per treatment to be added to make up 15.0 ml.

Example:

1 mg/ml x V_1 = 0.30 mg/ml x 15.0 ml

V_1 = 4.5 ml

15.0 ml - 4.5 ml = 10.5 ml media to be added to stock solution for treatment.

Treatments were prepared as indicated in Figures B.1, B.2 and B.3.

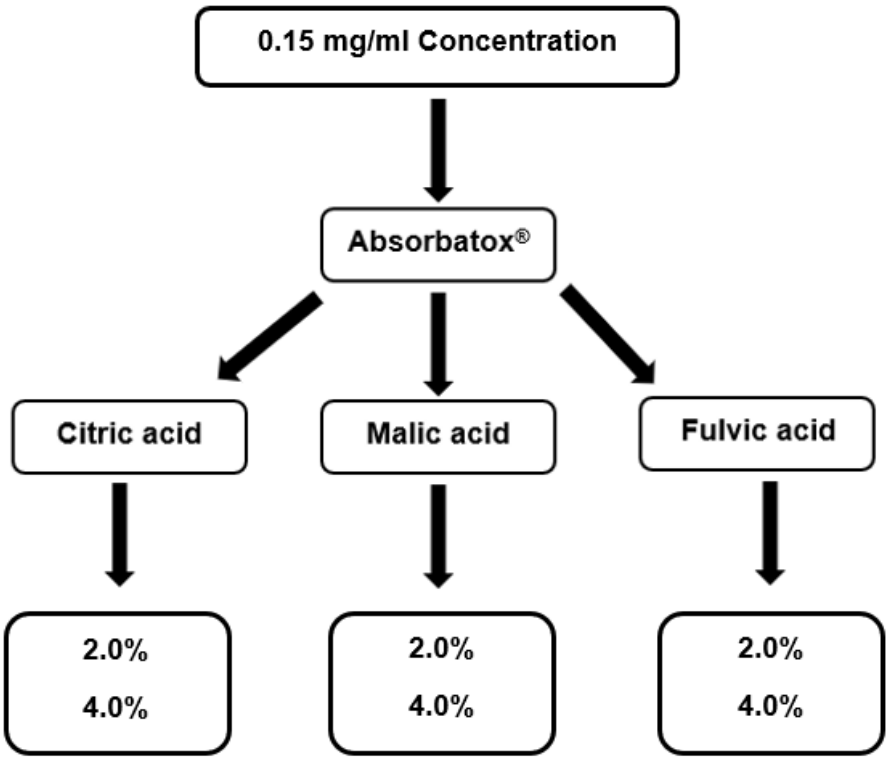

Figure B.1: Schematic representation on how the 0.15 mg/ml concentration treatments were prepared

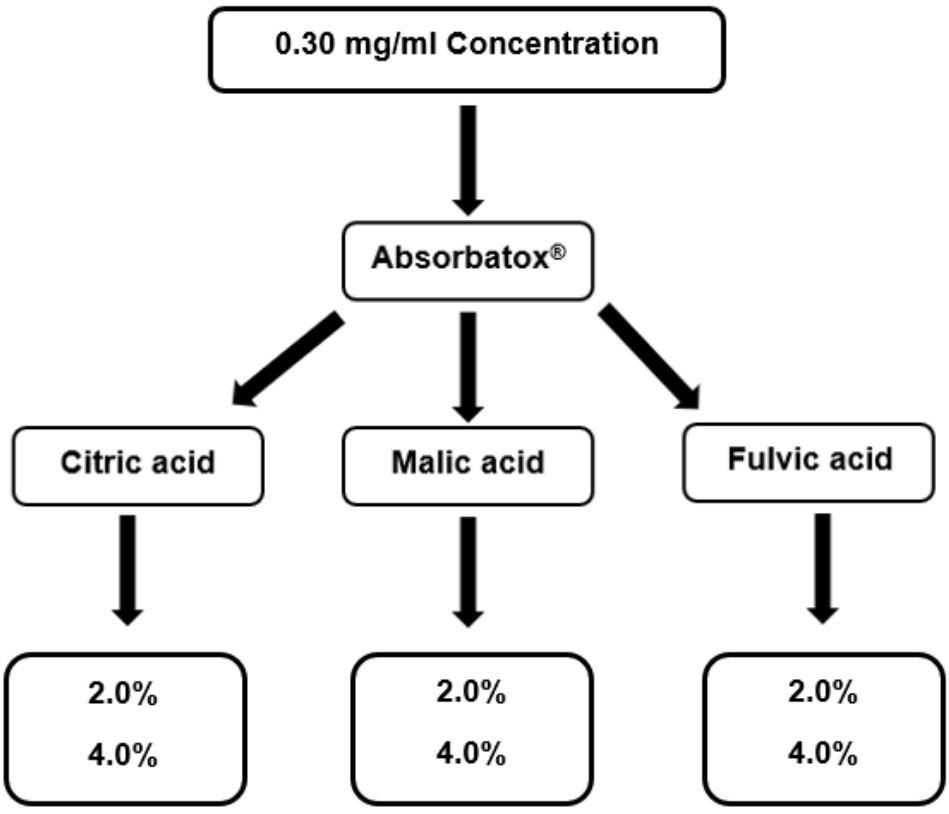

Figure B.2: Schematic representation on how the 0.30 mg/ml concentration treatments were prepared

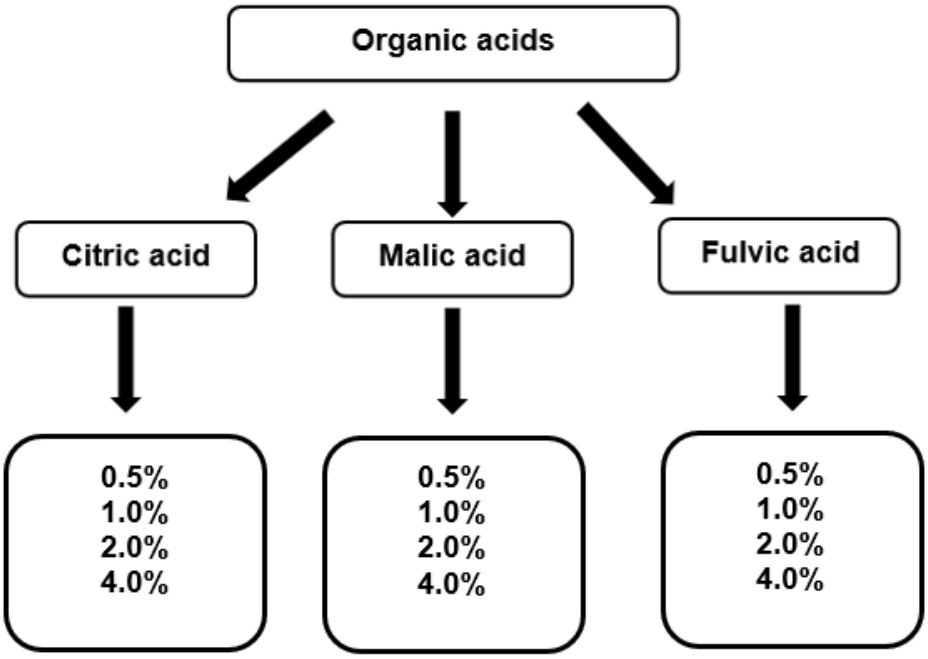

Figure B.3: Schematic representation 4on how the organic acid concentration treatments were prepared

B.2.3 *In vitro* cytotoxicity assays

Cultured HaCaT and 84BR cells were evaluated based on cell viability, when exposed to the Absorbatox® and organic acid formulations in this study. The cell lines were cultured to 80% confluence and trypsinised. The supernatant was discarded and the pellet re-suspended in 5 ml growth media. The new suspension was pipetted very well and 10 µl of the cell suspension added to the counting solution and counted immediately. The cell count was determined and cell suspensions with specific concentrations prepared and seeded in 96-well plates.

Cell counting was performed by means of the Trypan blue exclusion test using a haemocytometer (Figure B.4). The counting mixture was prepared as follows:

- The cell suspension (10 µl) was mixed with 25 µl Trypan blue (0.4%) and 15 µl PBS.
- After sufficient mixing of the components, 10 µl of the mixture was then loaded onto the coverslip of the haemocytometer, expelled and drawn into the chamber.
- The haemocytometer was then placed under a microscope and counted in terms of nine large squares on both sides dividing the surface of the haemocytometer. Only the cells present within the middle square and the corner squares were counted.
- The total cells after counting the five squares on both sides (ten squares in total) of the haemocytometer were then divided by two, which indicated the average viable cells on the two sides and thereafter divided by five to obtain the average per square.

- A dilution factor (5 x 10^4) multiplied with the final amount was used to determine the amount of viable cells present per millilitre of the cell suspension (C_1).
- This cell concentration was then multiplied with the total volume of cell suspension to obtain the total number of viable cells present in the cell suspension.
- The dilution required for seeding was then determined using Equation B.1.

Where:

C_1 = Amount of cells present per millilitre of the cell suspension or cell concentration (cells/ml)

C_2 = Required cell density (e.g. 15 000 cells/ml)

V_2 = Total volume of cell suspension needed

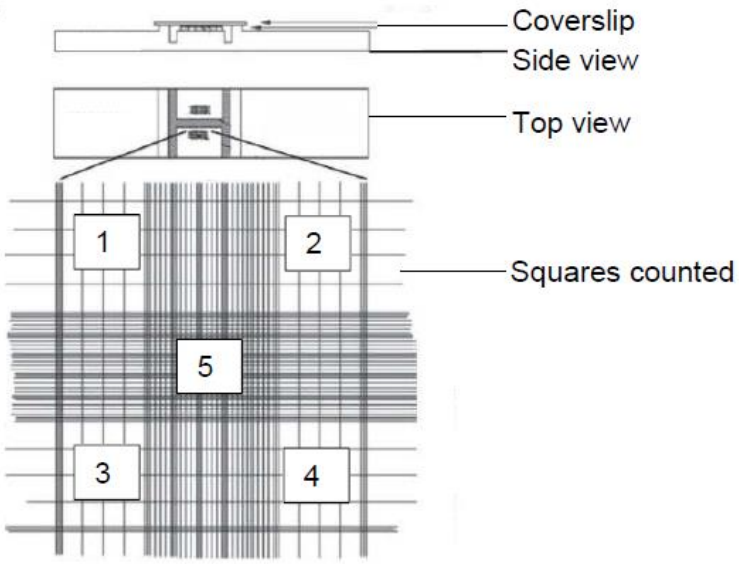

Figure B.4: Haemocytometer used to perform cell counting (adapted from BioTek, 2014:1).

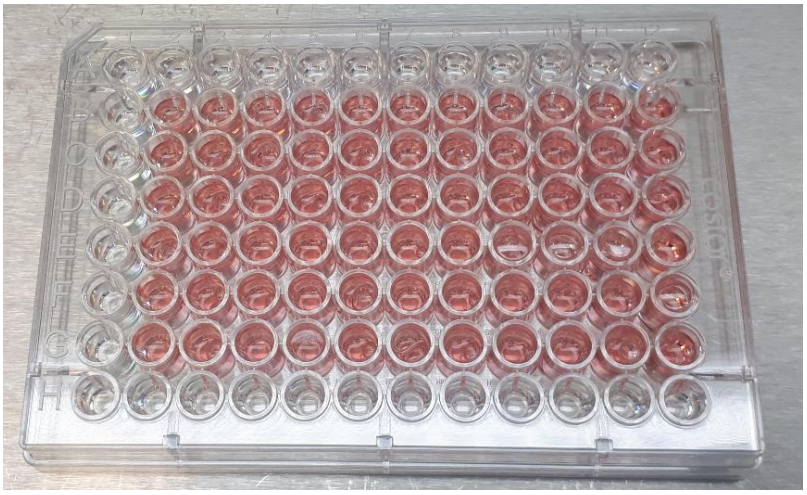

Figure B.5: Example of a 96-well plate, before adding the MTT-solution

The various cell suspensions were prepared and kept warm in the incubator, while loading 200 µl of each in the 96-well plate as indicated in Figure B.5. The plates were incubated for 24 h to allow the cells to adhere and recover from seeding.

The 84BR and HaCaT cells were seeded into clear 96-well plates at 50 000 cells/ml (10 000 cells/well) and 75 000 cells/ml (15 000 cells/well), respectively, and incubated for 24 h at 37 °C, 5% CO_2 and 95% humidity to ensure cells were properly adhered to the plate surface. The cells were then treated with various concentrations between 1% and 4%, of the organic acids separately, Absorbatox® alone and Absorbatox® bound to an organic acid. The *in vitro* cytotoxicity of the selected organic acids and Absorbatox® on the HaCaT and 84BR cells was determined by means of a colorimetric method, such as the MTT-assay.

B.2.3.1 Methylthiazol tetrazolium assay

The purpose of the MTT-assay was to measure the cell viability of the HaCaT and 84BR cell lines, after an exposure period of 24 and 48 h to the various treatment groups. Three different combinations of Absorbatox® bound to an organic acid concentration were tested, in addition to a negative control (dead cells obtained with Triton™ X-100) and a positive control group (untreated cells). All the treatments were conducted in six replicates. Blanks with DMSO were also included. For the organic acids, the highest tested concentration was also loaded separately to check for any possible interference with MTT.

The MTT-solution was freshly prepared (5 mg/ml) in PBS and stored at 4 °C until required. The cells were treated with the different dosages for a total period of 24 and 48 h, each. As soon as the cells were completely recovered from seeding (24 h), the media was removed and replaced with media containing the various dosages. This was noted as time 0 h. The media was then replaced daily and the plates incubated in between. At 24 and 48 h, the Absorbatox® bound to an organic acid containing media was removed and the cells were washed with PBS to get rid of the active ingredient. At this point, 180 µl non-additive medium together with 20 µl of MTT-solution was added to the wells. This resulted in a final MTT concentration on the cells of 0.5 mg/ml (Dwivedi *et al.,* 2015). The plate was shaken for 5 min on a plate shaker, followed by incubation at 5% CO_2 and humidified air at 37 °C for another 4 h until purple precipitate appeared as indicated in Figure B.6. The MTT-solution was removed and 200 µl DMSO added and mixed thoroughly (Dwivedi *et al.,* 2015). This dissolved the purple formazan crystals and generated a coloured solution, which was quantified by measuring the absorbance (Lee *et al.,* 2012:17).

The absorbance of the wells was recorded at 560 nm, as well as at 630 nm for background interference, using a microplate spectrophotometer. This absorbance was used as an indication of the viable cells capable of reducing the yellow colour of the MTT to purple formazan crystals. Such a colour change is a result of dehydrogenase enzymes present in the mitochondria of the

metabolically active cells (Fotakis & Timbrell, 2006:172). The calculation of the percentage cell viability was performed using Equation B.2; where the change in absorbance was measured as the absorbance difference between 560 nm and 630 nm with a SpectraMax® Paradigm® Multi-Mode Microplate reader as shown in Figure B.7.

$$\%\text{Cell viability} = \frac{\Delta\text{Absorbance of sample} - \Delta\text{ Absorbance of blank}}{\Delta\text{ Absorbance of untreated} - \Delta\text{ Absorbance of blank}} \times 100 \qquad \textbf{Equation B.2}$$

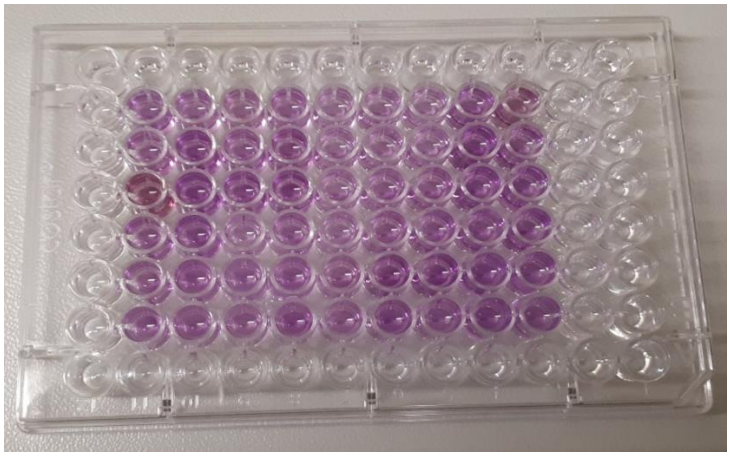

Figure B.6: Example of a 96-well plate after the addition of the MTT-solution, aspiration and addition of DMSO

Figure B.7: Example of a SpectraMax® Paradigm® Multi-Mode Microplate reader

B.2.3.2 Setup of 96-well MTT-plates

The 96-well MTT-plates were setup and dosed as indicated in Figure B.8.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			Untreated cells	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7	Treatment 8	Dead
C												Dead
D												Dead
E												Dead
F												Dead
G												Dead
H												

Figure B.8: MTT-plate layout indicating where the different treatments were loaded; light grey = PBS, dark grey = DMSO blank

B.2.4 Scratch wound healing assay

The purpose of the scratch assay was to measure the *in vitro* wound healing efficacy of Absorbatox® formulations on the HaCaT and 84BR cell lines, after inducing a scratch area. The scratch assay was performed using 12-well plates with the cultured HaCaT and 84BR cells seeded at 400 000 cells/ml and 200 000 cells/ml, respectively, and incubated for 24 h to form a monolayer. Thereafter, the monolayer was gently scratched across the centre of the well with a sterile 200 µl pipette tip in a straight line, noting that the long-axial of the tip is perpendicular to the bottom of the well as seen in Figures B.9 and B.10. The outer diameter of the end of the tip thus created the resulting gap. The wells were then washed four times with 1 ml non-additive medium to remove the detached cells after scratching.

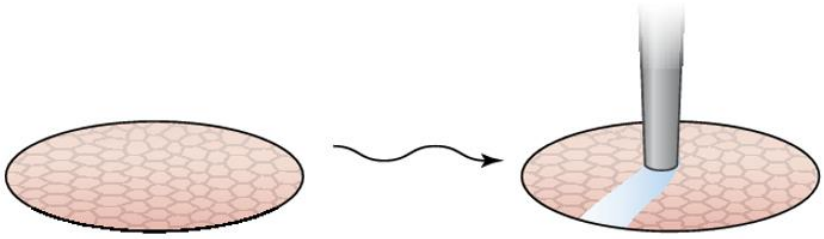

Figure B.9: A scratch made across the centre of each well with a pipette tip in a straight line (Adapted from Vedula *et al*., 2013: 370).

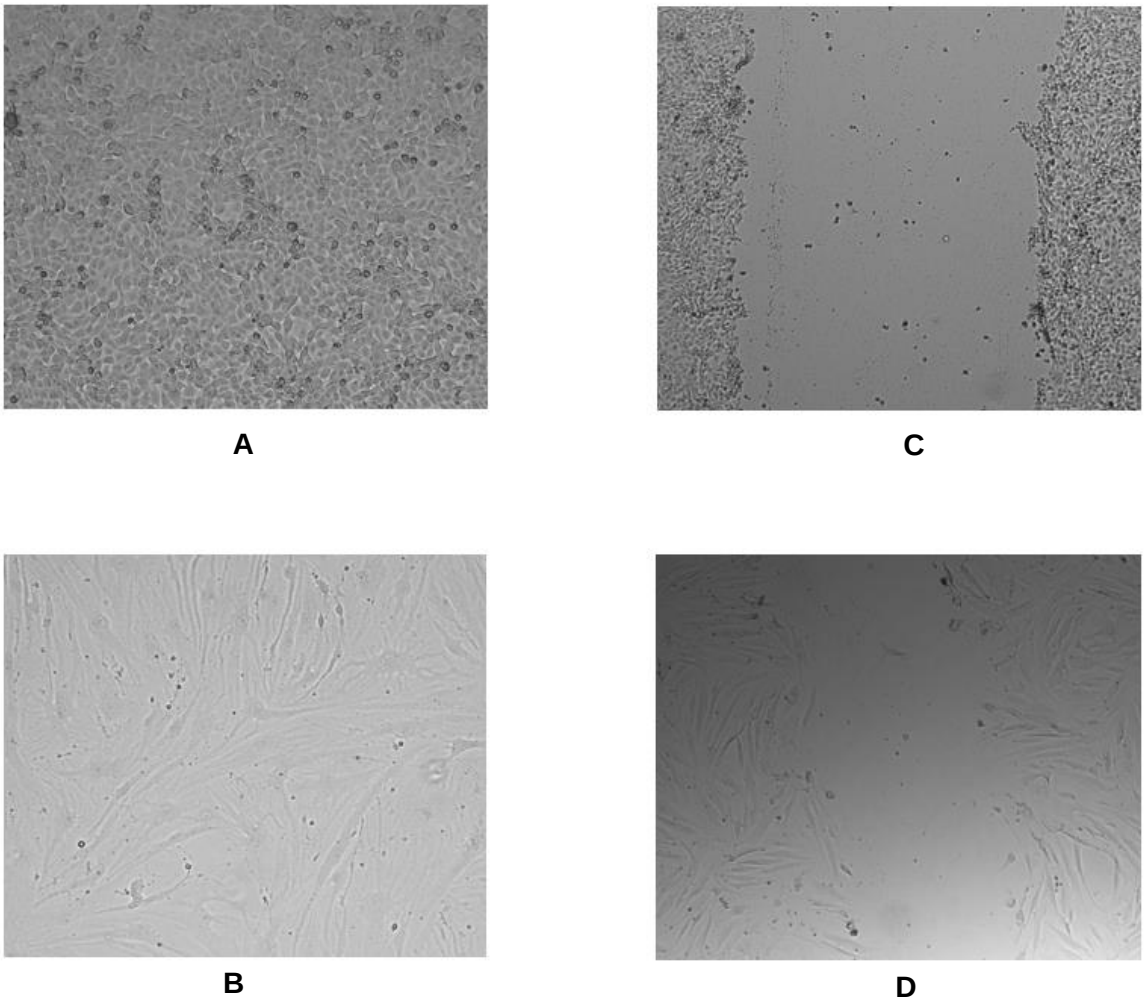

Figure B.10: Cell lines cultured as monolayers: A) HaCaT and C) 84BR, as well as immediately after the completion of a scratch: B) HaCaT and D) 84BR

B.2.4.1 Experimental groups

Three different Absorbatox® bound to an organic acid concentrations were tested, as well as an untreated group. Fresh growth medium containing the formulations were added to the scratched wells. The cells were then incubated for an additional 48 h during which photographs were taken of the designated wound area at 8, 16, 24, 32, 40 and 48 h intervals (Chen, 2012). The percentage wound closure of each wound was calculated according to Equation B.3:

$$\text{Wound closure\%} = \frac{(\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{(\text{Pre-migration})_{\text{surface area}}} \times 100 \qquad \textbf{Equation B.3}$$

The migration rate over time was calculated using Equation B.4:

$$\text{Migration rate } (\mu m^2/h) = \frac{(\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{\text{Time (hour)}} \qquad \textbf{Equation B.4}$$

B.2.4.2 Setup of 12-well wound healing plates

The 12-well wound healing plates were setup and dosed as indicated in Figure B.11.

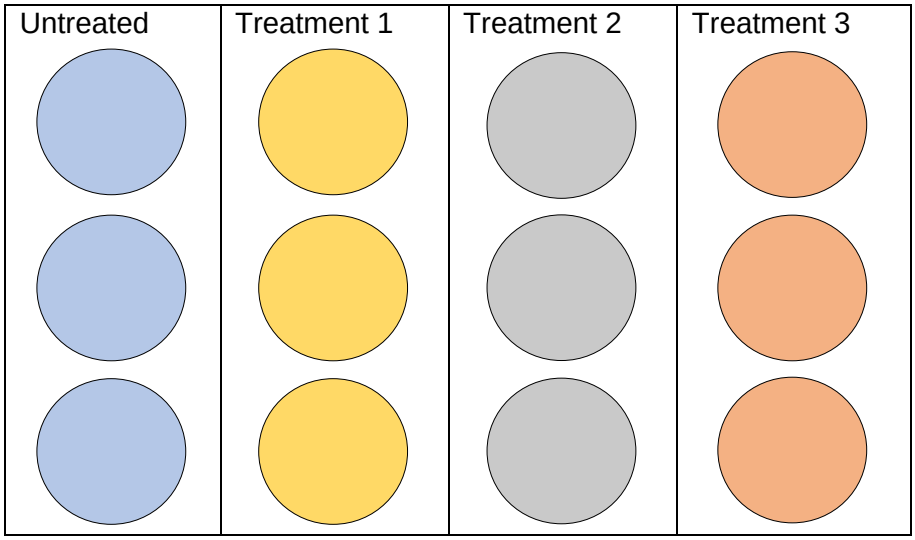

Figure B.11: Wound healing plate indicating where the different treatments were loaded

B.2.5 *In vitro* cell migration assay

The CytoSelect™ cell migration assay was performed on the HaCaT cells only. Firstly, the lower chambers of the migration plate were filled with 500 µl of DMEM supplemented with 10% (*v/v*) FBS. Serum free DMEM containing the selected treatment concentrations were used to make up cell suspensions of 1.0 x 10^6 cells/ml and aliquots of 300 µl were added to each insert of the 24-well plate. The untreated control consisted of cells in serum-free DMEM only. The plates were then incubated for 24 h at a temperature of 37 °C with 5% CO_2 and humidified air. Thereafter, the medium was carefully aspirated from the inside of the inserts. Cotton-tipped swabs were dampened with PBS and used to remove the non-migratory cells from the inside membrane of each insert. Each insert was then transferred to a new clean well containing 400 µl of a cell staining solution, followed by incubation at room temperature for 15 min. The stained inserts were washed thoroughly with PBS and allowed to air-dry. Each insert was then transferred to a well containing 200 µl of extraction solution and incubated for 10 min (Mazumder *et al.,* 2016). Lastly, the absorbance was measured with the microplate spectrophotometer at 560 nm. The percentage cell migration was calculated using Equation B.5.

$$\%\text{Cell migration} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \qquad \textbf{Equation B.5}$$

B.2.6 Statistical analysis

The Brown-Forsythe test was performed on the results in order to determine if the data collected was normally distributed. The analysis of variance (ANOVA) test, followed by Tukey's HSD and Kruskal-Wallis tests, were performed on the results to determine if there were statistically

significant differences among the various treatment groups. A p-value below 0.05 was considered as statistically significant.

B.3 Results and discussion

B.3.1 MTT-assay

The determination of the cytotoxicity of a treatment can be described in terms of the %cell viability, as seen in Table B.2. The results are cell line and assay specific (López-Garcia *et al*., 2014:44).

Table B.2: Classification of treatment cytotoxicity according to %cell viability

%Cell viability	Cytotoxicity
< 40%	Strong
40 – 60%	Moderate
60 – 80%	Weak
> 80%	Non-cytotoxic

Visual examination of the 96-well plates indicated a clear increase in purple colour intensity where the exposure to the Absorbatox® bound to an organic acid was favourable for cell proliferation, inducing a higher cell viability. Increased absorbance was a result of increased cell metabolic activity and consequently an increased amount of MTT formazan formed (Baluchamy et al., 2010:24770). The MTT results obtained after the 24 and 48 h exposure of the HaCaT and 84BR cells to various treatments of Absorbatox® bound to each organic acid is shown in Figures B.12 and B.13. The HaCaT cells resulted in a significantly increased cell viability when Absorbatox® bound to citric, malic or fulvic acid were applied. Formulations containing Absorbatox® bound to either citric or fulvic acid, gave the highest cell viability. According to López-Garcia *et al*. (2014), a compound with a %cell viability between 60 – 80% is weakly cytotoxic and a compound with a %cell viability above 80% is considered non-cytotoxic. Therefore, it can be concluded that the Absorbatox® bound to organic acids were not toxic to the selected keratinocyte cells and were thus safe for use in the formulation of new wound healing treatments. The results obtained from both the HaCaT and 84BR cells indicated no significant difference between treatment concentrations of 0.15 and 0.30 mg/ml. No significant difference was observed between the 2% and 4% concentrations of the organic acids bound to the Absorbatox®.

The MTT-assay indicated increased cell viability when treatments were applied to the 84BR cells. Absorbatox® bound to both citric and fulvic acid attained cell viability well over 100%, a clear indication of cell growth. Formulations containing Absorbatox® bound to both citric and fulvic acid, indicated the highest cell viability. The results obtained show direct correlation between 24 and 48 h cell viability, except in treatment with Absorbatox® bound to 2% citric acid. This is an indication that the Absorbatox® bound to organic acids are not toxic to fibroblast cells and are thus safe and effective for use in the formulation of new wound healing treatments.

The results obtained from Tukey's HSD test on the results of the HaCaT cells indicated statistical significant differences after 24 h exposure between the untreated control group and Absorbatox® (0.30 mg/ml). There were statistical significant differences between Absorbatox® (0.15 mg/ml) and Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 4% citric acid (0.15 mg/ml). Lastly, the test indicated statistical significant differences between all the treatment groups consisting of Absorbatox® (0.30 mg/ml) bound to 0.15 mg/ml concentrations of malic acid (2 and 4%), citric acid (2 and 4%) and fulvic acid (2 and 4%), as well as bound to 0.30 mg/ml concentrations of malic acid (2 and 4%), citric acid (2 and 4%) and fulvic acid (2 and 4%).

The results obtained from Tukey's HSD test on the results of the HaCaT cells indicated statistical significant differences after 48 h exposure between the untreated control group and Absorbatox® bound to 4% citric acid (0.15 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.15 mg/ml and 0.30 mg/ml). Statistical significant differences were seen between Absorbatox® (0.15 mg/ml) and treatments consisting of Absorbatox® bound to 4% malic acid (0.30 mg/ml), Absorbatox® bound to 2% citric acid (0.15 mg/ml), Absorbatox® bound to 4% citric acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% fulvic acid (0.15 and 0.30 mg/ml) as well as Absorbatox® bound to 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant differences for Absorbatox® bound to 2% citric acid (0.15 mg/ml), as well as Absorbatox® bound to groups containing 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml).

The results obtained from Tukey's HSD test on the results of the 84BR cells indicated statistical significant differences after 24 h exposure between Absorbatox® (0.30 mg/ml) and Absorbatox® bound to groups containing 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.30 mg/ml).

At 48 h, Tukey's test on the results of the 84BR cells indicated statistical significant differences between the untreated control group and Absorbatox® bound to 2% citric acid (0.15 mg/ml), as well as Absorbatox® bound to groups containing 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) showed statistical significant differences for Absorbatox® bound to 2% citric acid (0.15 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.30 mg/ml).

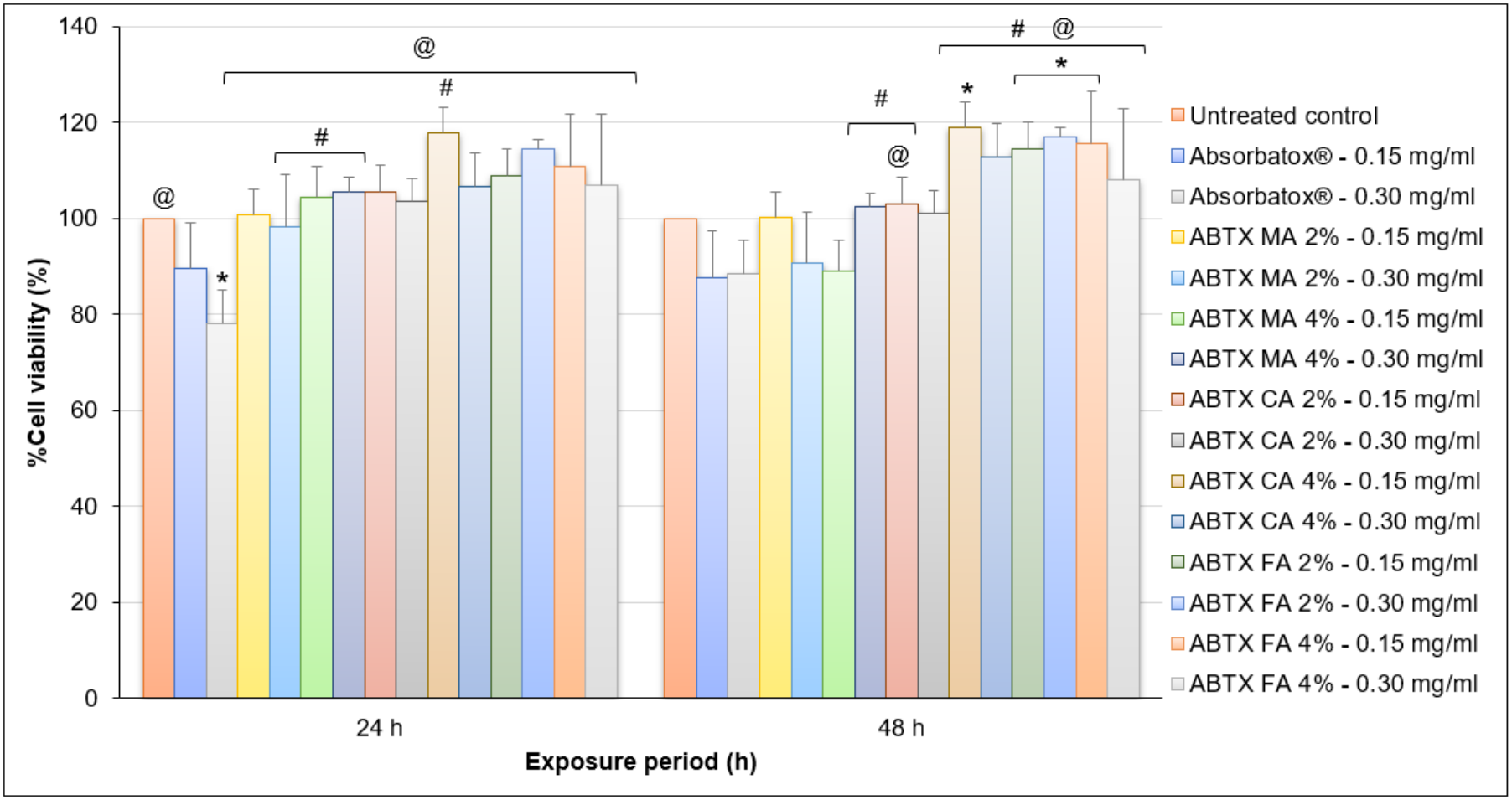

Figure B.12: %Cell viability of the HaCaT cell line treated with Absorbatox® bound to an organic acid for 24 and 48 h as determined by MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

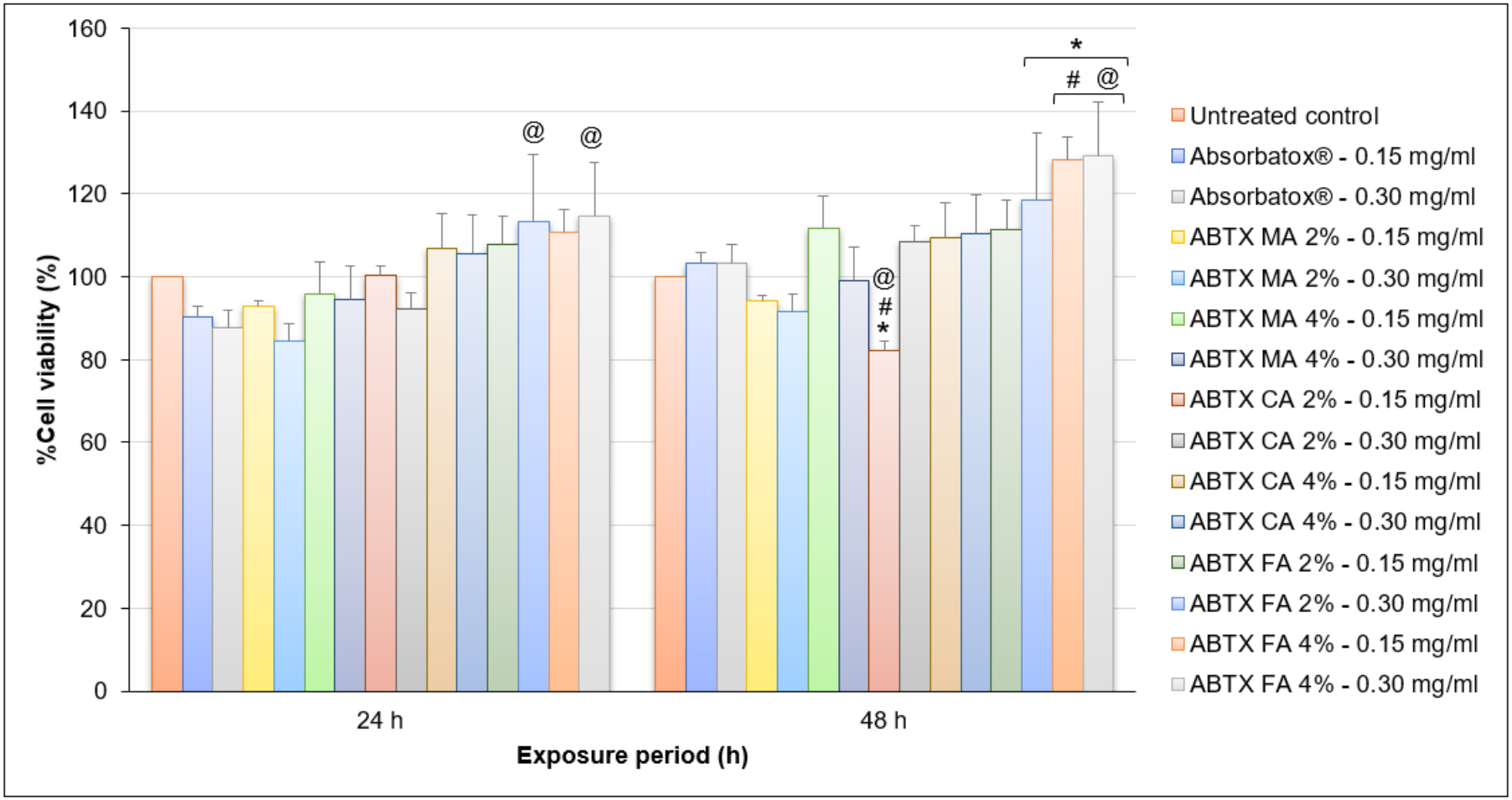

Figure B.13: %Cell viability of the 84BR cell line treated with Absorbatox® bound to an organic acid for 24 and48 h as determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

The MTT results obtained after the 24 and 48 h exposure of the HaCaT and 84BR cells to the various organic acid treatments alone are shown in Figures B.14 and B.15. The results showed a concentration dependent decrease in cell viability when the HaCaT and 84BR cells were exposed to the organic acids individually, however, no indication of cell death was observed. When compared to the other organic acids, fulvic acid proved to be the least toxic to both HaCaT and 84BR cells even though it still generated %cell viability values lower than 40%. This is considered as strongly cytotoxic to cells (López-García *et al.,* 2014:43) and not suitable for use in wound healing dressings. The results obtained show direct correlation between the HaCaT and 84BR cell lines when treated with organic acids alone. The organic acids caused a clear pH decrease when added to the media resulting in a distinct colour change, changing from a pink colour to bright yellow, malic and citric acid, and a dark brown-black colour when fulvic acid was used alone. Organic acids alone have been used extensively in dermatological procedures as peeling agents causing effects such as swelling, burning and pruritus of the skin, another strong indication that acids alone cannot be used in wound healing products. Organic acids function to promote cellular apoptosis (programmed cell death) (Tang & Yang, 2018:863).

Citric acid activates the death receptors and the BH3-interacting domain death agonist protein, increasing apoptosis-inducing factors. Malic acid has an antiproliferative effect on HaCaT cells by inhibiting the cell cycle progression. Malic acid has the ability to induce apoptosis through two molecular pathways: i) endoplasmic reticulum stress and ii) mitochondria-dependent signalling pathways. Although citric acid and malic acid have different structures, they activate the same apoptotic pathways. From the treatment of HaCaT cells with either citric or malic acid, clear apoptotic features are present including DNA damage, as well as apoptotic bodies (Tang & Yang, 2018:863).

From the cytotoxicity results, it was decided to not include the organic acids only for wound healing, seeing as a strong cytotoxic compound would not benefit the wound healing process.

Tukey's HSD test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate statistical significant differences between the untreated control groups compared to all three organic acids (citric, malic and fulvic aids) in concentrations of 0.5, 1.0, 2.0 and 4.0%. The Kruskal-Wallis test was performed on the results of both the HaCaT and 84BR exposure to organic acids alone over a 24 and 48 h period. The results obtained indicate statistical significant differences between the untreated control groups compared to treatments containing citric acid (2 and 4%) at a 48 h exposure period.

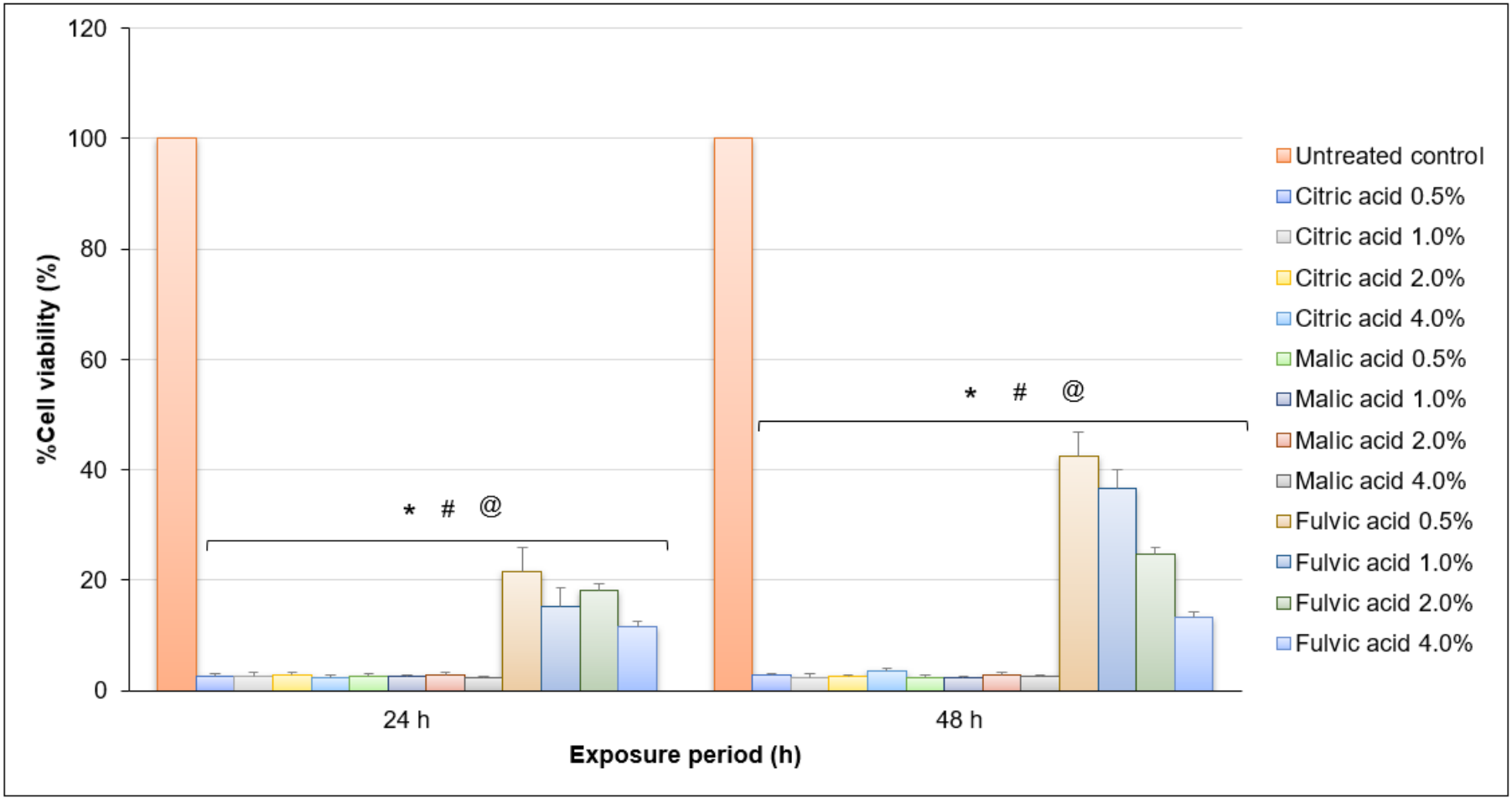

Figure B.14: %Cell viability of the HaCaT cell line treated with an organic acid for 24 and 48 h determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

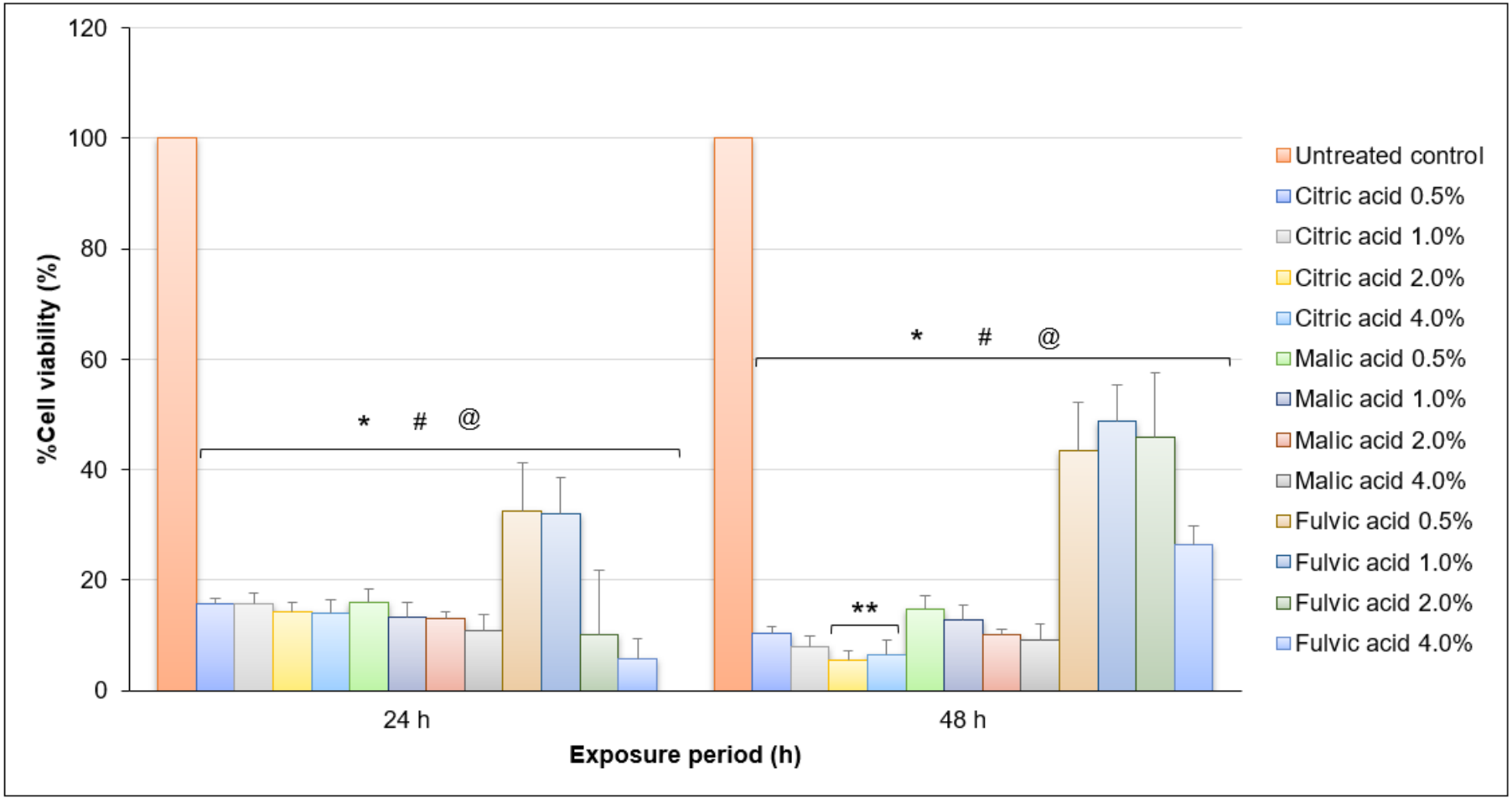

Figure B.15: %Cell viability of the 84BR cell line treated with an organic acid for 24 and 48 h determined with MTT (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid), Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml) and for Kruskal-Wallis test (** significantly different to the untreated control group)

B.3.2 Scratch wound healing assay results and discussion

Figures B.16 and B.17 indicate the %wound closure results obtained after 24 and 48 h exposures. The HaCaT cells exposed to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods showed a significant increase in improved wound closure when compared to the untreated control, as well as Absorbatox® alone, as shown in Figure B.16. HaCaT cells exposed to Absorbatox® bound to 2% citric acid and 4% fulvic acid, respectively, showed the highest percentage wound closure, with wounds indicating a percentage of over 90% closure after a 48 h exposure period. The 84BR cells exposed to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods showed a concentration dependent increase in improved wound closure when compared to the untreated control, as well as Absorbatox® alone, as shown in Figure B.17. 84BR cells exposed to Absorbatox® bound to 2% citric acid and 4% fulvic acid respectively, showed the highest percentage wound closure, with wounds indicating a 100% closure after a 48 h exposure period. Therefore, the Absorbatox® combinations with high acid ratios significantly improved the wound healing potential of Absorbatox® alone.

Previous studies have proved that *in vivo* wound treatment with Absorbatox® resulted in a rapid decrease of wound depth when compared to a control group with a conventional treatment. The results were apparent from day 7 of treatment, with the largest difference on day 10, where micronised and granular Absorbatox® resulted in 96% and 92% decrease in depth, respectively, versus only 88% for the control group (Anon, 2008).

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated group and Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml) and 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.15 mg/ml) indicated statistical significant differences for the treatments containing Absorbatox® bound to 2% malic acid (0.30 mg/ml) and Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) displayed statistical significant differences for the treatments containing Absorbatox® bound to 2% malic acid (0.30 mg/ml) and Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated group and Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml) 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented statistical significant differences for the treatments containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant

differences for the treatments containing Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated control group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% citric acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 4% citric acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% fulvic acid (0.15 and 0.30 mg/ml) and Absorbatox® bound to 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% citric acid (0.15 and 0.30 mg/ml), 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml) and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) displayed statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% citric acid (0.15 and 0.30 mg/ml), 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated control group and Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% citric acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 4% citric acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% fulvic acid (0.15 and 0.30 mg/ml) and Absorbatox® bound to 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% citric acid (0.15 and 0.30 mg/ml, 4% citric acid (0.15 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) indicated statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% citric acid (0.15 and 0.30 mg/ml), 4% citric acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.15 and 0.30 mg/ml) and fulvic acid (0.15 and 0.30 mg/ml).

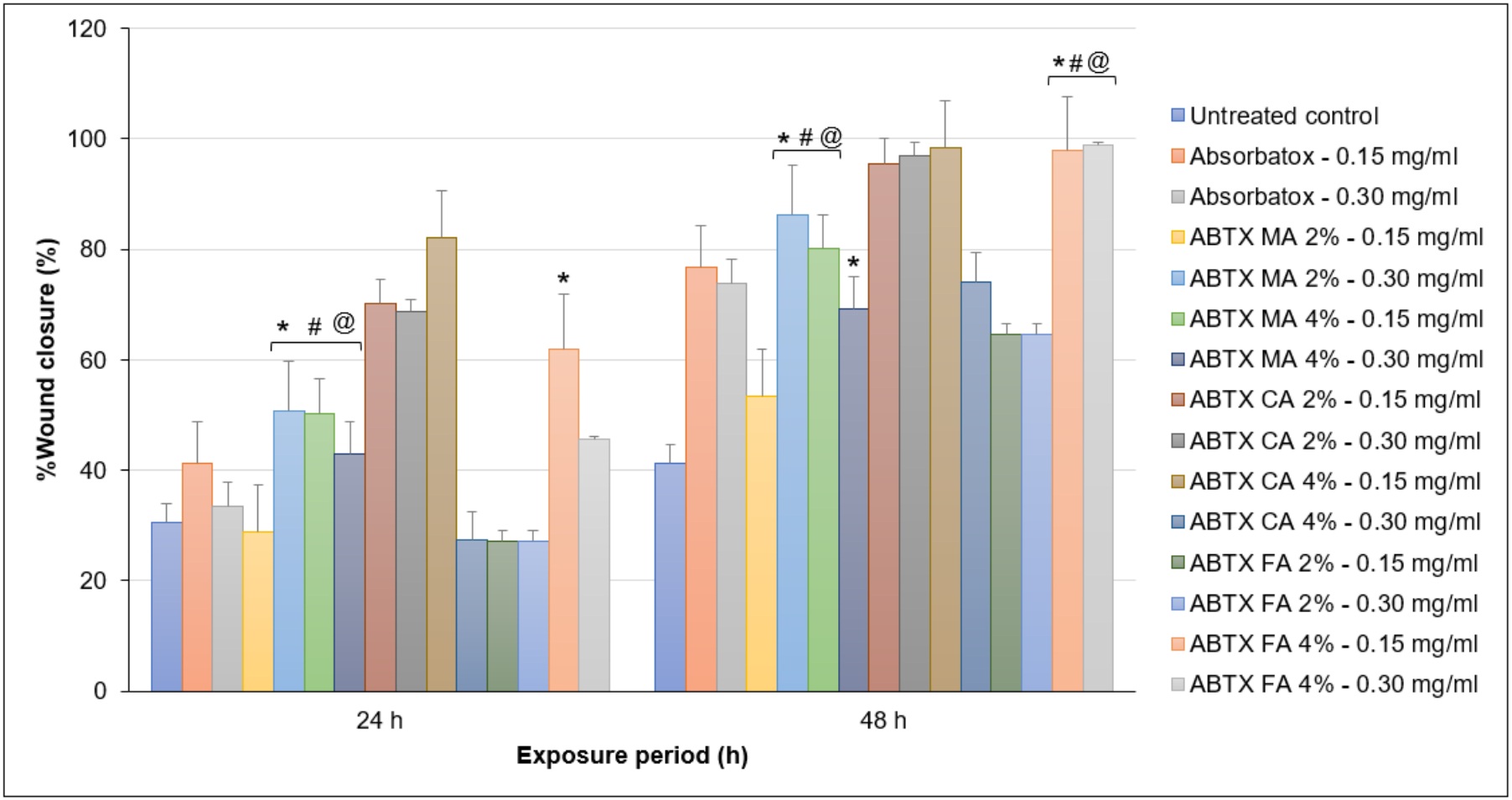

Figure B.16: HaCaT cell %wound closure results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

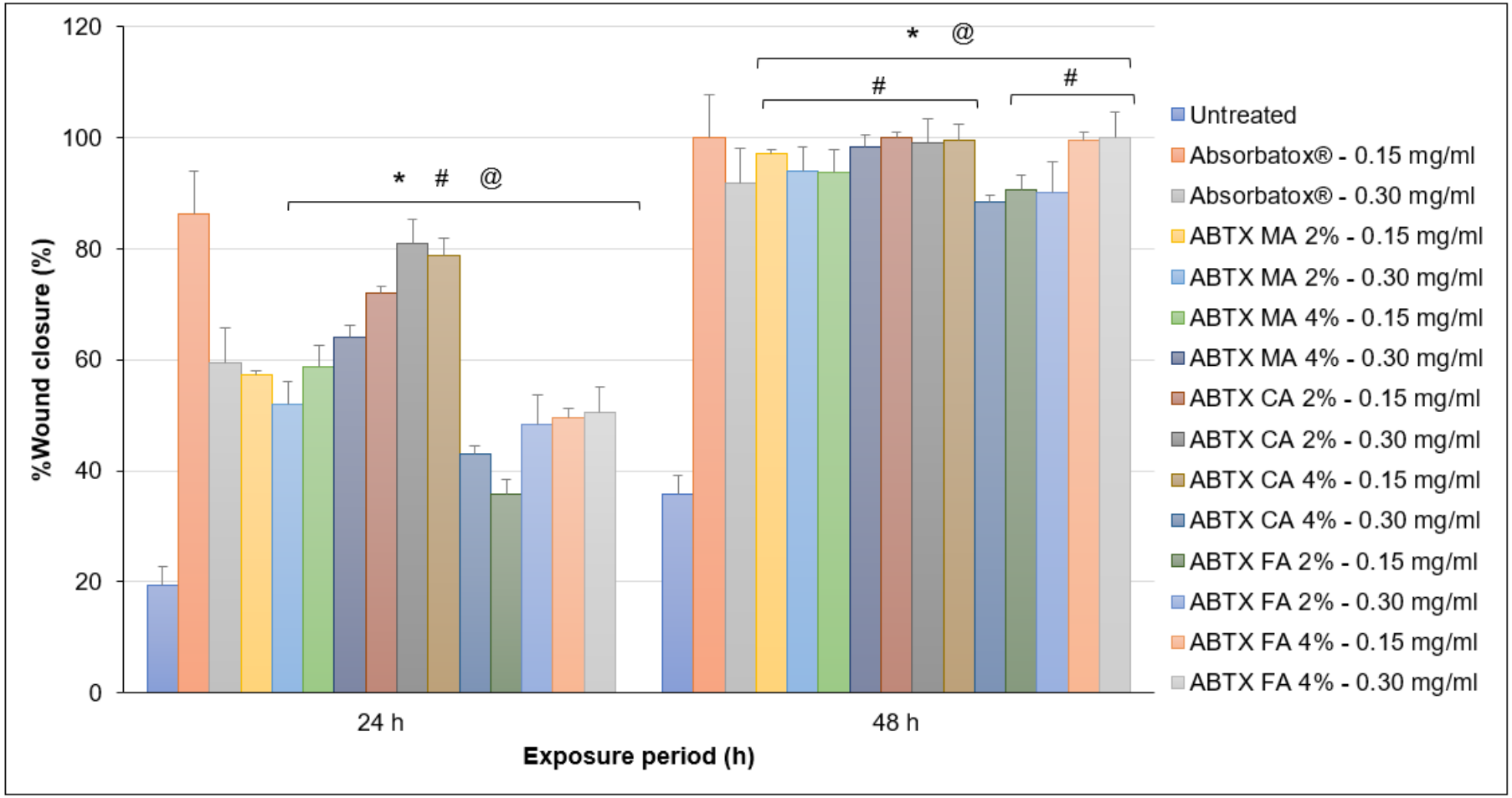

Figure B.17: 84BR cell %wound closure results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

The wound closure migration rate results obtained during the 24 and 48 h exposure of the HaCaT cells to various treatments of Absorbatox® bound to the organic acids are shown in Figures B.18 and B.19.

Figure B.18 indicates the HaCaT cell migration rate when exposed to Absorbatox® bound to each organic acid over a 24 and 48 h exposure period. The results obtained clearly indicated that the cells exposed to the various treatments showed an increased migration rate compared to the untreated control, as well as Absorbatox® alone. Cells treated with Absorbatox® bound to malic acid indicated a significant increase in migration rate compared to the untreated control group. Cells treated with Absorbatox® bound to both citric and fulvic acid indicated the highest significant difference in migration rate compared to the untreated control group. Figure B.19 indicates the 84BR cell migration rate when exposed to Absorbatox® bound to each organic acid over a 48 h exposure period. The 84BR cell migration rate when exposed to Absorbatox® bound to an organic acid over a 48 h exposure period indicated a clear increase. The results obtained, clearly indicated that the cells exposed to the various treatments show an increased migration compared to the control. Cells treated with Absorbatox® bound to malic acid indicate a notable increase in migration rate compared to the untreated control group. Cells treated with Absorbatox® bound to both citric and fulvic acid indicated the largest difference in migration rate compared to the untreated control group. The highest wound closure rate is observed within the first 24 h for both the HaCaT and 84BR cell lines, the closure rate slows after 24 to 48 h. This may be a result of cell contact inhibition; this occurs as a result of increased wound healing. As the cells move closer towards one another, they exert an inhibitory contact force, which slows migration as the space becomes smaller. This is evident on both Figures B.18 and B.19, as the plateau is reached. The results obtained indicate an average faster wound migration rate when treated with concentrations of 0.15 mg/ml of the Absorbatox® bound to organic acid treatments. The graphs indicate a faster migration rate observed with Absorbatox® bound to an acid in a concentration of 2% compared to a concentration of 4%.

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2% fulvic acid (0.30 mg/ml) , as well as Absorbatox® bound to 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.15 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.30 mg/ml) and 4% fulvic acid (0.15 mg/ml). Absorbatox® (0.30 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 2% fulvic acid (0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the HaCaT cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), as well as 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) displayed statistical significant differences for Absorbatox® bound to groups 2% malic acid (0.30 mg/ml), 4% malic acid (0.15 and 0.30 mg/ml), 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 24 h exposure between the untreated group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 2 and 4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) presented with statistical significant differences for Absorbatox® bound to groups containing 4% malic acid (0.15 and 0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).

Tukey's HSD test was performed on the results of the 84BR cells exposed to treatments indicated statistical significant differences after 48 h exposure between the untreated group and groups containing Absorbatox® bound to 2% malic acid (0.30 mg/ml), Absorbatox® bound to 4% malic acid (0.15 and 0.30 mg/ml), Absorbatox® bound to 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as Absorbatox® bound to 2 and4% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.15 mg/ml) displayed statistical significant differences for Absorbatox® bound to groups containing 4% malic (0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml) and 2% fulvic acid (0.15 and 0.30 mg/ml). Absorbatox® (0.30 mg/ml) showed statistical significant differences for Absorbatox® bound to groups containing 4% malic acid (0.30 mg/ml), 2 and 4% citric acid (0.15 and 0.30 mg/ml), as well as 2 and 4% fulvic acid (0.15 and 0.30 mg/ml).

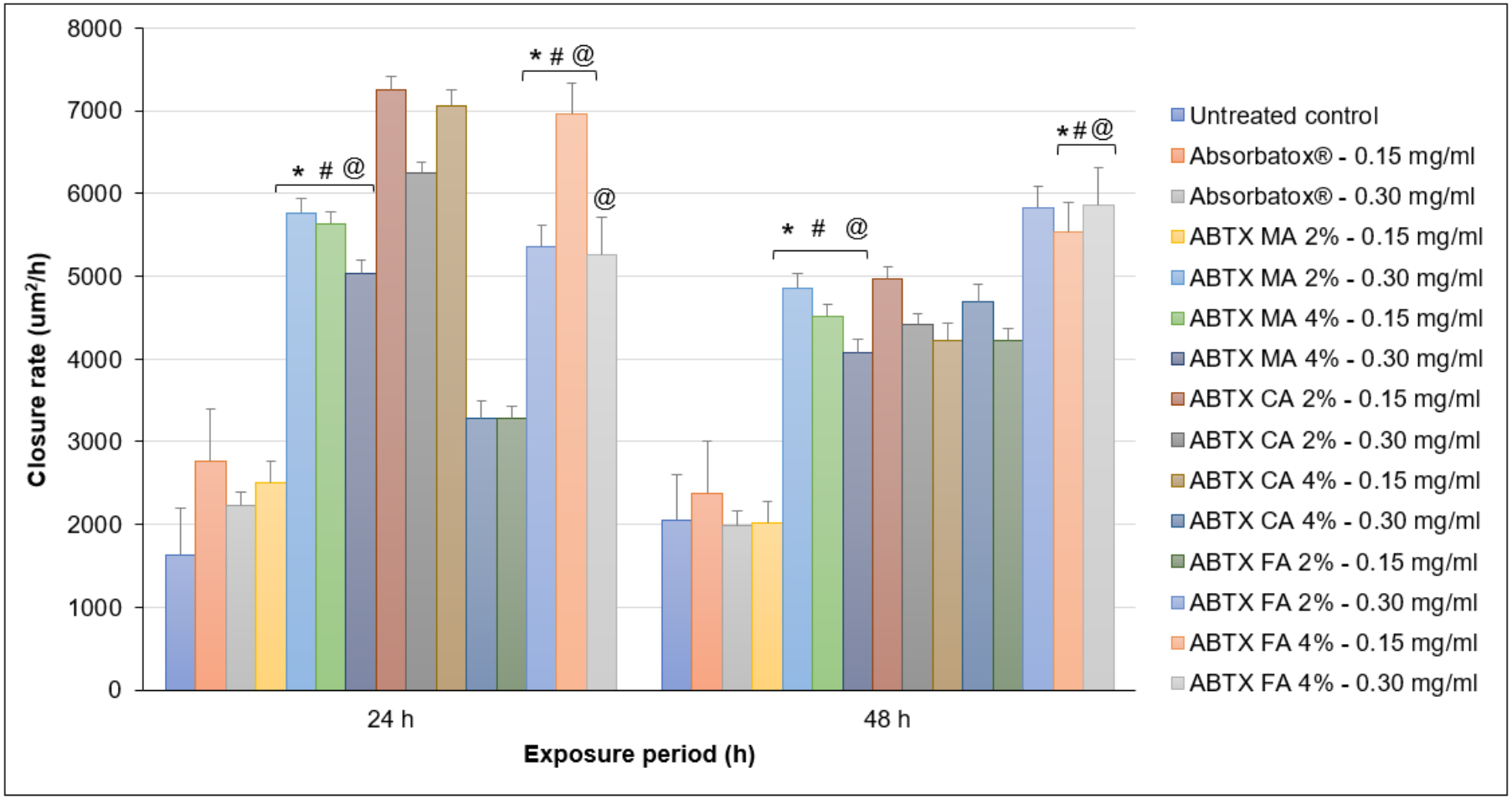

Figure B.18: HaCaT cell wound closure rate results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

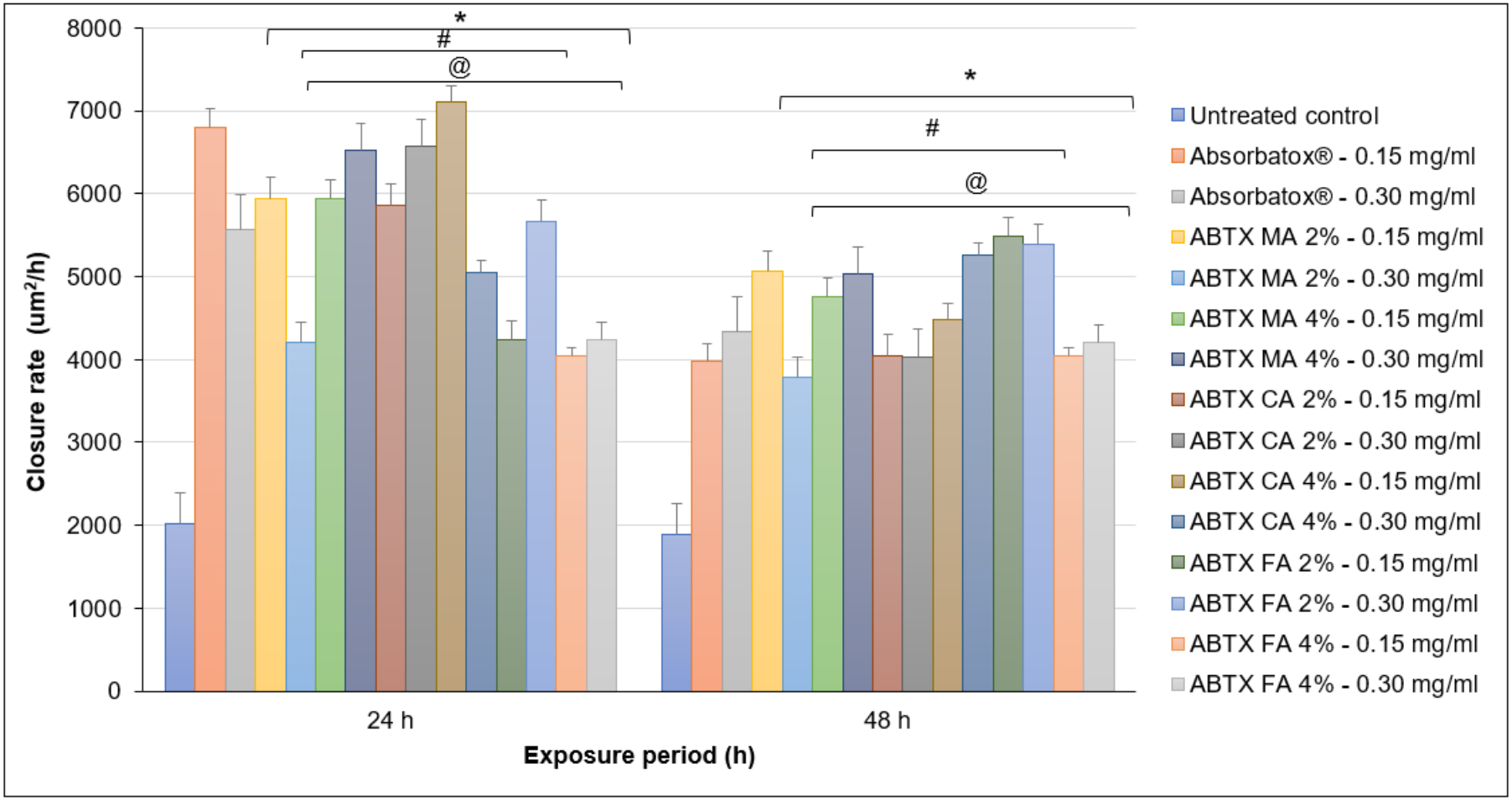

Figure B.19: 84BR cell wound closure rate results after exposure to Absorbatox® bound to an organic acid at 24 and 48 h treatment periods (with ABTX (Absorbatox®); CA (citric acid); MA (malic acid); FA (fulvic acid) and for Tukey's HSD test (* significantly different to the untreated control group; # significantly different to the ABTX 0.15 mg/ml, and @ significantly different to the ABTX 0.30 mg/ml)

B.3.3 *In vitro* cell migration assay results and discussion

The migration assay results of the HaCaT cells exposed to various concentrations of the Absorbatox® bound to organic acids for 24 h is illustrated in Figure B.20. The largest increase in cell migration was observed where the HaCaT cells were treated with Absorbatox® bound to 2% fulvic acid at a concentration of 0.30 mg/ml. This showed that cell migration may play an important role in wound healing at longer exposure time (i.e. 48 h), as seen during the scratch assay results in Section B.3.

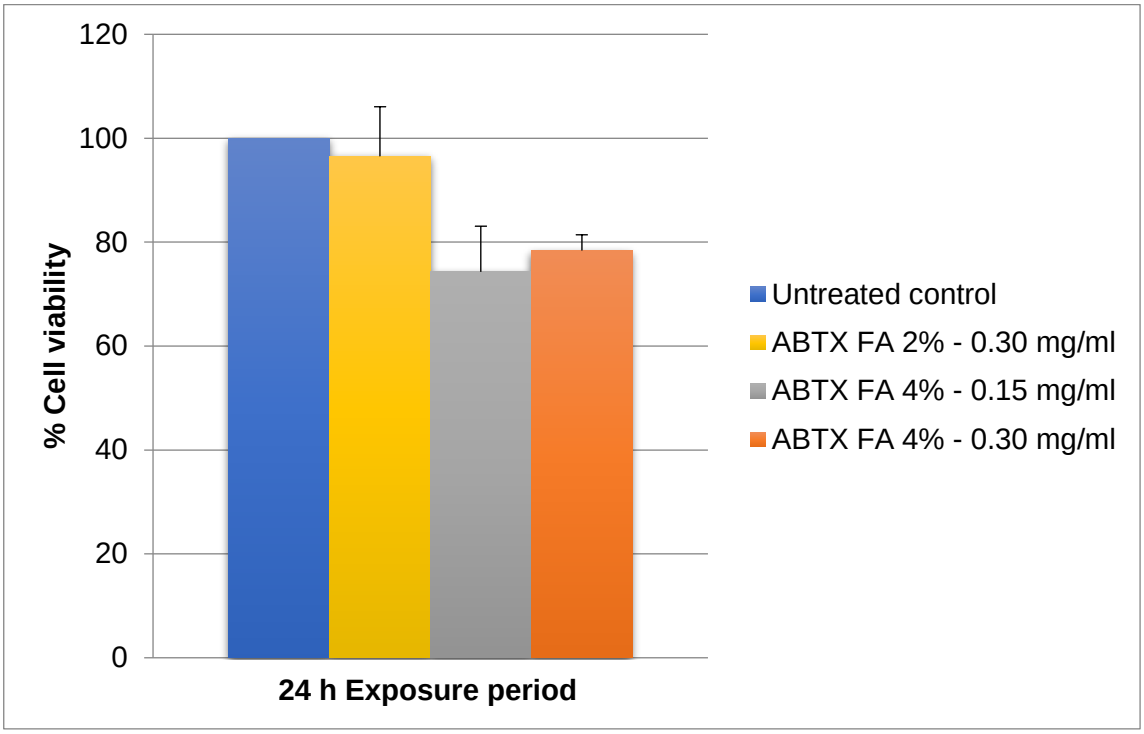

Figure B.20: Cell migration results of HaCaT cells treated with Absorbatox® bound to an organic acid (with ABTX (Absorbatox®) and FA (fulvic acid))

B.4 Conclusion

The MTT cytotoxicity assay was performed to determine if Absorbatox® bound to selected organic acids, as used in the formulations, as well as the Absorbatox® and organic acids separately posed cytotoxic effects on both HaCaT and 84BR cells. In this study, the results obtained from the MTT-assay were comparable between the two cell lines. The %cell viability of the HaCaT cells treated with the various combinations of Absorbatox® bound to an organic acid, gave similar results when compared to the 84BR cells over exposure periods of both 24 and 48 h. The organic acid treatments alone resulted in the lowest %cell viability, indicating strong cytotoxicity (< 40% cell viability) to both cell lines. In contrast, the cell lines treated with a combination of Absorbatox® bound to an organic acid, especially fulvic acid, resulted in a positive cell viability with results indicative of cellular proliferation (above 80% cell viability).

The scratch wound healing assay was performed to determine if Absorbatox® bound to selected organic acids, as used in the formulations, presented as possible wound healing agents. The scratch assay was performed on both the HaCaT and 84BR cell lines. The results obtained gave a clear indication that Absorbatox® bound to a selected organic acid proved to be advantageous for wound closure, as well as increasing the rate at which a wound would close. The results indicated that the best wound closure and fastest wound closure rate can be obtained when skin cells were treated with Absorbatox® bound to fulvic acid.

The results from both the MTT-assay, as well as the scratch assay indicated that Absorbatox® bound to selected organic acids would be beneficial to use as new wound healing agents. These combinations not only improved wound closure at an increased rate, but also provided an environment for optimal wound healing. This is a result of the unique combination of Absorbatox® bound to selected organic acids to provide a pH optimal for healing that protects against microbial infection, as well as creating a moist environment.

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APPENDIX C
FORMULATION OF A WOUND DRESSING USING ABSORBATOX® BOUND TO AN ORGANIC ACID AS ACTIVE INGREDIENT

C.1 Introduction

The modern development of wound dressings aims to facilitate the healing function of the wound environment rather than merely providing a cover. Wound dressings in the modern era are designed to focus on prevention of wound dehydration while promoting healing. Based on the type of wound, as well as the advancement of technology, there are currently many different wound dressing materials available with various properties aiming at creating an optimal healing effect (Degreef, 1998:365; Hunt, *et al,* 2000:6; Rivera & Spencer, 2007:40; Strecker-McGraw, *et al,* 2007:20).

Suitable wound dressing material must be used to aid in the healing of a specific wound. Thus, the selection of the dressing is based on its ability to maintain and provide a moist wound environment, enhance epidermal migration, promote angiogenesis, as well as synthesis of connective tissue, allow for effective gas exchange between the wound and the environment, maintain optimal tissue temperature, which allows for improved blood flow and epidermal migration, provide protection against bacterial infection, be non-adherent to the wound surface and easily removable, and/or provide debridement capabilities to improve leucocyte migration and support accumulation of enzymes and lastly, be sterile, non-toxic and non-allergenic (Dhivya, *et al.,* 2015:25).

Today, most wound dressings are developed to protect and provide moist wound healing environments. This aids in faster re-epithelialisation, collagen synthesis, as well as enhanced angiogenesis by creating hypoxia to the wound bed (hypoxia stimulates angiogenesis) as well as decrease the wound surface pH, allowing for a reduced risk of wound infection. Modern wound dressings deliver fundamental characteristics for effective healing, which provide moisture and absorb excessive fluids such as polyurethane foams. New synthetic dressings have expanded into various groups of products, which includes hydrogels, hydrocolloids, alginates, synthetic foam dressings, silicone meshes and vapour-permeable adhesive films (Jayesh, 2011:65-66; Sujatha, 2012:379).

During this study, the development of unique, new treatments for wounds such as burns and abrasions, acne and exuding wounds were formulated. Producing a variety of unique dressings aimed specifically at addressing some of the challenges in wound healing, i.e. exudate,

inflammation, infection and scarring, which was kept in mind during the formulation process. This study allowed for the development of three different types of wound dressings, which all include a unique combination of Absorbatox® with an organic acid to promote an optimal wound healing environment, which optimises moisture control and ensures protection from the risks of maceration, as well as microbial contamination (Potgieter *et al.,* 2007).

C.2 Development program for the formulation of a wound dressing

The development process for the different types of wound dressings, namely a silicone-based gel, hydrogel-based patch and a dry/sachet wound dressing consist of three phases, which include a pre-formulation, early formulation and final formulation phase. The pre-formulation phase was concluded by dermaV Pharmaceuticals (Pty) Ltd and Absorbatox (Pty) Ltd before the commencement of the study.

C.2.1 Pre-formulation

The pre-formulation phase consists of various studies that are to be conducted prior to the commencement of the final formulation development. This is done to ensure for the logical development of a stable, harmless, effective dosage form and is primarily concerned with classification of the physicochemical properties of the API. During pre-formulation studies, the final route of API administration is finalised (Walters & Brain, 2002:321).

C.2.2 Early formulation

The early formulation phase comprised of a “trial-and-error” approach. Formulations that were previously tested were used and adjusted accordingly to specific requirements.

C.2.3 Final formulation

The final formulation phase involved choosing the formulation which that best suited the API, as well as the purpose and type of formulation, which was then formulated in bulk for storage and stability testing. The stability testing of the final formulations was performed according to the International Conference on Harmonisation (ICH, 2003:5) at three different storage conditions (25 °C at 60% relative humidity (RH), 30 °C at 65% RH and 40 °C at 75% RH) for a time period of three months.

C.3 Silicone-based gel containing Absorbatox® bound to fulvic acid as active ingredient

C.3.1 Purpose and function of a silicone-based gel

A silicone gel has the ability to spread as an ultra-thin sheet (silky touch to the skin), which works for up to 24 h. It has a self-drying technology which allows for drying within 4 – 5 min (Quinn, 1987:13; Sawada & Sone, 1990:43). Silicone gels have many beneficial attributes; some of these include:

- Increase the hydration of the stratum corneum, which facilitates in the regulation of fibroblast production, as well as reduce collagen production. This results in softer and flatter scarring allowing the skin to "breathe".
- Silicone gel protects scar tissue from microbial invasion, while preventing bacterial-induced collagen production within scar tissue.
- It has the ability to modulate the expression of growth factors, fibroblast growth factor-β (FBF-β), as well as tumour growth factor-β (TGT-β). TGT-β is involved in the synthesis of collagen and fibronectin, whereas FGF-β normalises collagen synthesis in abnormal scarring and increases the level of collagenases which has the ability to breakdown excess collagen. This results in restored balance of fibrogenesis and fibrinolysis.
- Reduce itching, as well as discomfort associated with superficial abrasions.
- Easily administered onto sensitive skin or wound surface (Quinn, 1987:13; Sawada & Sone, 1990:43).

C.3.2 Formulation of a silicone-based gel

The purpose of this study was to formulate a silicone-based gel containing Absorbatox® bound to fulvic acid as the active ingredient for the effective treatment of superficial skin abrasions and burn wounds. The active ingredient chosen for the silicone-based gel was made on the results obtained from *in vitro* cytotoxicity studies, as well as wound healing studies (see Appendix B). From the results, the best active suited to the silicone-based gel was chosen.

C.3.2.1 Ingredients used in the manufacturing of a silicone-based gel

The ingredients used in the silicone gel formulation are given in Table C.1, along with the supplier and batch number of each ingredient.

Table C.1: Ingredients used in the selected silicone gel formula

Ingredient	Supplier	Batch number
Silky wax	Lycoderm Laboratories	0009021630
ST Elastomer	Lycoderm Laboratories	5410950100
Iris®	sonneborn® LLC.	601040
Jojoba oil	Lycoderm Laboratories	95831012017
Polysorbate 80	Lycoderm Laboratories	32662
Polysorbate 20	Lycoderm Laboratories	30953
Gransil ORB®	Grant Chemicals	980829A16
Absorbatox® bound to 2% fulvic acid	Absorbatox (Pty) Ltd (SA)	05/07/1672

C.3.2.2 Formula used during the manufacturing of a silicone-based gel

The formula of the silicone-based gel is given in Table C.2.

Table C.2: Formula of a silicone-based gel

Ingredient	%	Function
A:		
ST Elastomer	0.085%	Thickening agent
Silky wax	0.170%	Emulsifier
Iris®	0.204%	Emulsifier
Jojoba oil	0.136%	Emollient
Polysorbate 80	0.017%	Surfactant
Polysorbate 20	0.017%	Surfactant
B:		
Gransil ORB®	89.330%	Emulsifying elastomer base
C:		
Absorbatox® bound to 2% fulvic acid	10.040%	Active ingredient

C.3.2.3 Procedure to prepare a silicone-based gel

The procedure used to prepare the silicone-based gel was as follows:

- Add ingredients of mixture A together, heat to approximately 45 °C, and mix well using a mechanical stirrer at a speed of 1137 rpm for 15 min, until clear.
- Keep mixture A at approximately 45 °C.
- Add mixture A to mixture B with rigorous stirring. When mixture is completely mixed, add mixture C to mixture A/B.
- Continue to stir for 5 min at a speed of 752 rpm.

C.3.2.4 Outcomes of a silicone-based gel

The silicone-based gel applied easily to the skin, was not too oily and had a smooth, silky feel. The gel had a homogenous fig jam appearance with no odour (Figure C.1).

Figure C.1: Silicone-based gel formulation

C.4 Hydrogel-based patch containing Absorbatox® bound to citric acid as active ingredient

C.4.1 Purpose and function of a hydrogel-based gel

Hydrogels are formulated by crosslinking specific materials and have the ability to retain water and drug(s). Silicone gels can hold and retain wound exudate, as well as protect the wound against bacterial infection (Mohite & Adhav, 2017:82). Hydrogel-based patches allow for an increase in application time, thus resulting in prolonged delivery of active substance, as well as providing for longer intervals between dressing changes, which is desirable to prevent breaks in new epithelium. Owing to its success as an occlusive wound cover, it has the unique property to absorb exudate from wound cavities, as well as deliver drugs after being applied topically (Ladenheim *et al.,* 1996:48). Hydrogels are made of polymeric materials, which exhibit unique characteristics allowing for swelling and retention of a large volume of water with in its structure. The patch does not dissolve in water due to their polymeric backbone and cross-links between network chains. Hydrogel-based patches have a high degree of elasticity, much like that of natural tissue. The patches have a sustained and prolonged mechanism of action, decreased side-effects, as well as high patient compliance (Silna *et al.*, 2016:88).

C.4.2 Formulation of a hydrogel-based patch

The purpose of this study was to formulate a hydrogel-based patch containing Absorbatox® bound to citric acid as the active ingredient for the effective treatment of acne or similar lesions. The active ingredient chosen for the hydrogel-based patch was made on the results obtained from *in*

vitro cytotoxicity studies, as well as wound healing studies. From the results, the best active suited to the hydrogel-based patch was chosen.

C.4.2.1 Ingredients used in the manufacturing of a hydrogel-based patch

The ingredients used to formulate the hydrogel-based patch are given in Table C.3, along with the supplier and batch number of each ingredient.

Table C.3: Ingredients used in the selected hydrogel-based patch formula

Ingredient	Supplier	Batch number
Polyethylene	BASF	13/A457/23
Polyester	BASF	15/78/A45
Glycerine	BASF	25678FG7
Urethane	BASF	CF3478
Absorbatox® bound to 4% citric acid	Absorbatox (Pty) Ltd (SA)	05/07/1667

C.4.2.2 Formula used during the manufacturing of a hydrogel-based patch

The formula of the hydrogel-based patch is given in Table C.4.

Table C.4: Formula of a hydrogel-based patch

Ingredient	%	Function
Polyethylene	25%	Binder
Polyester	19%	Binder
Glycerine	35%	Humectant
Urethane	20%	Thickening agent
Absorbatox® bound to 4% citric acid	1%	Active ingredient

C.4.2.3 Procedure to prepare a hydrogel-based patch

The procedure used to prepare the hydrogel-based patch was as follows:

- Hydrogel-based patches were formulated by the cross-linking of urethane with glycerine and then using the “knife-over-roll” method.
- Patches were formulated to be 1 mm in thickness and buffered at a pH of 5.5.
- Formulation of the hydrogel-based patches was done at Electro Spyres Medical (Pty) Ltd manufacturing warehouse in Johannesburg, South Africa, under the supervision and guidance of Mr P Spyres.
- The hydrogel was then coated with 1% of the active ingredient.

- All processes and procedures of formulation are property of Electro Spyres Medical (Pty) Ltd.

C.4.2.4 Outcomes of a hydrogel-based patch

The formulated hydrogel-based patch adhered easily to skin, and has a smooth feel. It was slightly sticky to the touch, but was overall acceptable as a wound dressing. The colour was transparent and jelly-like. The adherent end of the patch comprised of a layer containing the active (Absorbatox® bound to citric acid), which gave it a sea sand-like appearance (Figure C.2).

Figure C.2: Hydrogel-based patch formulation

C.5 Dry dressing (sachet)

C.5.1 Purpose and function of a dry wound dressing (sachet)

Dry powder dressings in a sachet as a type of wound dressing are able to absorb wound exudate and associated bacteria and irreversibly bind to material that creates capillary suction forces, e.g. Cerdak™ (Kesavan, 2008:50). The latter dry ceramic wound dressing is able to improve the healing process by providing effective infection control by preventing microbial colonisation, as well as decreasing the risk of scar formation. The aim of the dry dressing is to produce an adequate, effective exudate, ad- and absorbing, wound dressing that maintains a moist environment at the surface of the wound and provides an environment for successful healing. The dry dressing is not frequently removed, as this could cause damage to the wound bed and increase the risk of contamination (Hampton, 2004). Lower leg ulcers, more often than not, produce large amounts of exudate due to their larger surface area, which may decrease growth factors available for cell proliferation and increase proteolytic activity (Muldoon, 2013; WUWHS, 2007).

C.5.2 Formulation of a dry wound dressing

The purpose of this study was to formulate a dry/sachet wound dressing similar to the ceramic dressing, which contains Absorbatox® bound to fulvic acid as the active ingredient for the effective treatment of exuding wounds such as diabetic leg ulcers. The active ingredient chosen for the dry/sachet wound dressing was made on the results obtained from *in vitro* cytotoxicity studies, as well as wound healing studies. From the results, the best active suited to the dry/sachet wound dressing was chosen.

C.5.2.1 Ingredients used in the manufacturing of a dry wound dressing

The ingredients used in the dry/sachet wound dressing formulation are given in Table C.5, along with the supplier and batch number of each ingredient.

Table C.5: Ingredients used in the selected dry/sachet wound dressing formula

Ingredient	Supplier	Batch number
Protofix®	Electro Spyres Medical (Pty) Ltd	12/12/1990
Reverse funnel	Electro Spyres Medical (Pty) Ltd	12/12/1876
Viscose	Electro Spyres Medical (Pty) Ltd	12/12/1776
Absorbatox® bound to 4% fulvic acid	Absorbatox (Pty) Ltd (SA)	05/07/1669

C.5.2.2 Formula used during the manufacturing of a dry wound dressing

The formula of the dry/sachet wound dressing is given in Table C.6.

Table C.6: Formula of a dry/sachet wound dressing

Ingredient	%	Function
Protofix®	15%	Adhesive layer
Reverse funnel	15%	Allows one-way absorption
Viscose	20%	Absorbent material
Absorbatox® bound to 4% fulvic acid	50%	Active ingredient

C.5.2.3 Procedure to prepare a dry wound dressing

The procedure used to prepare the dry (sachet) wound dressing was as follows:

- Treatment sachets were stitched from the viscose material with a size of: 35 x 40 mm (1 400 mm^2) with a 5 mm seam allowance. The stitched sachet was then filled with 2.5 g Absorbatox® bound to 4% fulvic acid.

- As the reverse funnel material is difficult to sew, the layers were adhered to the Protofix® layer (80 x 120 mm), which is sticky on one side. The reverse funnel layer should be cut larger to encase the sachet and to adhere to the Protofix® layer (50 x 50 mm) as seen in Figure C.3
- The layers (from wound surface outward), is as follows:
    - Reverse funnel
    - Viscose
    - Absorbatox
    - Viscose
    - Protofix®

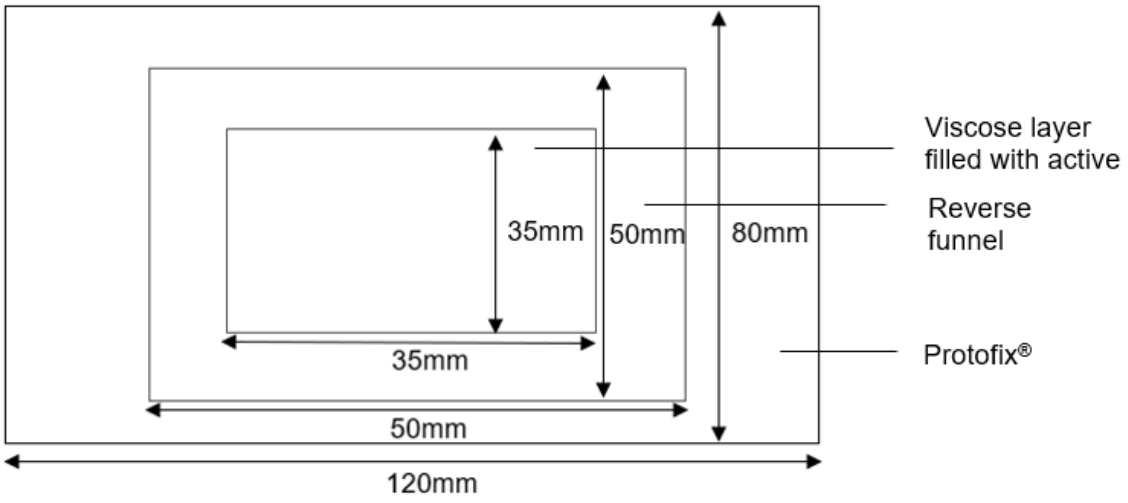

Figure C.3: Dimension diagram of dry/sachet dressing

C.5.2.4 Outcomes of a dry wound dressing

The dry/sachet wound dressing produced applied easily to skin, had a smooth, silky feel and did not become solid (cake) when in contact with Ringer's lactate, which is much like wound exudate. The dry dressing was formulated to have a clean, clinical appearance, like that of modern conventional wound dressings, as seen in Figure C.4.

Figure C.4: Dry/sachet wound dressing formulation

C.6 Conclusion

Three different wound dressing products were formulated, namely:

- a silicone-based gel containing Absorbatox® bound to 2% fulvic acid;
- a hydrogel-based patch containing Absorbatox® bound to 4% citric acid, and
- a dry/sachet wound dressing containing Absorbatox® bound to 4% fulvic acid

The final formulations of each product were prepared in adequate amounts for the three-month stability testing. The formulations were observed and inspected for appearance, as well as texture. All three formulations were found to be suitable before storage at three different storage conditions used throughout stability testing. The accelerated stability testing of each formulation will be discussed in Appendix D.

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APPENDIX D

STABILITY TESTING OF A SILICONE-BASED GEL, HYDROGEL-BASED PATCH AND A DRY/SACHET WOUND DRESSING FORMULATION

D.1 Introduction

The purpose of stability testing is to obtain presentable data and observe how the quality of a drug substance and/or drug product may change under the influence of a variety of different environmental factors such as humidity and temperature over time (ICH, 2003:5).

Possible transformations in the physicochemical properties of a specific formulation form part of the first phase of stability testing. These transformations can be observed according to the following characteristics:

- Chemical changes: colour change, colour fading, fragrance change, staining, as well as crystallisation.
- Physical changes: separation, sedimentation, aggregation, blooming, sweating, gelling, unevenness, evaporation, solidification, softening, as well as cracking (Mitsui, 1997:12).

According to the ICH, an intermediate stability study must be conducted with a minimum testing frequency of four time points, which includes the initial and final time of testing (ICH, 2003:12). To accurately perform the intermediate stability testing, formulations were stored at 25°C/60% RH (relative humidity), 30°C/65% RH and 40°C/75% RH.

The South African Health Products Regulatory Authority (SAHPRA), previously known as the Medicines Control Council (MCC), describes a significant change in a drug product as:

- a 5% change, form its initial value, in potency of the active ingredient assay;
- any degradation products, which exceed the acceptance criterion;
- failure to meet the accepted criteria for product appearance, which includes colour, phase separation, caking as well as hardness, and
- failure to meet the accepted pH criteria (MCC, 2012:14).

D.2 Methods of evaluation

D.2.1 API identification

API identification was performed using two different identification methods, namely FTIR, as well as thin layer chromatography (TLC). The methods to identify the API were dependent on the type of formulation evaluated.

FTIR spectroscopy is a technique used to obtain information on the structure and molecular conformation of a specific sample by measuring the vibration modes of bonded atoms. FTIR is a standard technique for the characterisation of compounds in the current context of solid materials (Bernstein, 2002; Rodriguez-Spong *et al.,* 2004).

Infrared (IR)-spectra was recorded on a Bruker ALPHA Platinum spectrophotometer (Bruker, Billerica, USA) across a range of 400 – 4 000 cm^{-1}. The ALPHA Platinum Module has the attenuated total reflection (ATR) accessory designed for minimal operator induced variations, sampling with no preparation and excellent reproducibility. OPUS software was used to analyse the data.

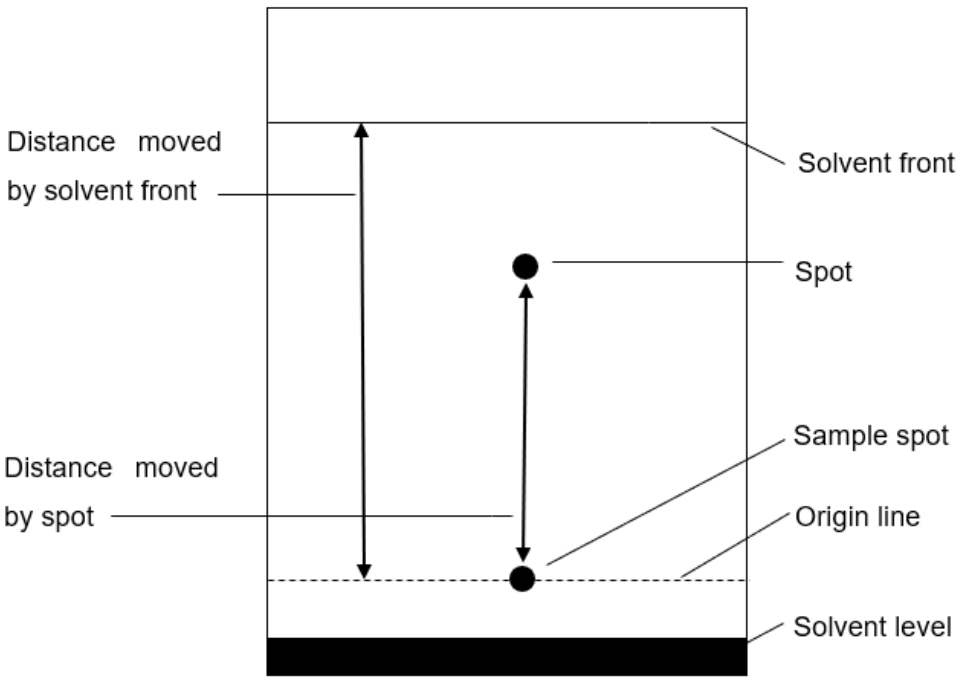


Figure D.1: Depiction of a standard TLC plate (Adapted from Hamilton & Hamilton, 1987).

TLC is classified as a flat-bed method, which is based on the principle to differentiate between different materials by detecting and measuring differences in distances moved by materials in a system. The system contains two separate phases, one of which is static and the other a mobile phase. Where the static phase is a solid absorbent porous material, normally a glass or

aluminium plate coated with silica gel and the mobile phase as the liquid (Gasparič & Churáček, 1978). The difference in absorption between various substances is a result of variation within the affinities of the substances analysed with the mobile phase and static phase.

Results obtained are seen as spots, as illustrated in Figure D.1 when the correct method of detection is used, either chemical; physical or biological. Each substance analysed will move a specific distance that is unique to that material and can then be used as a means of differentiating between, or identifying specific substances within in compound (Gasparič & Churáček, 1978).

The retention factor (R_f) is a quantitative value calculated, using Equation D.1, to describe the variation in the distance moved by a specific substance on the chromatogram.

$$R_f = \frac{\text{Distance moved by spot}}{\text{Distance moved by solvent front}} \qquad \textbf{Equation D.1}$$

The visualisation method used during this study was to expose the developed TLC plate to iodine (I_2) vapour. This is considered a semi-destructive method as complexation is reversible, and the iodine will be able to evaporate from the TLC plate, leaving the original compound behind. Theoretically it is thus possible to use another visualisation method once colouration fades; however, it is also possible that the compound may have also evaporated (Nichols, 2019). This method requires the development of an "iodine chamber", which is created by adding a few iodine crystals to a TLC chamber. When the TLC plate was developed, it was placed in the chamber and capped, the iodine then sublimes and reacts with the compounds on the plate forming yellow-brown spots. Colouration occurs as a result of the iodine forming coloured complexes with the organic compounds (Nichols, 2019). Samples were dissolved in ethanol and plotted on pre-coated aluminium TLC-sheets ALUGRAM® SIL G (Macherey-Nagel, Germany). The mobile phase consisted of acetonitrile and methanol in a ratio of 3:2 as indicated in Table D.1.

Table D.1: Excipients used in the mobile phase of TLC plate development

Ingredient	Volume	Supplier	Batch number
Acetonitrile	60 ml	ACE (Associated chemical enterprises)	8805
Methanol	40ml	ACE (Associated chemical enterprises)	32447

D.2.2 pH

A Mettler Toledo pH meter (Schwerenbach, Switzerland) equipped with a glass Mettler Toledo Inlab® 410 electrode was used to measure the pH of the silicone gel formulations. The pH meter was calibrated before the commencement of pH measurements with Mettler Toledo buffer solutions at pH 4.01, 7.00 and 10.01 at 25°C. The pH measurements of each formulation were performed in triplicate.

D.2.3 Viscosity

The definition of viscosity is the resistance to flow, which is caused by internal friction (Marriott, 2002:41; Brookfield, 1998:2). A means of classification of fluids and semisolids is determined by rheology, which is the science of the flow of matter (Marriott, 2002:41).

A viscometer is an instrument used to determine the viscosity of different fluids, semi-solids, as well as solid-suspensions. The instrument measures viscosity by determining the resistance of the formulation to a rotating spindle, which is immersed in the sample. A Brookfield Viscometer (Stoughton, Massachusetts, USA) was used to measure the viscosity of the formulations at different conditions.

Sample temperature was controlled by a Brookfield temperature controller which circulates water at 25°C in a water bath. The measurements were taken by immersing appropriate Helipath spindles (Stoughton, Massachusetts, USA) into the formulation at a specified rate. The rate at which the spindle turns is specified and measured in rpm. An LV Spindle (LV-4) was used to move the viscometer up and down in the different gel formulations. The viscosity of the formulations was determined at every 10 sec for 2 min at 10 rpm. The average viscosity was determined by using the readings obtained.

D.2.4 Mass loss

To determine if mass loss occurred, formulations were weighed on a calibrated Mettler Toledo balance (Schwerenbach, Switzerland). The mass of the formulations at each storage condition was determined in triplicate. The mass of each formulation was determined at the specified time intervals, by subtracting the original mass, the total mass loss was determined.

D.2.5 Particle size

The size and characteristics of particles within materials influence the stability, chemical reactivity, opacity, as well as the strength of the material.

In order to wet the samples of the formulations (stored at the different conditions), the sample (0.5 g) was mixed with approximately 3.0 ml distilled water to a uniform dispersion. After being made up with approximately 4.5 ml distilled water, the mixtures were injected into a Malvern Mastersizer 2000 (Worcestershire, United Kingdom). A Hydro 2000 SM is used to serve as the interface between the sample dispersion accessory and the optical unit. Triplicate measurements were analysed of each sample at a speed of 1 500 rpm.

The statistics of the distribution were then calculated from the results using the derived diameters D [m,n] – an international method of defining the mean, as well as other moments of particle size.

The results, as seen in Section D.3.1.5, indicates D (0.5), which refers to the size in microns (µm) where 50% of the sample is smaller and 50% of the sample is larger. This value indicates the Mass Median Diameter (MMD) (Malvern, 2000:6.3).

D.2.6 Colour and visual appearance

Visual assessment was made of each stored batch of the formulations. The visual appearance of formulation at each condition was compared to the initial colour and appearance by taking photographs, using a Canon E240 digital camera (Gauteng, SA).

D.2.7 Free swell capacity

The free swell capacity of absorbent wound dressings was performed on the dry/sachet. The test was performed by weighing the wound dressing (stored at the different conditions) using a calibrated Mettler Toledo balance (Schwerenbach, Switzerland). The dressings were then immersed in a tank containing Ringers lactate (SABAX Ringer-lactate, Adcock Ingram, Johannesburg) at 37°C where they remained for 30 min. The dressings were removed from the solution and transferred onto a grid where they could drain for 30 sec before re-weighing. All dressings were tested in triplicate and the mean absorbency was calculated (Cutting & Westgate, 2012:23). The dressings were left in the Ringers lactate for an additional 5 days after the experiment. The dressings were then re-weighed and the additional fluid absorbency was calculated.

D.3 Results and discussion of the silicone-based gel formulation

The following parameters were determined on months 0, 1, 2 and 3 for the silicone-based gel:

- API identification
- pH
- Viscosity
- Mass loss
- Particle size
- Visual appearance assessment

D.3.1 API identification

The presence of the active ingredient, i.e. Absorbatox® bound to 2% fulvic acid, within the silicone-based gel formulation was performed using the TLC method of identification. The gel was measured initially and then also at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.1. Results obtained are given in Table D.2 and Figure D.2.

Table D.2: R_f-values of API identified using TLC within the silicone gel formulation

Initial	Month 1	Month 2	Month 3
25°C/60% RH			
0.43	0.40	0.41	0.44
30°C/65% RH			
0.43	0.40	0.41	0.44
40°C/75% RH			
0.43	0.40	0.41	0.44

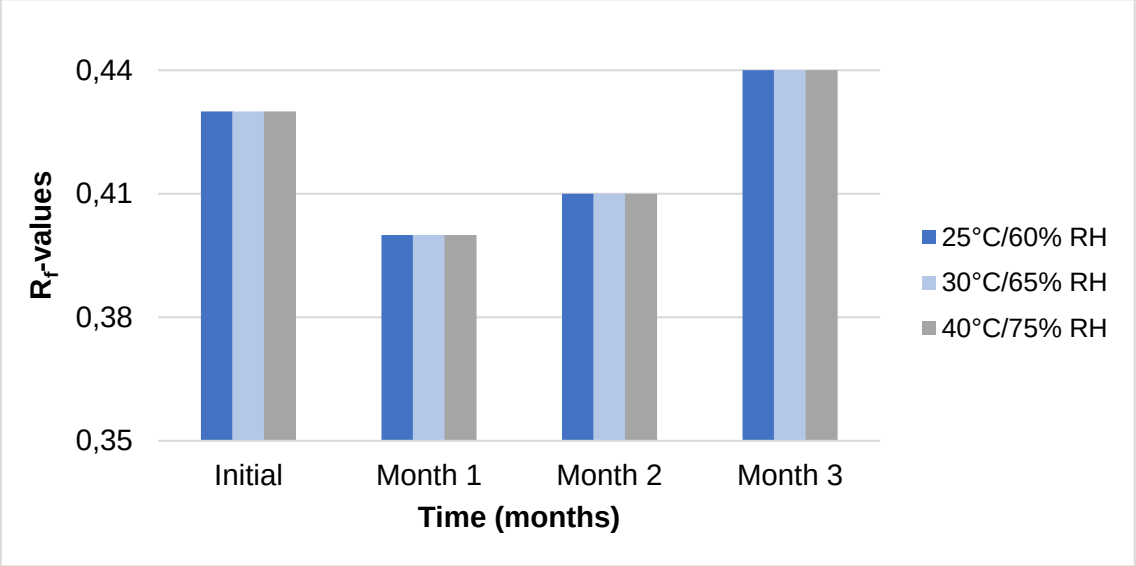


Figure D.2: Change in the R_f-values of the API identified using TLC within the silicone gel formulation.

The R_f-values of the API within the silicone-based gel did not show any significant changes throughout the 3-month testing period. The values recorded all showed a positive identification of the API within the formulation, which indicates the API remains in the formulation over a long period of time.

D.3.2 pH

The pH of the silicone-based gel was measured at month 0 (initial). The gel was then also measured at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.1.2. The pH, as well as the standard deviation (SD) values obtained during measurement are shown in Table D.3 and Figure D.3.

Table D.3: pH of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	5.923	4.328	4.571	5.928
2	5.923	4.564	4.546	5.561
3	5.960	4.572	4.556	5.114
Average ± SD	5.935 ± 0.021	4.488 ± 0.139	4.558 ± 0.013	5.534 ± 0.408
30°C/65% RH				
1	5.923	4.720	4.885	5.541
2	5.923	4.751	4.789	5.999
3	5.960	4.734	4.745	5.206
Average ± SD	5.935 ± 0.021	4.735 ± 0.016	4.806 ± 0.072	5.582 ± 0.399
40°C/75% RH				
1	5.923	4.636	4.578	5.610
2	5.923	4.641	4.503	4.980
3	5.960	4.638	4.493	5.008
Average ± SD	5.935 ± 0.021	4.638 ± 0.003	4.524 ± 0.046	5.199 ± 0.356

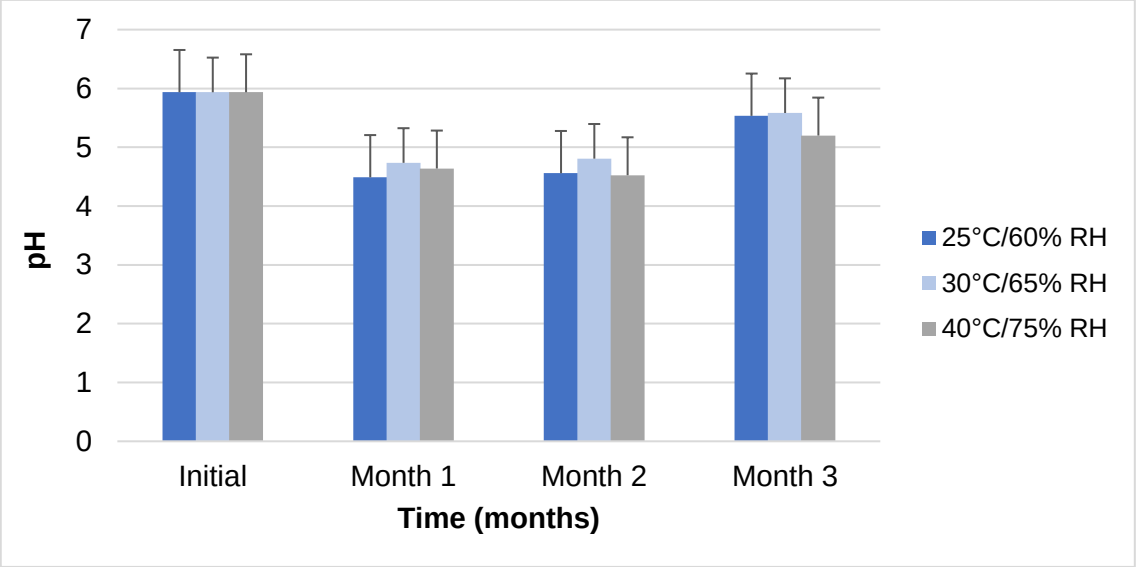


Figure D.3: Change in pH of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period.

The pH of the silicone-based gel formulation containing Absorbatox® with 2% fulvic acid did not remain constant over the 3-month period. The largest decrease in pH was observed between months 0 and 2, where the pH of the gel decreased in all three the different storage conditions. The pH values indicated a 22%, 19% and 23% decrease for the gel stored at 40°C/75% RH, 30°C/65% RH and 25°C/60% RH, respectively. Between months 2 and 3, the pH increased to values similar to the initial values measured. The reason for the decrease in pH can be attributed to the acid content of the gel, i.e. fulvic acid, which may cause for the sudden drop in pH where Absorbatox® has the ability to neutralise the acid, bringing the formulation pH back to its original

value. Therefore, the prediction can be made that the formulation will remain stable after a 3-month storage period.

D.3.3 Viscosity

The viscosity of the silicone-based gel was measured at month 0 (initial). Subsequently, the gel was measured at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.3. Viscosity readings, including SD and % relative standard deviation, obtained are indicated in Table D.4 and Figure D.4.

Table D.4: Viscosity (cP) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
Average	36710	54740	53000	56905
SD	430.739	229.307	405.597	605.137
%RSD	1.076	0.419	0.765	1.063
30°C/65% RH				
Average	36710	57585	55780	59010
SD	430.739	392.300	503.515	430.771
%RSD	1.076	0.681	0.903	0.730
40°C/75% RH				
Average	36710	58050	51010	57220
SD	430.739	1021.923	263.197	29.542
%RSD	1.076	1.760	0.516	0.052

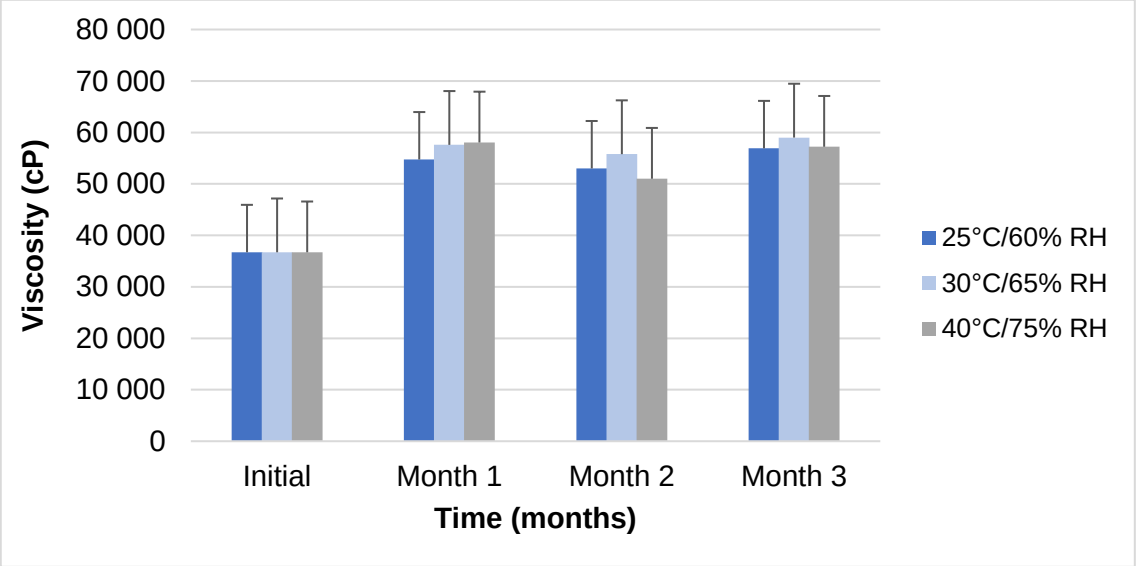


Figure D.4: Change in viscosity (cP) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period.

The viscosity of the silicone-based gel did not change radically over the three-month period. The gel showed an increase in viscosity from 36 710 cP (month 0) to 56 905 cP (month 3) at

25°C/60% RH, to 59 010 cP (month 3) at 30°C/65% RH and to 57 220 cP (month 3) at 40°C/75% RH.

An increase in viscosity can be a result of the settling process of the gel before the formulation reaches a state of equilibrium, as well as an average loss in moisture during stability testing over the 3-month period, which results in a higher viscosity value. The viscosity values obtained from months 1 to 3 showed no dramatic change and are similar for all three storage conditions, which indicates that the gel will be stable for a 3-month storage period.

D.3.4 Mass loss

The mass of the silicone-based gel was weighed at month 0 (initial). The gel was then also weighed at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.1.4. Table D.5 and Figure D.5 show the mass results obtained for the gel formulation over the 3-month period.

Table D.5: Mass (g) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	25.588	25.673	25.669	25.663
2	25.588	25.673	25.669	25.663
3	25.588	25.673	25.669	25.663
Average ± SD	25.588 ± 0.00	25.673 ± 0.00	25.669 ± 0.00	25.663 ± 0.00
30°C/65% RH				
1	25.083	24.982	24.971	24.963
2	25.083	24.982	24.971	24.963
3	25.083	24.982	24.971	24.963
Average ± SD	25.083 ± 0.00	24.982 ± 0.00	24.971 ± 0.00	24.963 ± 0.00
40°C/75% RH				
1	25.755	25.438	25.444	25.417
2	25.755	25.438	25.444	25.417
3	25.755	25.438	25.444	25.417
Average ± SD	25.755 ± 0.00	25.438 ± 0.00	25.444 ± 0.00	25.417 ± 0.00

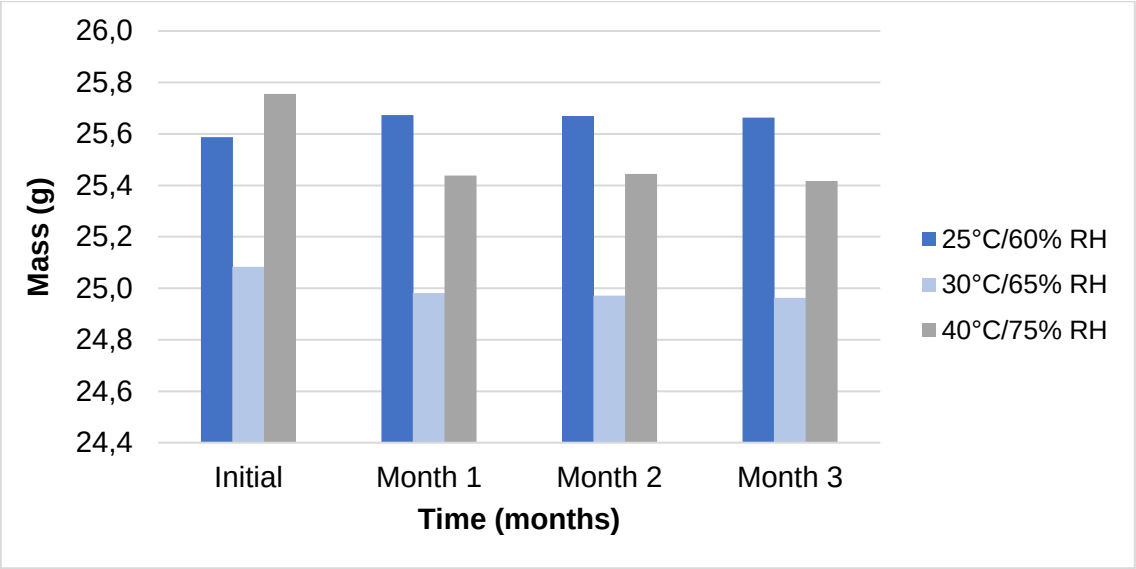


Figure D.5: Change in mass (g) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid over a 3-month period.

The loss in mass of the silicone-based gel remained relatively stable over the 3-month storage period with no significant change in mass. The largest decrease in mass is observed at storage conditions of 40°C/75% RH with a decrease of approximately 5.0%. The formulation stored at 30°C/65% RH confirmed a decrease in mass of approximately 0.5%, while the gel stored at 25°C/60% RH showed a slight increase in mass of approximately 0.3%. The results obtained may be result of insufficient container sealing, which allowed for evaporation as well moisture escaping the containers during storage periods.

D.3.5 Particle size

The particle size of the silicone-based gel was measured at month 0 (initial). The gel was then also measured at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.5.

Figure D.6 illustrates an overview of the changes in particle size between months 0 and 3. The results indicate D (0.5), as shown in Table D.6, which refers to the size in microns (µm) at which 50% of the sample is smaller and 50% of the sample is larger. The value is known as the MMD, as discussed in Section D.2.5.

Table D.6: Particle size (µm) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
D (0.5)	216.353	217.491	273.075	290.035
30°C/65% RH				
D (0.5)	216.353	250.814	217.534	273.776
40°C/75% RH				
D (0.5)	216.353	230.299	213.173	274.776

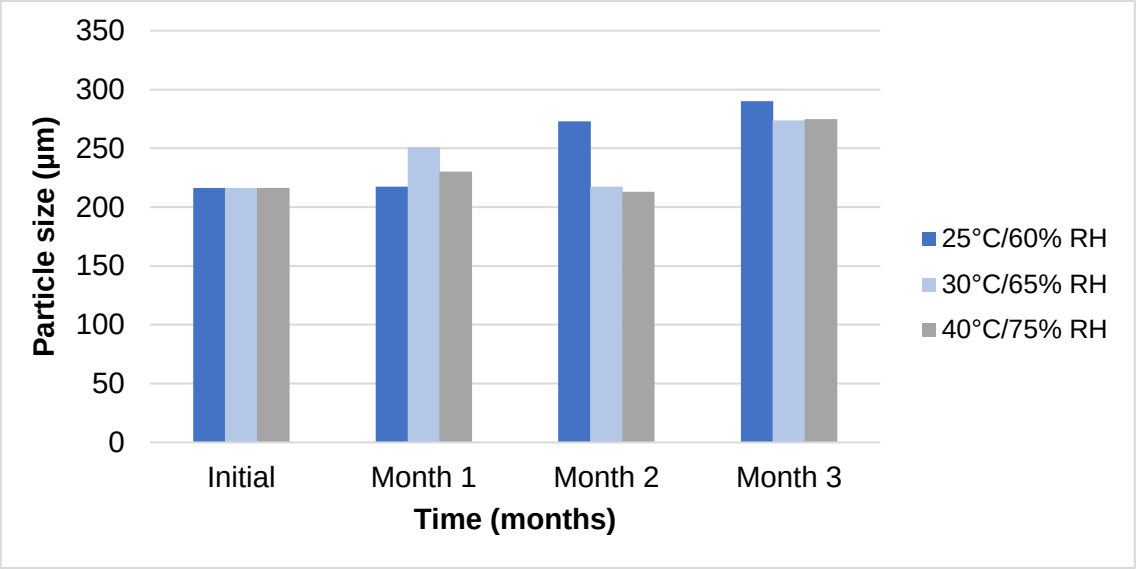


Figure D.6: Change in the average particle size (µm) of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid.

The average particle size of the Absorbatox® bound to 2% fulvic acid within the silicon-based gel increased after the 3-month period in all three stability-controlled conditions. The gel stored at 25°C/60% RH increased from an initial average particle size of 216.353 µm to a final average particle size of 290.035 µm after a 3-month period. This is an increase of approximately 74 µm. The gel stored at 30°C/65% RH demonstrated an increase of approximately 57 µm after three months of stability testing, while the formulation stored at 40°C/75% RH confirmed a final increase in average particle size of approximately 58 µm.

The results obtained from the particle size measurements confirmed the particles within the gel formulation demonstrate adherence to one another to form aggregates of successively increasing size. This phenomenon is referred to as flocculation and may occur as a result of the particles under the influence of gravity.

As the Absorbatox® containing 2% fulvic acid particles are not intended to dissolve within the gel formulation but rather remain suspended therein, the aggregation of particles does not render the

product unstable. The particles can once again decreased in size when the gel is in use with the use of mechanical stirring or rubbing onto the patient's skin. This will not cause the silicone-based gel to become less effective as the API, namely Absorbatox® containing 2% fulvic acid is bound together and is merely incorporated into the silicone-based gel as a carrier device for better patient compliance and more effective use of the product.

D.3.6 Colour and visual appearance

A visual assessment of the silicon-based gel formulation was performed on the initial gel, as well as at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.6. Indicated in Table D.7, the change in colour/visual appearance of the silicone-based gel during the 3-month period.

Table D.7: Change in colour/visual appearance of the silicone-based gel containing Absorbatox® bound to 2% fulvic acid

Initial	Month 1	*Month 2*	Month 3
25°C/60% RH			
			
30°C/65% RH			
			
40°C/75% RH			
			

The overall colour of the silicone-based gel formulation did not change over the 3-month stability testing period. A slight colour change is seen between month 0 and 1, this may be attributed to

the acid present in the formulation. No texture difference was observed within any of the formulations.

D.4 Results and discussion of the hydrogel-based patch formulation

The following parameters were determined on months 0, 1, 2 and 3 for the hydrogel-based patch:

- API identification
- Mass loss
- Colour and visual appearance

D.4.1 API identification

The API identification within the hydrogel formulation was performed using the TLC method of identification. The presence of the active ingredient of the hydrogel-based patch formulation was performed on the initial patch, as well as at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.1. Indicated in Table D.8 and Figure D.7 is the R_f-values obtained from the hydrogel-based patch formulation.

Table D.8: R_f-values of API identified using TLC within the hydrogel-based formulation containing Absorbatox® bound to 4% citric acid

Initial	Month 1	Month 2	Month 3
25°C/60% RH			
0.42	0.41	0.42	0.42
30°C/65% RH			
0.42	0.41	0.41	0.42
40°C/75% RH			
0.42	0.41	0.43	0.42

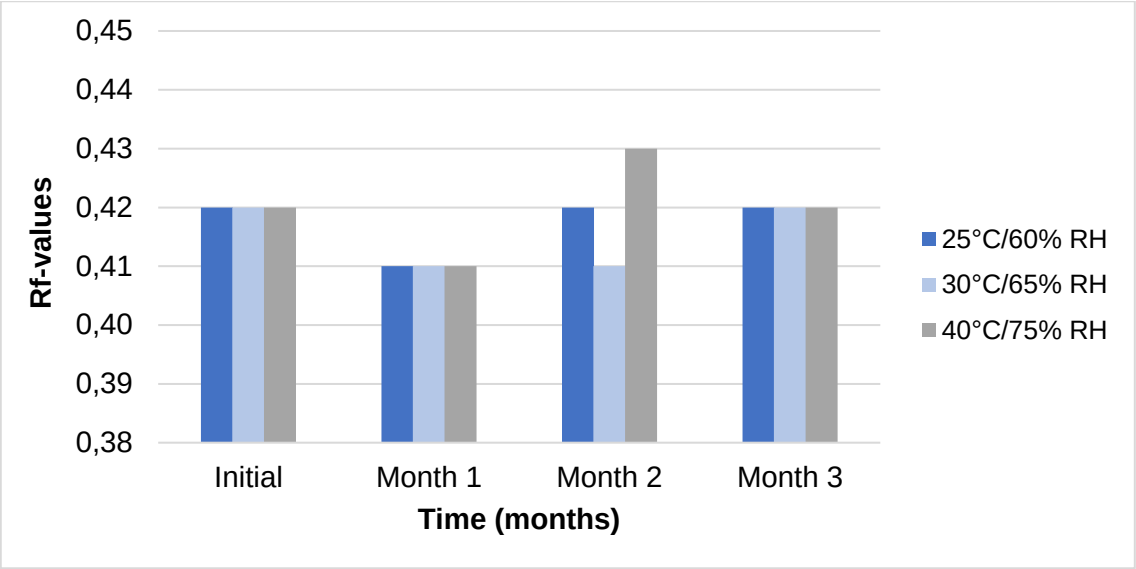


Figure D.7: Change in the R_f-values of the API identified using TLC within the hydrogel-based patch formulation.

The R_f-values of the API within the hydrogel-based patch did not show any large changes throughout the 3-month testing period. The values recorded all showed a positive identification of the API within the formulation which indicates the API remains in the formulation over a long period of time.

D.4.2 Mass loss

The mass of the hydrogel-based patch was weighed at month 0 (initial). The gel was then also weighed at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.4. The mass results obtained from the hydrogel-based patch are given in Table D.9 and Figure D.8.

Table D.9: Mass (g) of the hydrogel-based patch containing Absorbatox® bound to 4% citric acid

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	1.856	1.974	1.885	1.916
2	1.856	1.974	1.885	1.916
3	1.856	1.974	1.885	1.916
Average ± SD	1.856 ± 0.00	1.974 ± 0.00	1.885 ± 0.00	1.916 ± 0.00
30°C/65% RH				
1	1.942	1.969	1.980	1.995
2	1.942	1.969	1.980	1.995
3	1.942	1.969	1.980	1.995
Average ± SD	1.942 ± 0.00	1.969 ± 0.00	1.980 ± 0.00	1.995 ± 0.00
40°C/75% RH				
1	1.990	2.075	1.990	2.133
2	1.990	2.075	1.990	2.133
3	1.990	2.075	1.990	2.133
Average ± SD	1.990 ± 0.00	2.075 ± 0.00	1.990 ± 0.00	2.133 ± 0.00

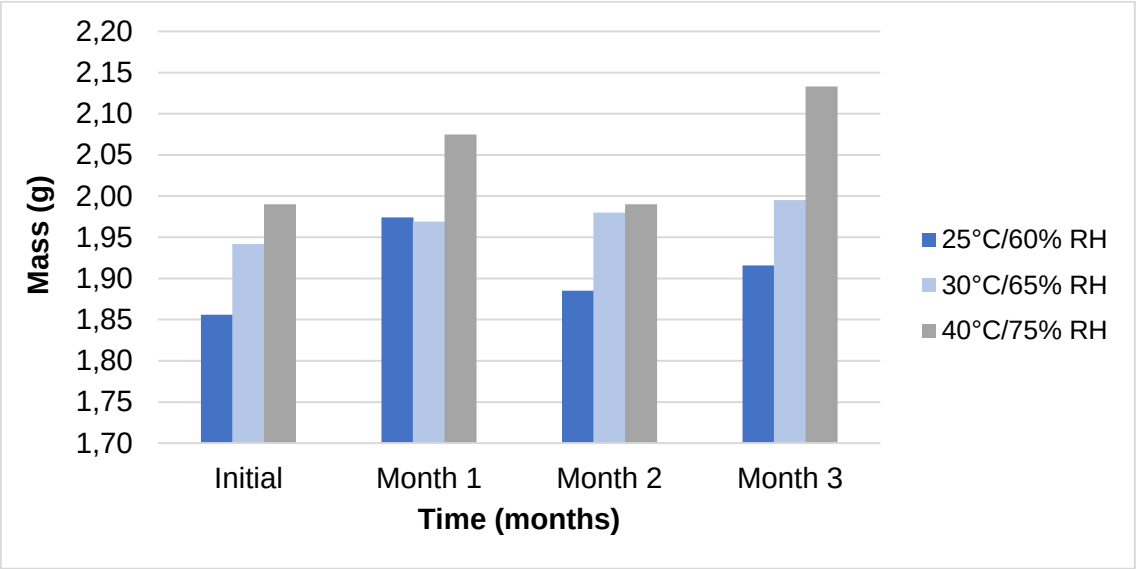


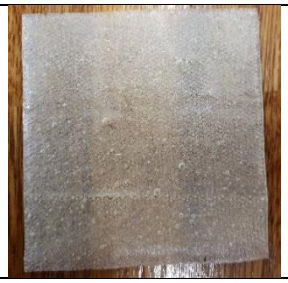
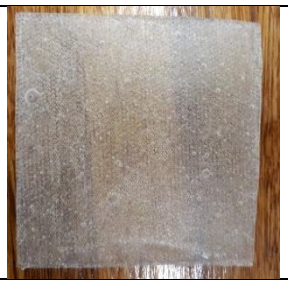
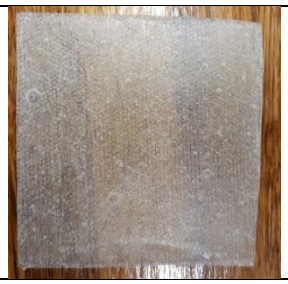
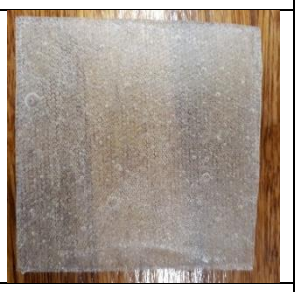
Figure D.8: Change in mass (g) of the hydrogel-based patch containing Absorbatox® bound to 4% citric acid over a 3-month period.

The mass of the hydrogel-based patch showed a slight increase over the 3-month storage period with no significant change in mass. The largest increase in mass was observed at storage conditions of 40°C/75% RH with an increase of approximately 7%. The patches stored at 30°C/65% RH and 25°C/60% RH both showed a slight increase in mass of approximately 3%. The aforementioned may be due to the insufficient sealing of the container, which allowed for swelling and increase in moisture content of the formulations during storage periods.

D.4.3 Colour and visual appearance

A visual assessment of the hydrogel-based patch formulation was performed on the initial patch, as well as at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.6. The change in colour/visual appearance of the hydrogel-based patch formulation during the 3-month period is indicated Table D.10.

Table D.10: Change in colour/visual appearance of the hydrogel-based patch containing Absorbatox® bound to 4% citric acid

Initial	Month 1	Month 2	Month 3
25°C/60% RH			
			
30°C/65% RH			
			
40°C/75% RH			
			

The visual appearance of the hydrogel-based patch formulation did not change over the 3-month stability testing period. No texture difference was observed within any of the formulations.

D.5 Results and discussion of the dry dressing (sachet) formulation

The following parameters were determined on months 0, 1, 2 and 3 for the powder dressing (sachet):

- API identification

- Mass loss
- Colour and visual appearance
- Free swell capacity

D.5.1 API identification

The presence of the active ingredient, i.e. Absorbatox® bound to 4% fulvic acid, within the dry (sachet) formulation was performed using FTIR. The dressing was measured initially and then also at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.1.

Table D.11 indicates the main absorptions of the IR spectrum of Absorbatox® raw material in comparison with Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid, whereas Table D.12 indicates the main absorptions (wavenumber (cm^{-1})) of the IR spectrum of Absorbatox® bound to 4% fulvic acid over the 3-month testing period.

Table D.11: Main absorptions of the IR spectrum of Absorbatox® raw material in comparison with Absorbatox® bound to 4% fulvic acid and the physical mix of Absorbatox® and 4% fulvic acid

	Wavenumber (cm^{-1})
Absorbatox®	1010 454
Fulvic acid	1561 1376 1182 1095 1034 604 525 467
Absorbatox® bound to 4% fulvic acid	1011 459
Physical mix of Absorbatox® and 4% fulvic acid	1026 453

Table D.11 indicates that there are no large differences in the absorption bands of the Absorbatox® bound to 4% fulvic acid compared to the physical mix of Absorbatox® and 4% fulvic acid.

Table D.12: Main absorptions (wavenumber (cm^{-1})) of the IR spectrum of Absorbatox® bound to 4% fulvic acid

Initial	Month 1	Month 2	Month 3
25°C/60% RH			
1011 459	1007 462	1008 463	1012 459
30°C/65% RH			
1011 459	1002 460	1008 457	1003 461
40°C/75% RH			
1011 459	1001 459	1011 459	1003 462

D.5.2 Mass loss

The mass of the dry (sachet) dressing formulation was weighed at month 0 (initial). The sachet was then also weighed at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.4. Table D.13 indicates the mass of the dry (sachet) dressing over the 3-month testing period. Figure D.9 illustrates the mass results.

Table D.13: Mass (g) of the dry (sachet) formulation containing Absorbatox® bound to 4% fulvic acid

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	4.035	4.505	4.504	4.503
2	4.035	4.505	4.504	4.503
3	4.035	4.505	4.504	4.503
Average ± SD	4.035 ± 0.00	4.505 ± 0.00	4.504 ± 0.00	4.503 ± 0.00
30°C/65% RH				
1	4.388	5.021	5.019	5.021
2	4.388	5.021	5.019	5.021
3	4.388	5.021	5.019	5.021
Average ± SD	4.388 ± 0.00	5.021 ± 0.00	5.019 ± 0.00	5.021 ± 0.00
40°C/75% RH				
1	4.436	4.746	4.700	4.587
2	4.436	4.746	4.700	4.587
3	4.436	4.746	4.700	4.587
Average ± SD	4.436 ± 0.00	4.746 ± 0.00	4.700 ± 0.00	4.587 ± 0.00

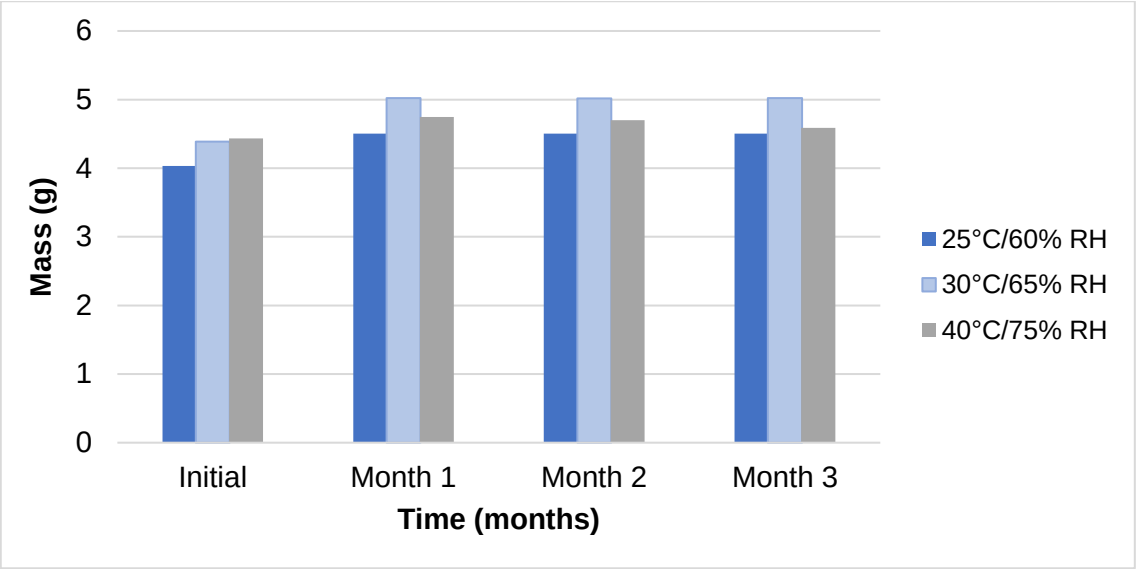


Figure D.9: Change in mass (g) of the dry (sachet) formulation containing Absorbatox® bound to 4% fulvic acid.

The mass of the dry dressing formulation remained relatively stable over the 3-month storage period with no significant change in mass. An increase in mass is observed at all three storage conditions of 40°C/75% RH, 30°C/65% RH, as well as 25°C/60% RH. The largest increase in mass is seen at storage conditions of 30°C/65% RH with a mass increase of 14%. The dry formulation stored at 40°C/75% RH indicates a mass increase of approximately 11%, while the formulation stored at 25°C/60% RH showed an increase in mass of approximately 3%. The sachets are designed to absorb moisture, thus results obtained may be due to insufficient container sealing, which allowed for moisture to enter the containers during storage periods.

D.5.3 Colour and visual appearance

A visual assessment of colour and appearance of the dry (sachet) formulation was performed on the initial dressing, as well as at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.6. The change in colour/visual appearance of the dry (sachet) formulation during the 3-month period is indicated in Table D.14.

Table D.14: Change in colour/visual appearance of the dry (sachet) formulation containing Absorbatox® bound to 4% fulvic acid

Initial	Month 1	Month 2	Month 3
25°C/60% RH			
			
30°C/65% RH			
			
40°C/75% RH			
			

The overall appearance of the dry (sachet) dressing formulation remained constant throughout the 3-month stability testing period. The colour and texture of all the sachets did not change over the testing period. No signs of moisture uptake are visible and the sachets remain intact.

D.5.4 Free swell capacity

The free swell capacity (fluid uptake) of the dry (sachet) formulation was measured at month 0 (initial). The sachet was then also measured at 25°C/60% RH, 30°C/65% RH and 40°C/75% RH on months 1, 2 and 3 as described in Section D.2.7. The results obtained from the fluid uptake study over the 3-month period are shown in Table D.15 and Figure D.10.

Table D.15: Mass (g) of the fluid uptake of the dry (sachet) formulation containing Absorbatox® bound to 4% fulvic acid

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	10.663	8.157	7.738	8.230
2	12.041	9.198	7.326	8.246
3	11.663	8.292	7.424	9.032
Average ± SD	11.456 ± 0.712	8.549 ± 0.566	7.496 ± 0.215	8.503 ± 0.458
30°C/65% RH				
1	10.663	7.483	8.753	8.543
2	12.041	8.028	10.767	7.721
3	11.663	8.569	8.454	9.158
Average ± SD	11.456 ± 0.712	8.027 ± 0.543	9.325 ± 1.258	8.474 ± 0.721
40°C/75% RH				
1	10.663	8.130	8.889	11.192
2	12.041	8.143	7.766	8.409
3	11.663	8.516	8.615	7.829
Average ± SD	11.456 ± 0.712	8.263 ± 0.219	8.423 ± 0.586	9.143 ± 1.798

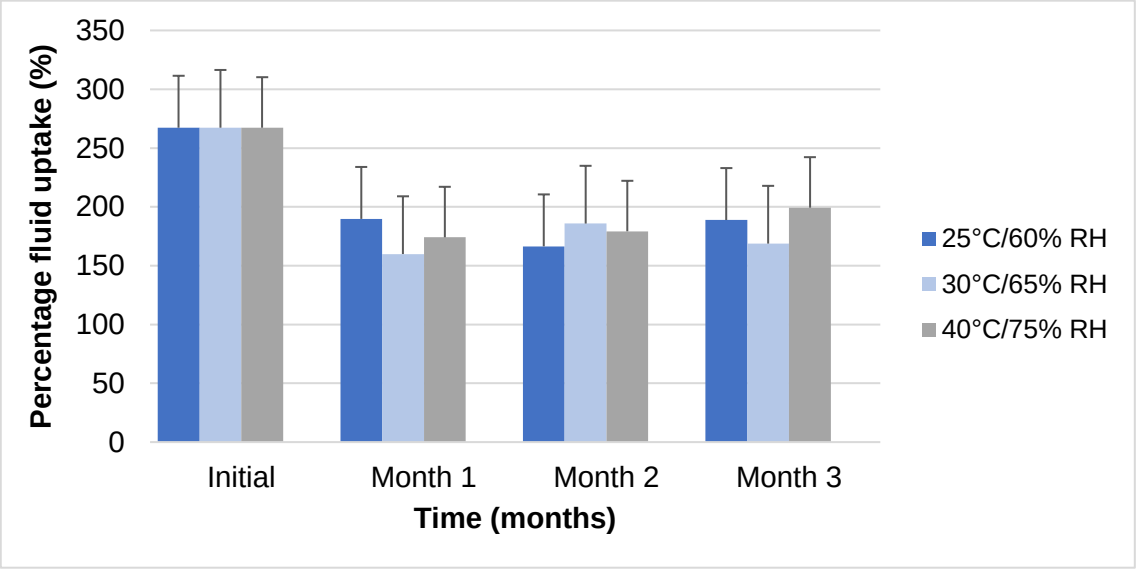


Figure D.10: Mass (g) of the fluid uptake of the dry (sachet) formulation containing Absorbatox® bound to 4% fulvic acid.

The dry (sachet) dressing indicated the most absorption capacity immediately after manufacture, absorbing a total of 267%. The absorption capacity of the sachet dressings decreased over the 3-month period. This result may be attributed to insufficient sealing of containers while storing, sachets may have been exposed to moisture prior to the free swell capacity testing. The dressings were formulated with absorption of fluid as a key factor, thus indicating a successful result.

The free swell capacity (fluid uptake) of the dry (sachet) formulation was measured as described above with additional measurements made after 5 days. The sachets were allowed to remain in

the Ringers lactate for an additional 5 days after the experiment. The dressings were then re-weighed and the additional fluid absorbency was calculated. Table D.16 tabulates the results obtained from the fluid uptake study after 5 days. The results are depicted graphically in Figure D.11.

Table D.16: Additional mass (g) of the fluid uptake of the dry (sachet) formulation (after 5 days in Ringers lactate) containing Absorbatox® bound to 4% fulvic acid

	Initial	Month 1	Month 2	Month 3
25°C/60% RH				
1	1.410	1.240	1.134	2.288
2	1.979	2.527	1.659	0.948
3	3.805	1.966	1.607	1.456
Average ± SD	2.398 ± 1.251	1.911 ± 0.645	1.467 ± 0.289	1.564 ± 0.676
30°C/65% RH				
1	1.410	2.366	2.135	0.527
2	1.979	2.671	0.615	2.176
3	3.805	2.071	1.369	3.000
Average ± SD	2.398 ± 1.251	2.369 ± 0.300	1.373 ± 0.760	1.901 ± 1.259
40°C/75% RH				
1	1.410	1.980	1.320	1.677
2	1.979	3.286	1.439	3.021
3	3.805	0.528	2.119	2.531
Average ± SD	2.398 ± 1.251	1.931 ± 1.380	1.626 ± 0.431	2.407 ± 0.680

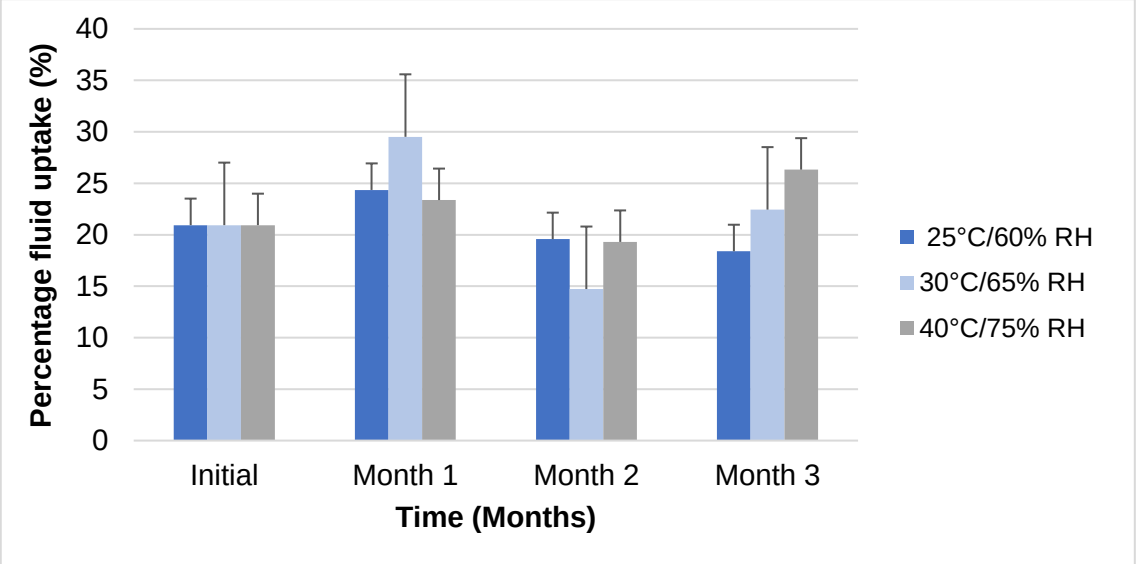


Figure D.11: Average free swell capacity percentage of the dry (sachet) dressing over a 3-month stability testing period.

The dry (sachet) dressing indicated an additional absorption capacity when left in the Ringer's lactate solution. The absorption capacity of the sachet dressings after 5 days remains stable over the 3-month period. This result may be attributed to insufficient sealing of containers while storing, sachets may have been exposed to moisture prior to the free swell capacity testing. The

dressings were formulated with absorption of fluid as a key factor, thus indicating a successful result.

D.6 Conclusion

The results obtained during the 3-month stability testing proved all three formulations to be stable and remain intact and active. The identification of the active ingredient namely, Absorbatox® bound to fulvic acid, as well as citric acid was successfully identified within all the formulations throughout the 3-month stability testing period under all three storage conditions.

The pH of the gel formulations may have an influence on the stability thereof. The silicone-based gel formulation is designed for the treatment of burn wounds and abrasions, as discussed in Chapter 2, the optimal wound healing environment is considered as a pH value of below 6, as most bacteria associated with infected wounds in humans require a pH above (Nagoba *et al.,* 2015:5). A decrease in pH, created by the presence of an organic acid, i.e. fulvic acid, thus aids in wound healing processes by providing infection control. A decrease in pH value leads to the Bohr-effect (i.e. increase in the amount of available oxygen of cells). This increase in the level of oxygen delivery to damaged tissue increases resistance to infection, as well as promote healing. An acidic environment also promotes epithelisation by boosting fibroblastic growth and neovascularisation, which increases microcirculation of wounds that in turn enables the formation of new healthy granulation tissue and ultimately faster wound healing (Nagoba *et al.,* 2015:5). The results obtained indicate that the silicone-based gel formulation met the stability criteria, proving to be a stable formulation.

The patch formulation is designed to have the ability to both swell and de-swell wound exudate in a reversible direction, which shows specific environmental stimuli-responsive e.g. temperature and pH (Kamoun *et al.,* 2017). Hydrogel wound patches can absorb as well as retain wound exudate which promotes fibroblast proliferation and keratinocyte migration, both of which are necessary for complete epithelialisation and wound healing (Kamoun *et al.,* 2017). This indicates that the swelling/moisture increase of the patch observed during the 3-month testing period is not considered an instability, but rather an indication that better care must be taken in future to sufficiently seal containers to prevent premature swelling of the patches before they are to come in contact with the wound surface.

The sachet dressings were formulated with absorption of fluid as a key factor, thus indicating a successful result. The overall results obtained from the dry (sachet) dressing are a clear indication of formulation stability.

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APPENDIX E

ANTI-MICROBIAL EFFICACY OF ABSORBATOX® RAW MATERIAL AND SELECTED ORGANIC ACIDS

E.1 Introduction

Modern research aims to develop new and improved anti-microbial agents from many different sources as a mechanism to prevent microbial resistance. The purpose of anti-microbial analysis is to determine the effectivity of a certain substance against specific micro-organisms and/or bacteria in order to formulate new drug products, which are selectively effective. This in turn decreases the risk of resistant organisms, as well as secondary infection and complications (Balouiri *et al*., 2015:71). In order to use organic acids in wound dressings, it is important to demonstrate antimicrobial activity of these selected acids against common pathogens typically found as wound contaminants, i.e. *S. aureus* and *E. coli*. Organic acids are known for their antimicrobial activity even against notoriously resistant organisms, such as *Pseudomonas aeruginosa* (Nagoba *et al.,*2013:67). Citric acid has been studied as a treatment for infected burn injuries (Nagoba *et al.,* 1998:481). Hence, it is beneficial to study organic acids for their potential use as antimicrobials in wound dressings.

E.2 Anti-microbial test results of Absorbatox®

Absorbatox® is used as a free radical scavenger to which organic acids can be bound in wound preparations and it is therefore essential to test whether it is sterile and free of microbial contamination before use in wound dressing products. The Absorbatox® raw material that was used in this study was obtained from Absorbatox (Pty) Ltd (SA). Citric acid, fulvic acid, as well as malic acid was obtained from ASAP Pharma Care (PTY) Ltd (SA). Testing for contamination of Absorbatox® raw material was outsourced to Ilse Simpson at Envirocare Laboratory (Potchefstroom). The objective of the analysis was to evaluate sterility of Absorbatox® raw material. Table E.1 reflects the results demonstrating that no microbial growth is observed on Absorbatox® raw material when tested as determined by the laboratory standard.

Cultures used included *S. aureus*, *E. coli* and *S. typhimurium*. Analysis was done on microtiter plates using Mueller Hinton Broth as media. The information from the test analysis results of Absorbatox® is shown in Table E.1 (Simpson, 2018a).

Table E.1: Analysis of microbial growth results of Absorbatox® raw material

Sample identification	Total plate count (Cfu*/1 g)
Absorbatox® Granular	< 10
Absorbatox® Micronised	< 20

*Cfu: Colony forming units; < 10: No units detected (Simpson, 2018a).

E.3 Anti-microbial efficacy of organic acids

The minimum inhibitory concentration (MIC) of a specific substance describes the lowest concentration of the agent which is bacteriostatic, i.e. prevents the visible growth of bacteria. The MIC of an agent is used to determine and evaluate the anti-microbial effectiveness of the agent by measuring the effect of decreasing concentrations of antibiotic/antiseptic over a defined period in terms of inhibition of microbial population growth (Goins, 2017). Table E.2 describes the MIC of the organic acids against *S. aureus*, *E. coli* and *S. typhimurium*.

Table E.2: Anti-microbial activity of citric, fulvic and malic acid

	Escherichia coli	*Salmonella typhimurium*	*Staphylococcus aureus*
Citric acid	1.50%	NG	NG
Fulvic acid	NG	NG	0.22%
Malic acid	3.50%	NG	NG

*NG: No growth at the lowest possible concentration (Al-Nabulsi *et al,* 2014; Fernandes *et al;* Kim *et al;* Liu *et al,* 2016; Matsuda *et al,* 1994).

E.4 Conclusion

As seen from the analysis results, Absorbatox® proves to be effective against the cultured organisms used during testing. The analysis results provide important anti-microbial efficacy data required to formulate optimal wound dressings. Fulvic acid tested to be the most effective against the organisms tested (Simpson, 2018b). Table E.2. describes the effectiveness against the selected organisms of the organic acids. Citric , fulvic, as well as malic acid provide effective anti-microbial activity, which may be effective in the use of new wound healing formulations in combination with Absorbatox®.

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Simpson, I. 2018b. Envirocare laboratory: Test report Organic acids. p1-2.

APPENDIX F

THE INTERNATIONAL JOURNAL OF PHARMACEUTICS:

GUIDE FOR AUTORS

F.1 Introduction

The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals. Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bio-adhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

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