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In vitro wound healing and cytotoxic activity of the gel and whole-leaf materials from selected aloe species

Lizelle T. Fox, Anisha Mazumder, Anupma Dwivedi, Minja Gerber*, Jeanetta du Plessis, Josias H. Hamman

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

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ABSTRACT

Ethnopharmacological relevance: *Aloe vera* is one of the most important medicinal plants in the world with applications in the cosmetic industry and also in the tonic or health drink product market. Different parts of *Aloe ferox* and *Aloe marlothii* are used as traditional medicines for different applications. Although wound healing has been shown for certain aloe gel materials (*e.g. A. vera*) previously, there are conflicting reports on this medicinal application of aloe leaf gel materials.

Aim of the study: The present study aimed at determining the wound healing properties of the gel and whole-leaf materials of *Aloe vera*, *Aloe ferox* and *Aloe marlothii,* as well as their cytotoxic effects on normal human keratinocyte cells (HaCaT).

Materials and methods: Nuclear magnetic resonance spectroscopy was used to chemically fingerprint the aloe gel and whole-leaf materials by identifying characteristic marker molecules of aloe gel and whole-leaf materials. An MTT assay was performed to determine the cytotoxicity of the various aloe whole-leaf and gel materials on HaCaT cells. Wound healing and *in vitro* cell migration were investigated with HaCaT cells by means of the CytoSelect™ assay kit.

Results: The *in vitro* wound healing assay suggested that all the aloe gel and whole-leaf materials examined, exhibited faster wound healing activity than the untreated control group. After 48 h, all the aloe gel and whole-leaf materials almost completely caused full wound closure, displaying 98.07% (*A. marlothii* whole-leaf), 98.00% (*A. vera* gel), 97.20% (*A. marlothii* gel), 96.00% (*A. vera* whole-leaf), 94.00% (*A. ferox* gel) and 81.30% (*A. ferox* whole-leaf) wound closure, respectively. It was noteworthy that the gel materials of all the three aloe species exhibited significantly faster ($p < 0.05$) wound healing actions when compared to their respective whole-leaf materials at 32 h.

Conclusion: The gel and whole-leaf materials of *A. vera*, *A. ferox* and *A. marlothii* have shown the ability to heal wounds at a faster rate and to a larger extent than untreated keratinocytes. The MTT assay results suggested that the gel and whole-leaf materials of all the selected Aloe species showed negligible toxicity towards the HaCaT cells.

1. Introduction

A wound can be described as a laceration or break of the skin surface caused by thermal or physical injury (Hashemi et al., 2015). Wound healing is a dynamic and multi-faceted process that can be divided into four phases, namely hemostasis, inflammation, proliferation (granulation and contraction) and re-modeling (maturation) (Orsted et al., 2004). Each phase of the wound healing process is characterized by the migration of specific cell types into the wound to interact with the environment and other cells (Topman et al., 2013).

The use of medicinal plants for the treatment of various skin conditions has been popular for decades. Some of these natural medicines are believed to possess considerable therapeutic potential and should therefore be investigated for use in the advancement of products in the treatment of skin burns and wounds (Serafini et al., 2014).

The medicinal properties of aloe plants, especially *Aloe vera* (*Aloe barbadensis* Miller) are well-known worldwide. It has been reported that *A. vera* possesses various therapeutic properties, specifically in promoting wound, burn, and frost-bite healing. Additionally, this

* Corresponding author.
E-mail addresses: Lizelle.Fox@nwu.ac.za (L.T. Fox), anishamajumdar@gmail.com (A. Mazumder), anupma.feb1@gmail.com (A. Dwivedi), Minja.Gerber@nwu.ac.za (M. Gerber), Jeanetta.DuPlessis@nwu.ac.za (J. du Plessis), Sias.Hamman@nwu.ac.za (J.H. Hamman).

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species has showed anti-inflammatory, anti-fungal, hypoglycemic and gastro protective properties (Choi and Chung, 2003). Several studies have shown the vulnerary activity of systemic or topically administered aloe gel material, but other studies indicated that it caused a delay in wound healing or no effect at all (Hamman, 2008).

Maenthaisong et al. (2007) concluded from a comprehensive literature survey that *A. vera* containing dosage forms may efficiently shorten the duration of wound healing of first and second degree burns. *A. vera* gel was found to increase the rate of epithelialization and thereby could contribute to successful wound healing. On the other hand, contradictory findings were documented by Schmidt and Greenspoon (1991), which included delayed wound healing after *A. vera* containing gel was applied on obstetric and gynecologic patients with wound complications.

An *in vitro* study by Topman et al. (2013) showed that *A. vera* had no effects on the migration kinematics of cultured fibroblasts after infliction of localized mechanical damage. However, due to the complexity of the wound healing process it was thought that *A. vera* may affect *in vivo* wound healing *via* alternative pathways.

Some of these contradictory results may be explained by the variation in chemical composition between plants from different regions as well as differing isolation techniques used for extracting the compounds (Hamman, 2008). Even though the wound healing properties of *A. vera* has been proven clinically and experimentally, additional studies are needed for confirmation (Hashemi et al., 2015).

Aloe ferox is another important aloe species that is often used for its curative properties (Coopoosamy and Naidoo, 2013). An investigation into the wound healing effect of *A. ferox* whole-leaf juice on incisional wounds in a rat model showed progression of wound closure as well as facilitation of the healing process. Treatment with *A. ferox* whole-leaf juice indicated an increase in the wound healing rate and a shortened time period for full or partial epithelialization (Jia et al., 2008).

Although anecdotal evidence exists for use of *Aloe marlothii* leave materials for wound treatment, no scientific evidence regarding the vulnerary activity of this aloe species could be found. Consequently, the aim of this study was to determine the *in vitro* wound healing and cytotoxic properties of the gel and whole-leaf materials of *A. ferox*, *A. marlothii* and *A. vera* on normal human keratinocyte cells (HaCaT).

2. Materials and methods

2.1. Materials

Methylthiazol tetrazolium [3-(4, 5-dimethylthiazol-2-yl)−2, 5-diphenyltetrazolium bromide] (MTT) was procured from Life Technologies (USA). Dimethyl sulfoxide (DMSO), phosphate buffer solution (PBS) and 0.4% trypan blue solution were obtained from Sigma-Aldrich (USA). CytoSelect™ wound healing and cell migration kits were obtained from Cell Biolabs, Inc., USA.

The dehydrated gel and whole-leaf materials (DaltonMax 700®) of *Aloe barbadensis* Miller (*Aloe vera* (L.) Burm.f.) were obtained from Improve USA, Inc. (DeSoto, Tx, USA, certified by the International Aloe Science Council)). Leaves of *Aloe ferox* Mill. were obtained from Organic Aloe (Pty) Ltd. (Albertinia, South Africa). *Aloe marlothii* A.Berger leaves were collected in the North-West Province of South Africa from natural populations near Koster (S25°47.100'; E026°46.725'). A specimen voucher (collection number: PUC 1151) was deposited in the North-West University's Herbarium. The names of the selected aloe species have been checked on www.theplantlist.org (date of access: 24/08/2016).

Human immortalized keratinocyte (HaCaT) is an immortalized non-tumorigenic human keratinocyte cell line, which was originally derived from normal human trunk skin and these cells are able to stratify. For this study, the cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, Switzerland), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Biochrom, Merck, Germany) and 1% penicillin/streptomycin (Lonza, Switzerland) at 37 °C in a 5% CO_2 humidified environment. PBS solution, free of calcium and magnesium (Lonza, Switzerland) was used in washing of cell monolayers.

2.2. Methods

2.2.1. Preparation of A. ferox and A. marlothii leaf materials

The traditional hand-filleting method was used to obtain the gel and whole-leaf materials from the *A. ferox* and *A. marlothii* leaves as previously published (Fox et al., 2015; Lebitsa et al., 2012; Ramachandra and Rao, 2008). In short, the base, tapering point and margins of the leaves were removed, followed by the removal of the rind from both the top and bottom side of the leaves. The remaining gel fillet was rinsed with water and liquidized in a kitchen blender, either alone or in combination with pieces of the green rind to obtain the gel and whole-leaf material, respectively. Thereafter; the aloe leaf materials were lyophilized with a freeze dryer (VirTis, UK) and the obtained dried powder passed through a sieve (150 – 180 μm).

2.2.2. Chemical characterization of aloe leaf materials

Nuclear magnetic resonance (^{1}H NMR) fingerprinting was performed on the selected aloe leaf materials as previously described to identify the presence of marker molecules (*i.e.* aloverose, glucose, malic acid and isocitric acid) that can confirm the origin of the aloe gel and whole-leaf materials (Fox et al., 2015). In short, solutions containing approximately 30 mg of the aloe gel and whole leaf materials were prepared with D_2O (1.5 ml) and subsequently filtered through cotton wool. Thereafter, a small quantity of 3-(trimethylsilyl) propionic acid-D4 sodium salt was added and the H^1-NMR spectra recorded with an Avance III 600 Hz NMR spectrometer (Bruker, Rheinstetten, Germany).

2.2.3. Passaging of human immortalized keratinocytes (HaCaT) cell line

HaCaT was cultured at 37 •C in a 5% CO2 humidified environment and maintained in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, South Africa), with 10% (v/v) fetal bovine serum and 0.1% mixture of streptomycin and penicillin (Sigma, USA). The monolayer of cells were washed twice with PBS and then treated with trypsin-EDTA solution for 5−10 min at 37 C to remove the cells from the substratum. Cell pellets were harvested at 1000 rpm centrifugation for 5 min, then the supernatant was discarded and the cell pellet was re-suspended into the fresh medium. The culture medium was changed every two to three days and the concentration of cells were determined by utilizing trypan dye exclusion Mazumder et al. (2015).

2.2.4. In vitro cytotoxic assay

The cytotoxicity of the selected aloe gel and whole-leaf materials was performed by means of an MTT assay. The MTT solution (2 mg/ml) was prepared in a serum free DMEM medium. Solutions of all the selected aloe leaf powder materials were prepared in PBS at a concentration of 5 mg/ml and PBS was used as the control. All the samples were filtered using 0.45 μm filters. A total of 2×10^4 HaCaT cells were seeded into 96-well plates, incubated for 24 h and allowed to grow as a monolayer. The cells were then treated with different concentrations (0.40, 0.66 and 1.30 mg/ml) of gel and whole-leaf materials of aloe species and PBS. After 24 h, the test solutions were removed and discarded from the 96 well plates and 50 μl of the MTT was added to the well plates. The plate was then incubated at 5% CO_2, 37 °C, for 1.5 h. Thereafter, the MTT solution was discarded and 200 μl of DMSO was added to each well and mixed gently Dwivedi et al. (2015). The absorbance of each well was subsequently recorded at 560 nm utilizing a VERSA max microplate reader (Lab system Multiskan RC, USA). The absorbance gives an indication of the viable cells because they reduce the yellow MTT to purple formazan. The

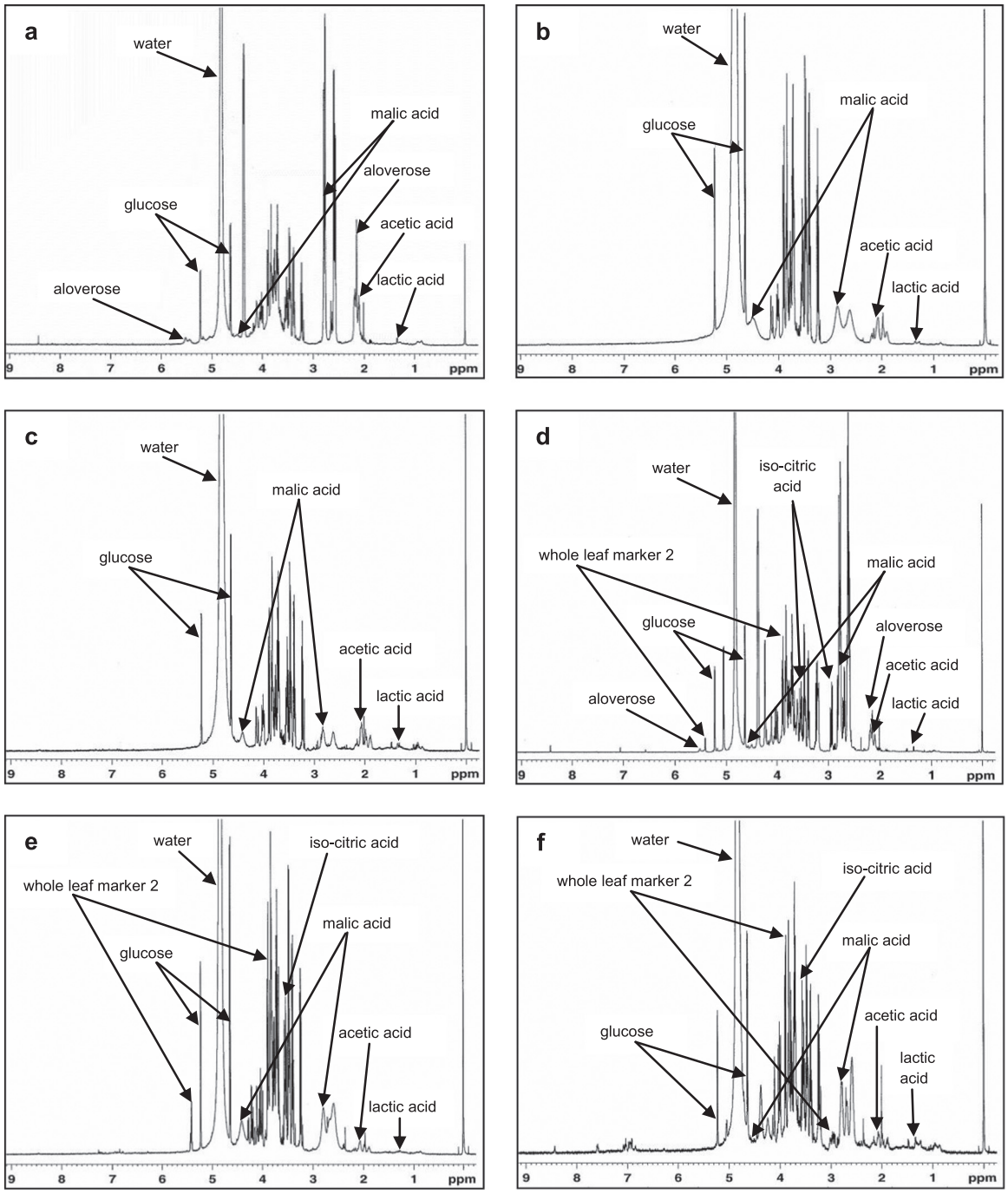

Fig. 1. Nuclear magnetic resonance (^{1}H NMR) spectra of (a) *A. vera* gel, (b) *A. marlothii* gel, (c) *A. ferox* gel, (d) *A. vera* whole leaf, (e) *A. marlothii* whole leaf and (f) *A. ferox* whole leaf.

insoluble purple formazan was dissolved in DMSO to give a colored solution (pink to purple), which was quantified by measuring its absorbance of UV light. The percentage cell viability was calculated by using Eq. (1).

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of the untreated cells}} \times 100 \quad (1)$$

2.2.5. In vitro *wound healing assay*

The CytoSelect™ wound healing assay was performed on normal human keratinocyte (HaCaT) cells. The aloe gel and whole-leaf materials were dissolved in serum free DMEM, vortexed for approximately 2 min and subsequently filtered through 0.45 μm filters. The untreated cells (PBS) served as the control group.

Sterile forceps were used to place the inserts (which were used to form wound areas) in the well plates to ensure that they made firm contact with the bottom of the well plate and in such a way that all wound fields were aligned in the same direction. A cell suspension consisting of 1.0×10^6 cells/ml in DMEM containing 10% v/v FBS was prepared (as per CytoSelect™ kit instructions).

Aliquots of the cell suspension (500 μl) were added to each well and incubated for 24 h to obtain a monolayer where after the inserts were gently removed without disturbing the edges of the formed wound area. The media was slowly aspirated from wells, discarded and the wells were washed with serum free media 4–6 times until the debris and dead cells were all removed. After washing was completed, fresh media with 10% FBS was added and the aloe leaf material samples (dissolved

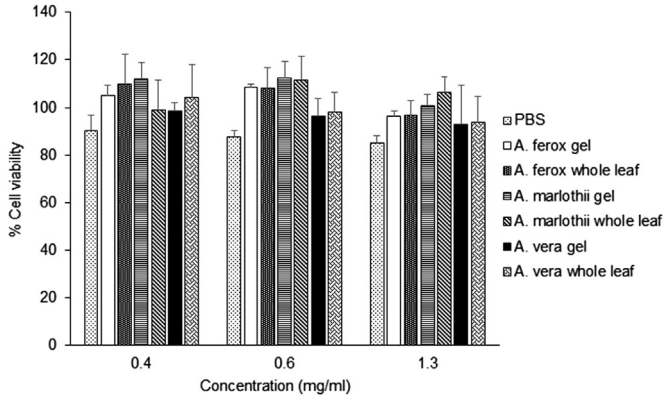

Fig. 2. Percentage cell viability of various concentrations of aloe gel and whole-leaf materials and the control group (PBS) on HaCaT cells. Each value represents the mean and SD of 3 experiments.

in serum free medium) were added to their respective wells (Mazumder et al., 2015). The cells were then incubated at 37 °C, with 5% CO_2 while pictures were captured with light microscopy at the following time intervals: 6, 12, 24, 32 and 48 h. The medium in each well was discarded and 200 µl of cell staining solution (as per CytoSelect™ kit) was added to each well. The well plates were then incubated for about 10 min, washed several times with PBS where after the images were taken at 10x magnification with an inverted light microscope (Nikon eclipse TS100) fitted with a Nikon camera. By utilizing Image J software, the wound closure (diameter in µm) was measured and the percentage wound closure was calculated by using Eq. (2).

$$\%\ \text{Wound closure} = \left[\frac{(\text{Pre-migration})_{\text{diameter}} - (\text{Migration})_{\text{diameter}}}{(\text{Pre-migration})_{\text{diameter}}}\right]\times 100 \quad (2)$$

Where $(\text{pre-migration})_{\text{diameter}}$ is the initial wound diameter (µm) at time 0 h and $(\text{migration})_{\text{diameter}}$ is the wound length at a particular time (h). The migration rate was calculated by using Eq. (3).

$$\text{Migration rate (µm/h)} = \left[\frac{(\text{Pre-migration})_{\text{length}} - (\text{Migration})_{\text{length}}}{\text{Time (h)}}\right] \quad (3)$$

2.2.6. In vitro *cell migration*

DMEM (500 µl) supplemented with 10% v/v FBS was added to the lower wells in 24 well plates of the CytoSelect™ assay kit. A cell suspension (300 µl) containing 1.0×10^6 cells/ml in serum free DMEM was added to the inside of the inserts. The well plates were incubated for 24 h at 37 °C with 5% CO_2. After 24 h, the medium from the inside

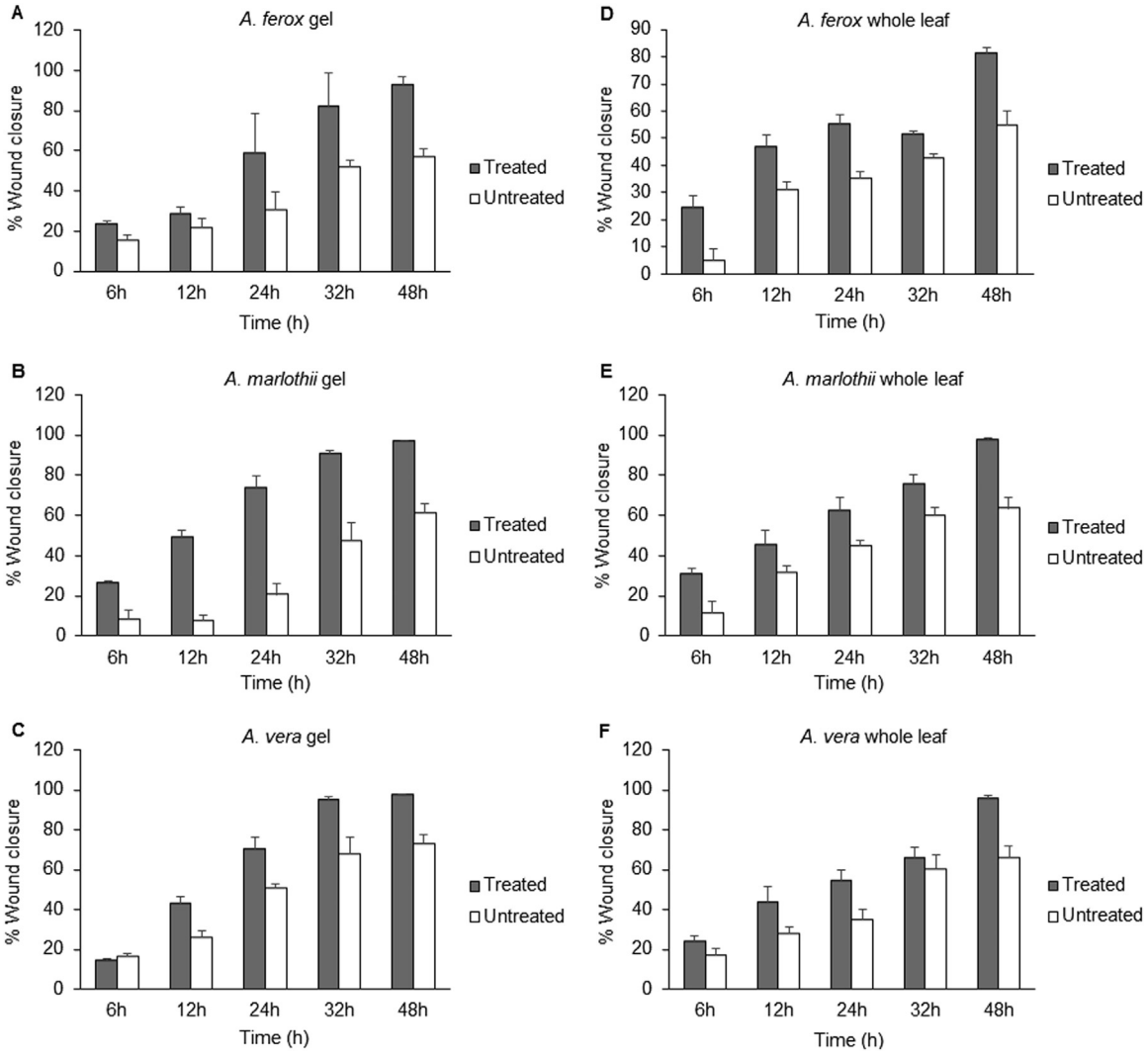

Fig. 3. Percentage wound closure after treatment with gel and whole-leaf materials of selected aloe species at different time periods on HaCaT cells. Untreated cells were used as a control for each time period. Each value represents the mean and SD of 3 experiments. (A) *A. ferox* gel, (B) *A. marlothii* gel, (C) *A. vera* gel, (D) *A. ferox* whole leaf, (E) *A. marlothii* whole leaf and (F) *A. vera* whole leaf.

Table 1
Percentage wound closure 32 h after treatment with aloe gel and whole-leaf materials on HaCaT cells. Each value represents the mean and SD of 3 experiments.

Aloe leaf material	%Wound closure	
	Treated	Untreated
A. ferox gel	82.41 ± 16.30	52.04 ± 3.39
A. ferox whole-leaf	51.77 ± 0.75	42.88 ± 1.23
A. marlothii gel	90.73 ± 2.82	47.73 ± 8.34
A. marlothii whole-leaf	76.06 ± 4.10	59.94 ± 4,24
A. vera gel	95.52 ± 1.33	67.80 ± 8,74
A. vera whole-leaf	66.10 ± 4.87	60.47 ± 6.78

of the inserts was gently removed and discarded.

Cotton-tipped swabs were wetted with PBS and used to gently swab the interior of the inserts in order to remove the non-migratory cells from the inside perimeter of each insert. Each insert was then transferred to a clean well containing 400 µl of cell staining solution and then incubated for 15 min at room temperature. The stained inserts were washed several times with PBS and allowed to air-dry. Thereafter, each insert was transferred to empty wells with 200 µl of extraction solution (included in the CytoSelect™ assay kit) and incubated for about 10 min (Mazumder et al., 2015). The absorbance was recorded at 560 nm in the microplate reader (Lab Systems Multiskan RC, USA). The percentage cell migration was calculated by using Eq. (1).

2.2.7. Statistical analysis

All experiments performed during this study were done in triplicate and the data were analyzed using GraphPad Prism™ software (version 5.01, USA). Statistical analysis was carried out by means of a one-way analysis of variance (ANOVA, non-parametric analysis) with Tukey's multiple comparison test. Data are shown as the mean ± standard deviation (SD), with significance accepted when $p < 0.05$.

3. Results and discussion

3.1. Chemical characterization of aloe leaf materials

The ^{1}H NMR spectra obtained for the aloe gel and whole-leaf materials are shown in Fig. 1 (Fox et al., 2015). The ^{1}H NMR spectra showed that aloverose (partly acetylated polymannose/acemannan), the major marker molecule to identify *A. vera* gel material, was present in both the *A. vera* gel and whole-leaf materials. All of the selected aloe leaf materials contained glucose and malic acid; whereas an additional whole-leaf marker (*i.e.* isocitric acid) was found to be present in the whole-leaf materials of all three aloe species (Fox et al., 2015).

3.2. In vitro *cytotoxic assay*

The results obtained from the MTT assay on the HaCaT cells are given in Fig. 2 and revealed that the control group (PBS) as well as the aloe leaf material samples (at all the concentrations tested) exhibited negligible cytotoxicity towards the HaCaT cells. This is in agreement with the results obtained in a previous study where the cytotoxicity of leaf materials of the same aloe species showed very low reduction in the viability of human hepatocellular (HepG2), human neuroblastoma (SH-SY5Y) and human adenocarcinoma epithelial (HeLa) cells (Du Plessis and Hamman, 2014). The results from this *in vitro* toxicity assay therefore indicate that the gel and whole-leaf materials from all three the aloe species investigated are safe to use on skin tissue.

3.3. In vitro *wound healing*

The epidermis is a stratified epithelium consisting of many layers of keratinocytes, which provides a physical barrier between the environment and the organism. This barrier protects the organism from external agents and pathogens as well as restricting the loss of fluids. As keratinocytes are known to be an important cellular component of the epidermis, they play a critical role in barrier maintenance and

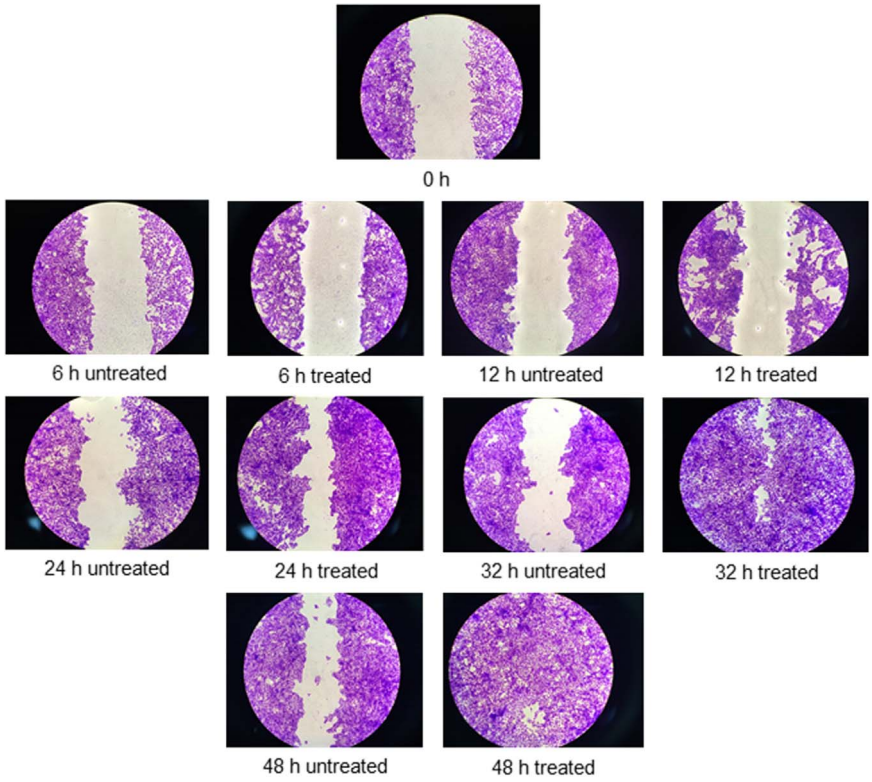

Fig. 4. Images of the wound healing activity of *A. vera* gel and untreated (control) HaCaT cells at selected time periods. Images were captured at 10×magnification.

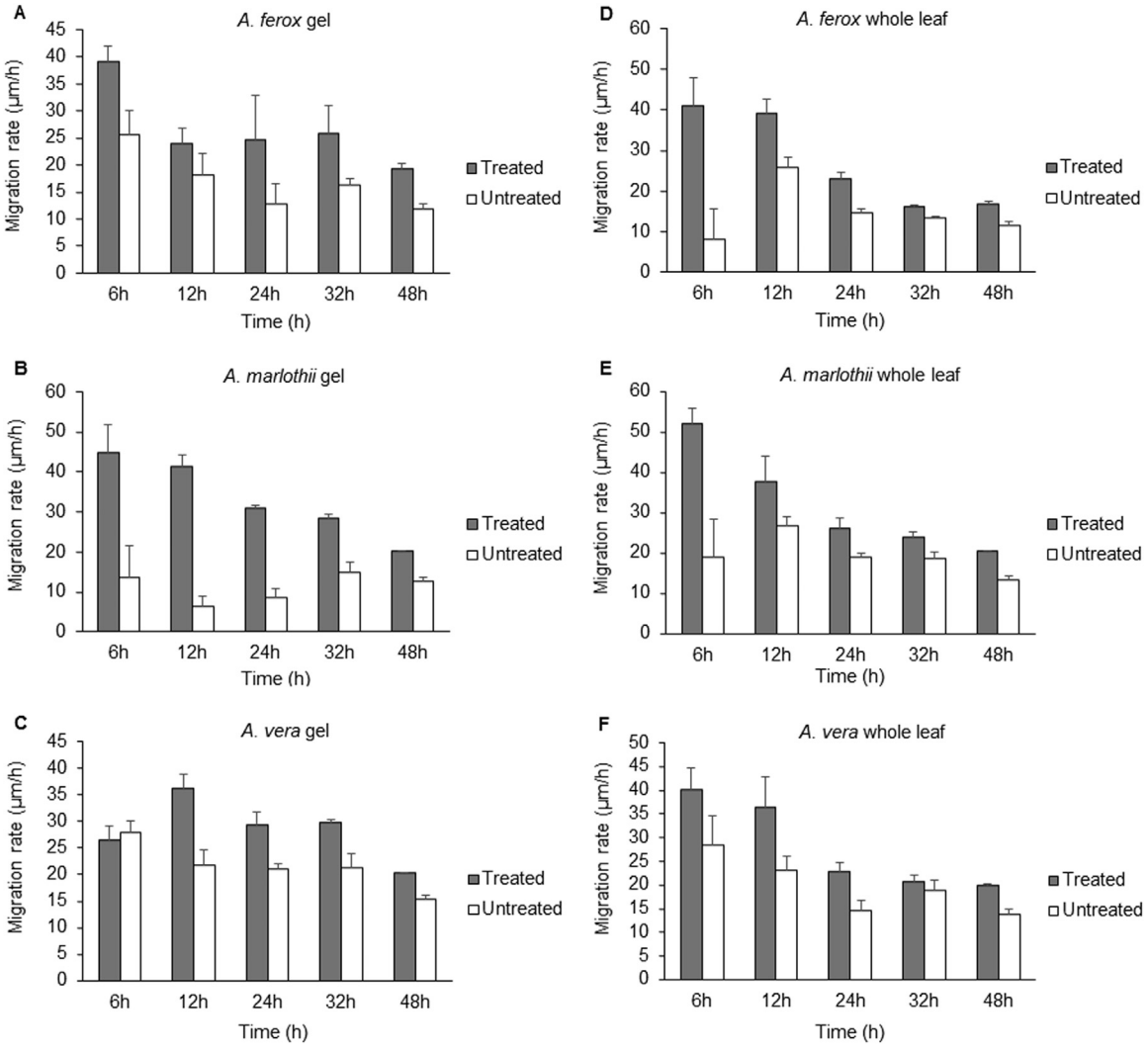

Fig. 5. The migration rate (µm/h) of HaCaT cells after treatment with gel and whole-leaf materials of aloe species at different time periods. Untreated cells were used as a control for each time period. Each value represents the mean and SD of 3 experiments. (A) *A. ferox* gel, (B) *A. marlothii* gel, (C) *A. vera* gel, (D) *A. ferox* whole leaf, (E) *A. marlothii* whole leaf and (F) *A. vera* whole leaf.

Table 2
Percentage cell migration caused by the aloe gel and whole-leaf materials on HaCaT cells, with standard deviation.

Aloe leaf material	%Cell migration
A. ferox gel	126.15 ± 15.86
A. ferox whole-leaf	93.30 ± 7.67
A. marlothii gel	148.17 ± 20.23
A. marlothii whole-leaf	99.48 ± 3.42
A. vera gel	143.37 ± 9.34
A. vera whole-leaf	108.99 ± 4.77

restoration upon injury through a process of epithelialization. They are involved in the complex mechanisms of wound healing (Pastar et al., 2014). Re-epithelialization is an important step of the wound healing process and is characterized by increased proliferation and migration of keratinocytes over the wounded area (Sivamani, 2014).

The wound closure data obtained from the CytoSelect™ wound healing assay are shown in Fig. 3. For all the aloe gel and whole-leaf materials, it was observed that the wound healing activity increased with time. The results also showed that all the aloe leaf materials tested, exerted significant wound healing effects in comparison to the untreated cells at all the tested time periods. Additionally, it was revealed that all the gel material of aloe species showed better wound healing effects than the whole-leaf materials. After 48 h, all the aloe gel and whole-leaf materials almost completely cured the wound, displaying 94% (*A. ferox* gel), 97.2% (*A. marlothii* gel), 98% (*A. vera* gel); 81.3% (*A. ferox* whole-leaf), 98.07% (*A. marlothii* whole-leaf) and 96% (*A. vera* whole-leaf) closure, respectively.

Table 1 shows the percentage wound closure caused by the aloe gel and whole-leaf materials after 32 h. It was noticed that the gel material displayed better wound healing action when compared to whole-leaf materials. *A. ferox* gel (82.4%), *A. marlothii* gel (90.72%) and *A. vera* gel (95.5%) all exhibited excellent wound healing activity, although there was differences between them in their wound healing properties (in terms of percentage wound closure).

A statistical significant difference ($p < 0.05$) was seen in the wound closure capabilities of the aloe whole-leaf materials: *A. ferox* whole-leaf =51.7%, *A. marlothii* whole-leaf =76.05% and *A. vera* whole-leaf =66.09% wound closure. The images in Fig. 4 depict the wound healing closure of *A. vera* gel, which exerted excellent wound healing activity.

The speed at which cells migrate towards the wound area is known as the migration rate. The cell migration rate of the wound areas treated with the various aloe gel and whole-leaf materials are represented in Fig. 5.

It was necessary to calculate the migration rate (µm/h) as it indicates how fast the wound healing agent causes the wound area to fill. It was observed that the migration rate gradually decreased over

time as wound healing progressed for both the gel and whole-leaf aloe materials, due to a decreased wound area.

An earlier study by Chithra et al. (1998) reported the influence of *A. vera* gel on the collagen content and its characteristics in the matrix of healing dermal wounds in rats. *A. vera* gel showed a positive influence on the collagen content and stability in wound curing. This corresponds to findings by Heggers et al. (1996), which showed that a high molecular weight polypeptide constituent from the aloe gel promotes wound contraction caused by increased collagen activity.

A glycoprotein fraction from *A. vera* leaf material was found to increase wound healing in a monolayer of human keratinocytes and was furthermore capable of increasing the expression of proliferation markers at the immunohistochemical level (Choi et al., 2001). A study by Davis and Maro (1989) showed that *A. vera* (carbohydrate fraction of the gel) inhibited inflammation in a dose-response manner and improved wound healing actions in diabetic mice. Furthermore, the effects of oral and topically administered *A. vera* gel indicated that it decreased the wound diameter on mice, stimulated and enhanced vascularity near the wound area. It was also suggested that *A. vera* gel assisted by increasing the supply of oxygen to the wound (Davis et al., 1989).

This study not only confirmed the wound healing properties of *A. vera* gel and whole-leaf materials, but also showed that *A. marlothii* and *A. ferox* gel and whole-leaf materials have the ability to speed up the healing of wounds in keratinocytes.

3.4. In vitro *cell migration*

The polycarbonate membranes of the CytoSelect™ cell migration assay kit, act as a barrier to discriminate migratory cells from non-migratory cells. The data displayed in Table 2 indicate that the percentage cell migration caused by the aloe gel materials was higher than that caused by the whole-leaf materials. All of the aloe gel materials exhibited statistically significantly higher % cell migration than their respective whole-leaf materials. This experiment also substantiated the results from the wound healing experiments that the gel material overall exhibited a better wound healing activity than the whole-leaf material of the various aloe species.

4. Conclusion

The aim of the current study was to evaluate the *in vitro* cytotoxic and wound healing properties of the gel and whole-leaf materials of *A. vera*, *A. ferox* and *A. marlothii* on HaCaT cells. The results suggested that all the aloe leaf materials studied exerted minimal toxicity towards the HaCaT cells. The gel material of the aloe species exhibited faster wound healing effects than the whole-leaf materials at 32 h, as indicated by the percentage wound closure. Furthermore, it was seen that in general the migration rate of the cells in the wound area was higher in the presence of the different aloe materials as compared to the control group and the aloe gel materials caused a higher percentage cell migration than the aloe whole-leaf materials.

The data obtained during this study indicate that gel materials from the selected aloe species act as wound healing therapies and may be included in wound treatment products. Although *A. vera* gel has been extensively used and studied for wound healing and other skin care applications, the results of the current study indicate that *A. marlothii* and *A. ferox* gel materials are also good candidates for use in wound healing. Future research on the vulnerary activity of the selected aloe gel materials should include *in vivo* studies on a suitable animal model and possible human clinical trials.

Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Declaration of Interest

The authors declare no conflict of interest. The NRF only provided financial support for the research and had no involvement in the design of the study; the experimental work conducted, or the analysis and interpretation whereof; in writing the publication or in choosing to which journal the manuscript will be submitted.

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