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A biochemical comparison of the *in vivo* effects of *Bulbine frutescens* and *Bulbine natalensis* on cutaneous wound healing

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ABSTRACT

Aim of the study: In South Africa the local population relies extensively on indigenous plants in the formulation of traditional medicines to treat skin ailments. The scientific merits of many of these plants used to treat wounds and burns are yet to be validated. *Bulbine natalensis* and *Bulbine frutescens* of the Asphodelaceae family are indigenous to only southern Africa and are widely used as a skin remedy. This study aimed to explore the scientific value of these plants through investigating the *in vivo* biochemical effects of *Bulbine natalensis* and *Bulbine frutescens* on cutaneous wounds.
Material and methods: Excisional and incisional wounds treated with either *B. natalensis* or *B. frutescens* and mirrored control wounds were created on the back of 12 domestic pigs. Wound contraction was recorded daily. The excisional wounds, biopsied at days 2, 4, 7, 10 and 16, were used to analyse the biochemical composition of the wounds by estimating the total amount of protein, DNA, collagen and hexosamine present. The incisional wounds, biopsied at day 16, were used to test the tensile strength of the healed wounds using a tensiometer.
Results: Wound contraction following treatment with *Bulbine natalensis* on days 2, 4 and 10 ($p = .004$, 0.007 and 0.03, respectively), and *Bulbine frutescens* on day 4 ($p = 0.004$) increased significantly when compared to the corresponding untreated wounds. The tensile strength of the wounds treated with the leaf gels was significantly stronger than that of the untreated wounds. There was also a significant increase in the collagen, protein and DNA content of the *Bulbine natalensis*- and *Bulbine frutescens*-treated wounds compared with that of the untreated wounds (collagen content: $p = 0.014$ and 0.018; protein content: $p = 0.03$ and 0.04; DNA content $p = 0.04$ and 0.04; respectively) over the 16-day experimental period. Treatment with both leaf gels followed the same pattern in hexosamine content with a maximum hexosamine content on day 4 followed by a steady decrease to day 16. No significant difference between the hexosamine content of the wounds of animals treated with either *Bulbine frutescens* or *Bulbine natalensis* was found.
Conclusions: These findings validate the traditional use of the leaf gel extracts of *B. frutescens* and *B. natalensis* in the treatment of wounds and may warrant further investigation towards producing a low-cost effective topical treatment for wounds.

1. Introduction

Wound healing commences immediately following injury and proceeds in a complicated but well organised sequence involving the interaction of many cell types and cytokines (Lin et al., 2003). The main objective of wound management is to heal the injury in the shortest possible time with minimal pain and discomfort to the patient. At the site of the wound, a flexible fine scar with maximal tensile strength is desired (MacKay and Miller, 2003).

Humans have always been faced with the dilemma of how to treat wounds (MacKay and Miller, 2003). Many diverse and interesting approaches have been applied throughout medical history. Although scientific proof was lacking, some wound care therapies applied by 'adventurous' physicians are still considered valuable today, e.g. honey and sugar paste were used for scarless healing and *Symphytum officinalis* (comfrey) was initially applied externally to the skin to heal fractures (MacKay and Miller, 2003). In the quest to accelerate healing, scientists continue to use medicinal plants to promote the various stages of wound healing (e.g. coagulation, inflammation, fibroblast proliferation, collagen forma-

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tion and deposition, re-epithelialisation and wound contraction). Some of these plants include *Aloe vera* (Chithra et al., 1998a,b; Choi et al., 2001; Subramanian et al., 2006), *Centella asiatica* (Shukla et al., 1999) and *Calotropis procera* (Rasik et al., 1999). All three of these plants have been shown to promote fibroblast proliferation and collagen formation.

The present study examined the *in vivo* biochemical effects of the leaf gel extracted from *Bulbine natalensis* and *Bulbine frutescens* on wound healing. The leaf of both these plants of the Asphodelaceae family (Van Wyk and Gericke, 2000) are filled with a clear gel similar in appearance and consistency to the *Aloe vera* gel. The gel of several species of *Bulbine* is commonly used by both traditional healers and the local population for the treatment of wounds, burns, rashes, itches, ringworm, cracked lips and herpes (Hutchings et al., 1996). Although the use of these plants in traditional medicine for wound healing is well entrenched, their effects have not previously been scientifically validated. In traditional medicine, the fresh leaf gel is rubbed directly on to the skin wound to promote healing or used in the form of a warm poultice (Hutchings et al., 1996). According to Hutchings et al. (1996), the species of *Bulbine* which are used in South Africa by both people of African and European descent for their (skin) wound healing properties are: *Bulbine asphodeloides*, known also *as* copaiva, 'geelkatstert', 'ibhucu', 'intelezi' and 'mmele'; *Bulbine frutescens*, known also as burn jelly plant, 'ingelwane', 'ibhucu', 'ithethe elimpofu'; and *Bulbine natalensis*, *known also as* 'rooiwortel', 'ibhucu', 'ibucu'.

In the present study, the application of the fresh leaf gel was used to mimic the use of the gel as applied in traditional healing practices.

2. Materials and methods

2.1. Plant material

Plant material was sourced from the Walter Sisulu Botanical Gardens in Roodepoort, Johannesburg, South Africa, following confirmation of the taxonomic identity of the plants by the principal horticulturalist. Voucher specimens (voucher numbers: *Bulbine frutescens*, J95815, and *Bulbine natalensis*, J95814) have been deposited in the C.E. Moss Herbarium of the School of Animal, Plant and Environmental Sciences of the University of the Witwatersrand, Johannesburg.

The fresh gel from the mature leaves of *Bulbine frutescens* and *Bulbine natalensis* was extracted from the leaf under sterile conditions.

The water content of the fresh leaf from the plants was estimated by lyophilising harvested plant leaves. Briefly, plant leaves were harvested, washed in cold water, trimmed and weighed. The washed leaves were then immersed in boiling water for 1 min before blending in a liquidizer. The solution was then decanted into sterile specimen jars and frozen at −80 °C for 3–4 h before lyophilising in a lyophiliser. The lyophised product yielded from each plant species was then weighed: 100 g of *Bulbine natalensis* and *Bulbine frutescens* leaves yielded 3.32 g and 3.89 g respectively.

2.2. Experimental animals

Animal ethics clearance for this study was granted by the Animal Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa (AESC #2006 2804). Post-weaning female pigs (*Sus scrofa domestica*) were chosen for this study due to the similarities of pig and human skin. A total of twelve post-weaning pigs, which weighed between 20 and 30 kg, were used in this study.

2.3. Wound creation

As skin thickness, wound contraction and healing can depend on the site of injury, mirror-image control (untreated) wounds were used. Mirror-image wounds have the advantage of providing a control site at an anatomically similar position to the 'experimental' site with regard to location and orientation (Gonçalves et al., 2007). The animals were divided into two groups of six animals each viz. a *Bulbine frutescens* and a *Bulbine natalensis* group. Prior to wounding, the animals were anaesthetised with halothane. The skin on the dorsal aspect of all the animals was shaved. Each of the animals had two sets of mirror-imaged wounds created on either side of the dorsal midline as follows: (a) 2 cm × 2 cm full-thickness incisional wound, closed using interrupted sutures (used for testing the tensile strength of the repaired skin); (b) 5 × 0.4 mm full-thickness excisional wounds made with a biopsy puncture (used for biochemical and histological analysis). The wounds were treated daily with 1 ml of the corresponding leaf extract. All wounds were covered with Opsite Flexigrid® and a veterinary sock. Opsite Flexigrid® is transparent, adhesive film that is moisture vapour permeable, conformable and extensible. It is clinically proven to provide an optimal milieu for the moist healing of superficial wounds (Mennen and Wiese, 1993; Foster et al., 1994). The excisional wound together with a uniform perimeter of surrounding tissue was harvested on days 2, 4, 7, 10 and 16 following wounding. The outer margin of the excisional wounds was traced on transparent film immediately before performing a biopsy. The excised tissue was then bisected; one half was stored at −80 °C for biochemical analysis and the other half was fixed in 10% buffered formalin for histological analysis. At the time of harvesting, all wounds were photographed.

2.4. Rate of contracture and period of re-epithelialisation

The time taken for full re-epithelialisation of the wound biopsies was noted. The time for the excisional wounds to 'close' was calculated from the digital photographs using the ImageJ® software program and from the wound tracings. The change in wound size over a period of 16 days was then calculated as the percentage of the original wound area.

2.5. Measurement of tensile strength

The tensile strength of the treated and untreated incisional wounds harvested on day 16 was examined using a method adapted from Shukla et al. (1999) and Cho Lee and Moon (2003). The harvested 16-day incisional wounds were trimmed into strips 20 mm long and 2 mm wide, with the area of the original wound lying lengthwise in the centre of the sample. The TA. XT. plus Texture Analyser® system was used to measure the maximum force and time taken for the skin samples to snap or break.

2.6. Biochemical analyses of the excisional wounds

The excised tissue was biochemically analysed to estimate the total amount of collagen, hexosamine, protein and DNA present in the treated and untreated wound tissue as detailed below. Each of these tests was repeated six times.

2.6.1. Estimation of collagen (hydroxyproline)

Hydroxyproline is a basic constituent of collagen (Shukla et al., 1999). The collagen content of the granulation tissue was determined by estimating hydroxyproline content, as described by Woessner (1961) and modified by Switzer (1991).

Briefly, the wet tissue samples removed on each of the biopsy days were weighed, homogenised in saline and NaOH and autoclaved. The hydrolysis solution was incubated with chloramine-T

reagent at room temperature and then with freshly prepared p-dimethylamino-benzaldehyde (Ehrlich's reagent) solution at 60 °C. Following incubation, the solution was read at an absorbance of 550 nm using a spectrophotometer. Hydroxyproline content was then determined by comparing the absorbance obtained from the skin samples with a standard curve obtained from hydroxyproline analysis.

2.6.2. Estimation of hexosamine

Hexosamine levels will decrease as collagen accumulates (Dunphy and Udupa, 1955). This factor is therefore used to deduce the increase in collagen concentrations in the tissue. The hexosamine content of the granulation tissue was estimated by a modified Elson–Morgan reaction developed by Rondle and Morgan (1955) and modified by Levvy and McAllan (1959).

Briefly, the weighed samples for each of the biopsy days were homogenised in 50 μl saline and then vortexed with 50 μl NaOH. This solution was then autoclaved at 120 °C for 20 min. Thereafter, 100 μl of the solution was transferred into Eppendorf tubes and 20 μl of potassium tetraborate was added. This solution was then vortexed and heated for 3 min before cooling to room temperature. Following cooling, 600 μl of Ehrlich's reagent was added and then incubated at 37 °C for 20 min. The absorbance of the solution was then measured at 530 nm using a spectrophotometer. The amount of hexosamine of each sample was extrapolated using a standard curve for hexosamine obtained by using serial dilutions of 0.1% N-acetylglucosamine.

2.6.3. Estimation of protein (Lowry protein assay)

Sample tissue removed on each of the biopsy days was weighed and then homogenised with 5 ml of homogenising buffer with DTT and protease inhibitor cocktail stock (Sigma–Aldrich Co., USA). The homogenised solution was centrifuged at 12 000 rpm for 20 min at 4 °C. The supernatant was collected. The total protein content of supernatant of each sample was then determined by using the Lowry protein assay. The assay is based on the addition of the folin phenol reagent which forms a complex with protein and copper thus resulting in a colour change (from yellow to blue) (Hartree, 1972). On each biopsy day, 200 μl of sodium carbonate with copper sulphate and sodium potassium tartrate was added to 50 μl of the diluted protein sample. This solution then was incubated for 10 min at room temperature on a shaker. Thereafter, the solution was incubated for 30 min with 50 μl of folin reagent. The absorbance of the solutions in the microtiter plate was read at an absorbance of 690 nm. The protein content of each sample was extrapolated from a standard curve obtained using bovine serum albumin (BSA).

2.6.4. Estimation of total DNA content

The protein and DNA content of granulation tissue indicates the levels of protein synthesis and cellular proliferation. Higher protein and DNA content will indicate cellular proliferation and suggest an increase in the synthesis of collagen. According to Chithra et al. (1998a), the collagen/DNA ratio in granulation tissue may be taken as the index of the synthesis of collagen per cell in the wound area.

DNA content was extracted from samples for each of the biopsy days using the phenol–chloroform method. Weighed tissue was incubated overnight on a heat block at 55 °C with 200 μl lysis buffer and 20 μl of Proteinase K. The solutions were then heated on the heat block at 85 °C for 10 min. Thereafter, 400 μl of a phenol–chloroform mixture was added and vortexed. This solution was centrifuged for 3 min at 12 000 rpm. The upper phase in each Eppendorf tube was then carefully harvested. In this step, it was important not to remove the protein layer at the interface. The phenol–chloroform mixture was then added and the mixture centrifuged for 3 min at 12 000 rpm. Again, the aqueous upper phase was carefully pipetted into a sterile labeled Eppendorf tube containing 40 μl of 3 M sodium acetate and 280 μl isopropanol. The Eppendorf tubes were then centrifuged at 14 000 rpm for 30 min. The small pellet visible at the bottom of each Eppendorf tube was harvested. Thereafter, 500 μl of 70% ethanol was added and the tube was centrifuged for 2 min. The ethanol was removed from the tube and the pellet dried at 65 °C. The pellet was then resuspended in 100 μl MilliQ™ water and incubated on a heat block at 65 °C for 10 min. The total amount of DNA in each of the samples was then measured in triplicate using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

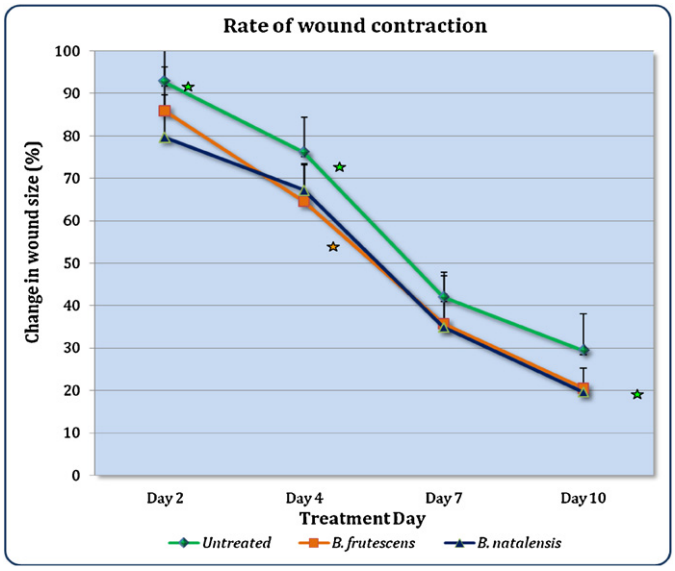

Fig. 1. Percent of contraction for *Bulbine natalensis*-, *Bulbine frutescens*-treated and untreated wounds. ⋆ indicates significant differences with untreated wounds.

3. Results

3.1. Rate of contracture and period of re-epithelialisation of wounds

Both treated and untreated wounds of all the pigs were fully re-epithelialised by day 7 (i.e. *Bulbine natalensis*: $n = 6$ and *Bulbine frutescens*: $n = 3$ pigs; untreated wounds, $n = 12$). In three pigs treated with *Bulbine frutescens*, complete re-epithelialisation occurred earlier, that is, by day 4. The area of the wounds treated with the leaf extracts was indistinguishable from the surrounding normal skin by day 10. However this occurred later in the untreated wounds, i.e. on day 16.

The percent of wound contraction for untreated wounds and *Bulbine natalensis*- and *Bulbine frutescens*-treated wounds is shown in Fig. 1. There was a significant increase in wound contraction in the *Bulbine natalensis*-treated wounds when compared to the untreated wounds on days 2, 4 and 10 ($p = 0.004$, $p = 0.007$ and $p = 0.03$, respectively). In the *Bulbine frutescens*-treated wounds, a significant increase in wound contraction was evident only on day 4 ($p = 0.004$) (Fig. 1) when compared to that of the untreated wounds. There was no significant difference in the rate of contraction of the wound on any of the treatment days between the *Bulbine natalensis*- and *Bulbine frutescens*-treated wounds.

3.2. Tensile strength of wounds

The tensile strength of the wounds treated with *Bulbine frutescens* and *Bulbine natalensis* was significantly higher than that of the untreated wounds ($p = 0.002$ and $p = 0.019$, respectively), with the *Bulbine natalensis*-treated wounds being stronger than the *Bulbine frutescens*-treated wounds ($p = 0.02$).

Table 1
Summary of collagen concentration (mg/100 ml) per day following treatment with *Bulbine* gel.

Day	*Bulbine natalensis*-treated animal group			*Bulbine frutescens*-treated animal group		
	Treated wounds	Untreated wounds	Treated:untreated	Treated wounds	Untreated wounds	Treated:untreated
2	20.31 ± 2.07	13.34 ± 2.56	1.52	15.97 ± 3.91	12.91 ± 1.40	1.24
4	16.27 ± 0.97	15.58 ± 5.75	1.04	15.29 ± 0.96	12.30 ± 2.01	1.24
7	21.32 ± 4.86	16.15 ± 0.65	1.32	19.87 ± 6.63	13.92 ± 3.12	1.43
10	26.54 ± 5.44	8.47 ± 1.41	3.13	16.33 ± 0.49	7.66 ± 3.82	2.13
16	16.89 ± 2.32	10.91 ± 2.04	1.54	12.81 ± 1.75	9.10 ± 5.31	1.41
Mean for the 16 day period			1.71	Mean for the 16 day period		1.49

3.3. Collagen (hydroxyproline) content of wounds

The collagen content in the granulation tissue of the treated and untreated wounds for *Bulbine natalensis* and *Bulbine frutescens* is shown in Table 1. The mirror-imaged untreated wounds for each treatment group showed no significant difference ($p = 0.386$) to each other. The collagen content of the untreated tissue gradually increased from day 2 and reached a peak on day 7. On day 10 there was a sharp decrease in collagen content with an increase again on day 16.

Treatment with *Bulbine natalensis* caused a significant difference in the collagen content of the wound tissue compared to that of the untreated wounds ($p = 0.014$). The collagen content in the *Bulbine natalensis*-treated group increased to peak at day 10, when it was 3.13 times more than in the untreated wounds (Table 1). Although this was followed by a decrease on day 16, the collagen content was still greater (1.54 times) than that of the untreated wounds on the same day. Over the duration of the 16 days of treatment, the average collagen content of the *Bulbine natalensis*-treated wounds was 1.71 times greater than that of the untreated wounds (Table 1). Thus *Bulbine natalensis* caused a later but larger peak in hydroxyproline content of the wounds.

Similarly, treatment with *Bulbine frutescens* resulted in a significant difference between the collagen content in the *Bulbine frutescens*-treated and untreated wounds ($p = 0.018$). In this treatment group however, the collagen content of the treated wounds peaked earlier than in the *Bulbine natalensis*-treated group, i.e. on day 7, being 1.43 times that of the untreated wounds (Table 1). Although the collagen content was lower on day 10 than on day 7 in the *Bulbine frutescens*-treated group, the difference in the concentration between the treated and untreated wounds was at its greatest (treated wounds were 2.13 times the collagen content compared to the untreated wounds). Over the duration of the 16-day treatment period, the collagen content of the *Bulbine frutescens*-treated wounds was 1.49 times that of the untreated wounds (Table 1).

There was no significant difference between the *Bulbine natalensis* and *Bulbine frutescens*-treated groups ($p = 0.089$), although collagen content of the treated wounds peaked on different days (day 10 and 7, respectively). In both groups, the highest ratio of the collagen concentration of the treated:untreated wounds occurred on day 10.

3.4. Hexosamine content of wounds

There was no significant difference in the hexosamine content of the untreated wounds of the two treatment groups ($p = 0.55$). In the untreated wounds of both groups, the content of hexosamine was at its maximum on day 4 followed by a gradual decrease to day 16. There was also no significant difference between the *Bulbine natalensis*- and *Bulbine frutescens*-treated groups ($p = 0.64$). Treatment with either leaf gel followed the same pattern in hexosamine content (Table 2) as the untreated wounds, with maximum hexosamine content on day 4 followed by a steady decrease to day 16. In both the treated and untreated wounds, there was an overall decrease in hexosamine content between day 2 and day 16. Over the duration of the 16-day treatment period, the hexosamine content (mean) of the *Bulbine natalensis*- and *Bulbine frutescens*-treated groups was 1.08 and 1.07 times greater than that of the untreated groups, respectively (Table 2). In both treatment groups the highest ratio of treated:untreated wounds occurred on day 7 with a gradual decrease to day 16. The lowest ratio was prevalent in wounds on day 16.

3.5. Protein content of wounds

Over the 16-day treatment period, the untreated wounds for both treatment groups displayed no significant difference from each other ($p = 0.79$) (Fig. 2a and b). In these groups, a rapid increase in the protein content from day 4 to day 7 preceded a sharp decrease to day 16. Both groups of treated wounds followed a similar pattern in protein content to that of the untreated wounds, in that the highest protein content was prevalent on day 7 and then gradually decreased to day 16 (Fig. 2a and b).

Treatment with *Bulbine natalensis* appeared to significantly increase the protein content of the wounds over that of the untreated wounds ($p = 0.03$). Over the duration of the 16-day treatment period, there was an increase in the protein content in all of the treated wounds. This increase followed the same pattern as the untreated wounds until day 7. On day 10, a major decrease in the amount of protein was noted in the untreated wounds compared to the treated wounds (Fig. 2a). *Bulbine frutescens* treatment, similarly to *Bulbine natalensis* treatment, increased the protein content of the wounds significantly over that of the untreated wounds ($p = 0.04$) over the 16-day treatment period. This increase followed the same pattern as the untreated wounds until day 7. On day 10,

Table 2
Summary of hexosamine concentration (mg/100 ml) per day following treatment with *Bulbine* (B.) gel.

Day	*Bulbine natalensis*-treated animal group			*Bulbine frutescens*-treated animal group		
	Treated wounds	Untreated wounds	Treated:untreated	Treated wounds	Untreated wounds	Treated:untreated
2	54.96 ± 0.66	57.70 ± 4.21	1.05	51.51 ± 1.44	52.53 ± 11.61	1.02
4	73.66 ± 13.38	83.93 ± 8.14	1.14	69.38 ± 13.62	79.32 ± 20.51	1.14
7	57.72 ± 12.10	71.63 ± 2.05	1.24	53.43 ± 11.54	61.72 ± 14.58	1.16
10	49.16 ± 4.19	48.65 ± 15.80	0.99	43.02 ± 2.18	45.56 ± 13.09	1.06
16	46.41 ± 1.80	41.41 ± 3.36	0.89	43.13 ± 1.41	39.11 ± 2.44	0.91
Mean for the 16 day period			1.08	Mean for the 16 day period		1.07

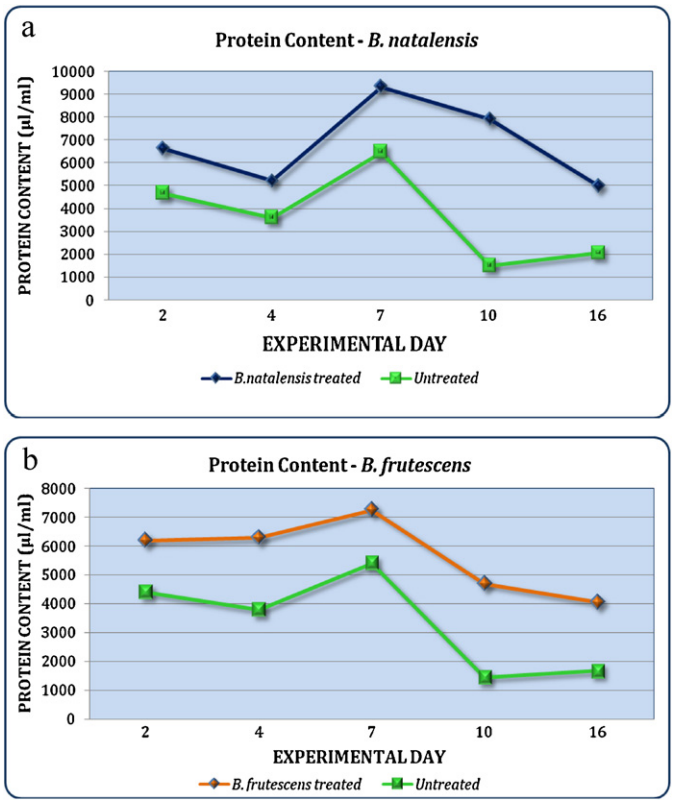

Fig. 2. (a) Protein content of *Bulbine natalensis*-treated and untreated wounds. (b) Protein content of *Bulbine frutescens*-treated and untreated wounds.

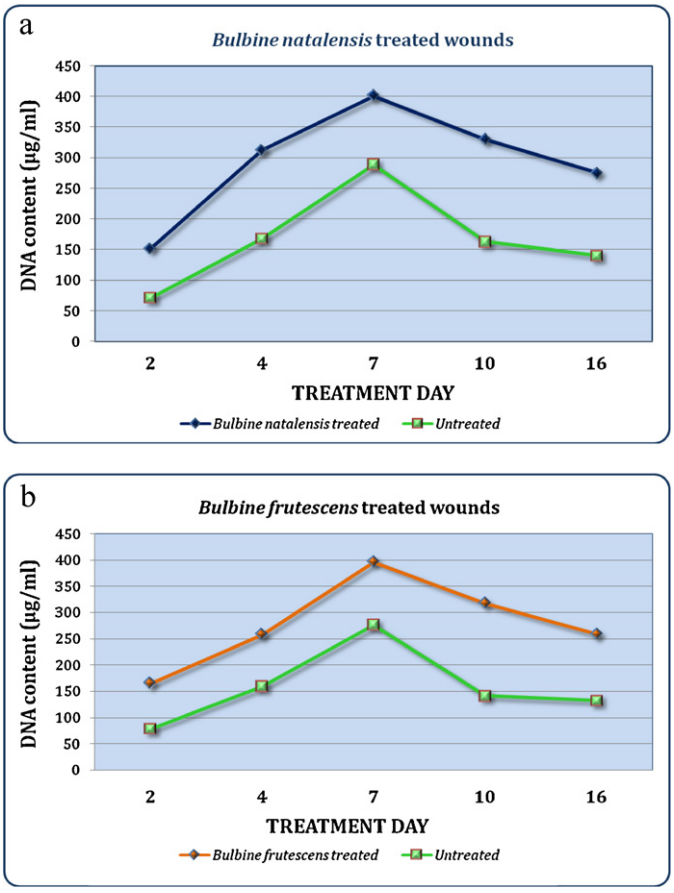

Fig. 3. (a) DNA content of *Bulbine natalensis*-treated and untreated wounds. (b) DNA content of *Bulbine frutescens*-treated and untreated wounds.

the decrease in the amount of protein in the untreated wounds was slightly greater than in the treated wounds.

There was no significant difference in the protein content between the two treated groups ($p = 0.30$). The mean ratio of protein in the *Bulbine natalensis*- and *Bulbine frutescens*-treated groups compared to the untreated wounds was 2.38 and 2.02, respectively.

3.6. DNA content of wounds

The total DNA content of the untreated wounds of both groups displayed a similar pattern (Fig. 3a and b). There was an increase in DNA content to day 7, followed by a sharp decrease to day 10. There was no significant differences between the untreated groups ($p = 0.87$).

Treatment with both leaf gels significantly increased the DNA content of the wounds compared to the untreated wound (*Bulbine natalensis*, $p = 0.04$ and *Bulbine frutescens*, $p = 0.04$). The effect of *Bulbine natalensis* and *Bulbine frutescens* was not significantly different from each other ($p = 0.80$). Over the 16 day treatment period, there was a noticeable increase in DNA content in both sets of treated groups compared to the untreated wounds (*Bulbine natalensis*, 1.78 and *Bulbine frutescens*, 1.77 times that of the control). However, in the group treated with *Bulbine natalensis* there was a more gradual increase in the DNA content from day 4 to day 7 than in the *Bulbine frutescens* group.

3.7. Collagen: DNA ratio of wounds

The ratio of collagen content to DNA content is said to be an indication of the synthesis of collagen (Chithra et al., 1998a). The ratio of collagen:DNA content for both treatment groups and the untreated control wounds is given in Table 3. There was no significant difference between treatment with either *Bulbine natalensis* or *Bulbine frutescens* compared to the untreated wounds ($p = 0.57$ and $p = 0.37$, respectively). There was no statistically significant difference between both untreated groups.

4. Discussion

In this study, treatment of the wounds with either of the leaf gels appeared to initiate wound contraction on days 2 and 4 which is earlier than in the untreated wounds. These results are comparable to the effect of *Aloe vera* on diabetic wounds in the male Wistar rat (Chithra et al., 1998a), where wounds treated with topical *Aloe vera* contracted 10% faster than the untreated wounds. Similarly, Rasik et al. (1999) demonstrated increased wound contraction in guinea pig skin treated with 1% *Calotropis procera* (a well known plant of the family Asclepiadaceae, used in Ayurvedic medicine).

Wound contraction is fibroblast-dependant and involves the deposition and maturation of collagen. The role of collagen in wound healing commences immediately the wound is formed and continues for months after it appears to have healed. Collagen is the predominant extracellular protein in the granulation tissue of wounds (Chithra et al., 1998b). Immediately following injury, there is an increase in the synthesis of collagen in the wound area. Collagen plays a role in haemostasis and in providing strength and integrity to the wound matrix. It is also essential for re-epithelialisation and cell–cell and cell–matrix interactions (Raghow, 1994; Chithra et al., 1998a).

Hydroxyproline is the major constituent of collagen and is found almost exclusively in collagen. The estimation of hydroxyproline is an accepted method of biochemically evaluating the total collagen content of a sample (Lin et al., 2003) and is also used as a marker

Table 3
Summary of collagen:DNA content per day following treatment with *Bulbine (B.)* gel.

Day	*Bulbine frutescens*-treated animal group		*Bulbine natalensis*-treated animal group	
	Treated	Untreated	Treated	Untreated
2	0.096	0.163	0.133	0.186
4	0.059	0.077	0.052	0.093
7	0.050	0.050	0.053	0.056
10	0.051	0.054	0.080	0.052
16	0.049	0.069	0.061	0.078

of collagen synthesis (Rasik et al., 1999). A biochemical analysis of the excisional wound tissue of both *Bulbine natalensis*- and *Bulbine frutescens*-treated wounds demonstrated a significant increase in the total collagen content compared to that of the untreated wounds. In all three groups, the total collagen content reached a maximum on day 7 but was higher in the treated groups. Chithra et al. (1998a) reported a similar pattern in their biochemical estimation of collagen content (with maximal collagen content on day 8) in the wounds of diabetic rats treated with *Aloe vera* and their corresponding untreated control wounds. According to Chithra et al. (1998a,b) and Lin et al. (2003) an increase in collagen may be attributed to an increase in collagen synthesis or an increase in the proliferation of fibroblasts which synthesise collagen, or both.

Glycoaminoglycans and proteoglycans are synthesised by fibroblasts in the wound area. These substances form a hydrated gel-like ground substance (the provisional matrix) on which collagen is deposited. As the collagen content increases, hexosamine levels decrease (Dunphy and Udupa, 1955; Chithra et al., 1998b). Estimation of hexosamine therefore, estimates the amount of ground substance in a wound (Chithra et al., 1998b). From the biochemical estimation of hexosamine in the present study, it was found that treatment with *Bulbine natalensis* and *Bulbine frutescens* resulted in a maximal concentration of hexosamine on day 4 followed by a steady decrease to day 16. This was associated with a concomitant increase in total collagen content in both treated groups on day 7. This indicates replacement of granulation tissue in the wound area by collagen.

Collagen imparts tensile strength and elasticity to healed skin. An increase in collagen in the treated wounds corresponds with the significantly increased tensile strength in both treated groups of incisional wounds over that in the untreated wounds. According to Singer and Clark (1999), wounds gain 20% of their final strength in the first 3 weeks post-wounding. Thereafter, the rate at which the wounds gain tensile strength is reduced. Scar tissue will only ever reach 70% of the strength of normal unwounded tissue over a period of 2 years (Singer and Clark, 1999). The tensile strength of a wound can be related to its collagen formation and maturation. As the wound heals, collagen molecules are synthesised and laid down at the wound site. These molecules become cross-linked to form fibres. The strength of the repaired wound tissue is a result of the remodelling of collagen and the formation of stable intra- and inter-molecular cross linking.

These results may imply that the leaf gel extracts are able to increase collagen synthesis and possibly even aid in formation of cross linkages as the collagen matures. Shukla et al. (1999) demonstrated a 53% increase in tensile strength of wounds treated with 0.2% asiaticoside (isolated from *Centella asiatica*, a plant widely used in eastern traditional medicine for its wound healing properties) compared to untreated wounds in a guinea pig wound healing experiment. Chithra et al. (1998b) confirmed the earlier results of Davis et al. (1987) who showed that treatment of wounds with *Aloe vera* increased collagen concentration and tensile strength when compared with the untreated wounds. Chithra et al. (1998b) attributed this to an increase in the aldehyde groups of collagen fibres responsible for forming cross-linkages and therefore resulting in greater tensile strength of the *Aloe vera* treated wounds. Subramanian et al. (2006) studied rabbit skin excisional wounds (harvested on days 7 and 14 post-wounding) treated with *Aloe vera* and concluded that *Aloe vera* increased wound contraction and collagen synthesis and significantly increased protein and DNA synthesis. This was attributed to mannose-6-phosphate known to be present in *Aloe vera* leaf gel (Subramanian et al., 2006). Mannose containing products have been shown to increase macrophage activity and therefore stimulate fibroblast activity (Tizard et al., 1989; Davis et al., 1994).

The protein and DNA content of granulation tissue is said to indicate the levels of protein synthesis and cell proliferation (Rasik et al., 1999). Bourguignon and Bourguignon (1987) state that an increase in protein content is due to an increase in collagen synthesis. In the present study, the total protein and DNA content of both treatment groups were similar. They reached a maximum on day 7 followed by a decline to day 16 and were significantly greater than in the untreated wounds. Chithra et al. (1998a,b) suggests that when the protein and DNA content of treated wounds are greater than in the untreated wounds, it implies that the treatment stimulates cell proliferation. Thus, it is suggested here that the *Bulbine natalensis* and *Bulbine frutescens* leaf gels appear to stimulate cell proliferation. Shukla et al. (1999) demonstrated a significant increase in DNA content but no increase in protein content in guinea pig skin wounds treated with a 0.2% asiaticoside isolated from *Centella asiatica*. They attributed this discrepancy to the specificity of the assays used for the estimation of the different amino acids.

Collagen:DNA ratios are said to indicate the collagen produced per cell. In the present study, the collagen:DNA ratios showed no significant difference between the untreated wounds and the two treatment groups. There may be several reasons for the similarity of the collagen:DNA ratios in these three groups, e.g. a rapid cell proliferation initiated by the inflammatory response or an initial exponential increase in cell numbers, followed by a stationary phase. *In vivo* studies on wound healing are generally carried out over a period of 7 days post-wounding. The 16-day experimental period in the present study using the pig model was essential to sample the different phases in wound healing. It is possible that increasing the experimental time even further may demonstrate collagen remodelling more clearly. In wound experiments on the red Duroc pig, Zhu et al. (2005) demonstrated that skin is restored to its normal structure in 6–8 weeks. New collagen formation continues for up to 6 weeks post-wounding. Thereafter, the fibroblasts stop producing collagen and undergo apoptosis. Remodelling of the collagen fibres by degradation and re-synthesis allows the wound to gain strength. Remodelling continues for up to 2 years and the resulting scar is less cellular than normal skin and never achieves the same tensile strength as uninjured skin. A longer experimental period would provide insight into the long term effects of the leaf gel on tensile strength and collagen maturation.

The present study demonstrated that the fresh leaf gels of *Bulbine frutescens* and *Bulbine natalensis* have a beneficial effect on collagen synthesis and hence on wound contraction resulting in faster healing than in the untreated wounds. Data from a histological study of these extracts on pig skin (Pather, 2009) demonstrated

enhanced wound contraction with a corresponding increase in collagen deposition. In addition, there was an increase in the presence of myofibroblasts. These results suggest that these leaf gels promote wound healing by increasing cell proliferation and collagen deposition. Widgerow and Chait (2000) describe the use of a microporous tape treated with both *Bulbine frutescens* and *Centella asiatica*. They claim that the *Bulbine frutescens* increases hydration by 'leaving a layer of fatty vesicles of glycoprotein on the skin surface which also has anti-bacterial properties'. This claim was previously purported by Briggs (1995). While this may be so *in vivo*, it does not account for the significant increase in collagen and protein content seen in the present study which suggest that both leaf gels stimulate collagen deposition and possibly increase fibroplasia. A similar reason for the efficacy of *Aloe vera* in wound healing ws (1980), Fox (1999), Marshall (1990) and rth (1997) however showed that *Aloe vera* ating oral wounds that were always moist. The present study proposes that the leaf gel of both *Bulbine natalensis* and *Bulbine frutescens* plants contain active ingredients (yet to be isolated) that impacts keratinocyte and fibroblast proliferation (Pather, 2009) and collagen deposition which could be the major contributor to increased ECM deposition and wound healing. In addition, both leaf gels increase wound contraction by increasing the differentiation of fibroblasts into myofibroblasts (Pather, 2009).

Increase collagen maturation, not fibroplasia! therefore quicker healing

Naturally derived compounds are purported to be the most relevant compounds for future drug discovery, yet the biological activity of much of the world's biodiversity remains scientifically untested (Harvey, 2001, 2008). This is particularly true for many of the indigenous traditional medicines of South Africa. Despite the anecdotal evidence of the use of *Bulbine* species as a traditional wound healing agent and more recently its appearance locally in a variety of 'healing' lotions and creams, the literature does not present any scientific data supporting the wound healing capabilities of either *Bulbine frutescens* or *Bulbine natalensis*. In rural communities in South Africa, skin lesions arising from bruises, cuts and scratches amongst others are sometimes untreated at the initial stages, especially in children (Grierson and Afolayan, 1999). In most cases such wounds become infected and septic. Primary health care in these situations is often sought from traditional healers and herbalists. The validation of herbal remedies is therefore essential and in addition to preserving the indigenous heritage, has an enormous beneficial value to these communities.

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