

Topically applied *Terminalia sericea* (Combretaceae) leaf extract and terminoic acid on infected wounds lead to better wound healing outcomes than gentamicin in an animal model

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Plants are well-known sources of compounds with biological activity and thus have been used by mankind for centuries as medicines. *Terminalia sericea* occurs widely in southern Africa in sandy soils. It has been used traditionally to treat abscesses and wounds.

This study investigated the antibacterial properties and wound healing activity of an acetone leaf extract of *Terminalia sericea* and terminoic acid, an isolated compound. The backs of 10 Wistar rats were shaved and incisions made in four different areas to which the two treatments, a positive control (gentamicin) and no treatment, were applied 48 hours after the wounds were superficially infected with *Staphylococcus aureus*. The wounds were monitored for exudate formation, erythema and size, daily for five days. The leaf extract and terminoic acid treatment had positive effects on the exudate production and erythema, superior to gentamicin treatment and the negative control over the treatment period. All treatments had an effect on the size of the wounds after five days. The effects of the treatment may be attributed to the modulation of wound healing by pathways other than, or in addition to, antibacterial action of phytochemical constituents. This study shows that the extracts have the potential for pharmaceutical development into topical formulations for possible use in wound treatment.

Introduction

The emergence of antimicrobial resistance (AMR) is now recognised as a public health emergency with the United Nations convening Interagency Coordination Group on Antimicrobial Resistance (IACG) formed to address the problem. In Europe an estimated 25 000 people die each year from antibiotic-resistant bacteria (Control & Agency 2009), while in the US, 90 000 infections of methicillin-resistant *Staphylococcus aureus* (MRSA) are reported, with 21% of the patients dying annually (Carlet, Jarlier *et al.* 2012; Carlet & Mainardi 2012).

Antimicrobial resistance arises as a result of inappropriate antibiotic prescription and use (Weber 2005) and the use of antimicrobials to maintain and increase feed efficacy in animals (McEwen & Fedorka-Cray 2002; Durso & Cook 2014). The lack of investment in the antibiotic drug discovery pipeline may also play a role (Spellberg, Powers *et al.* 2004). There is therefore a need for new antimicrobial agents. Bacterial infections frequently occur when a wound is infected.

Wounds are a disruption of normal skin anatomy and function which may result from physical, chemical, thermal, microbial, or immunological causes (Strodtbeck 2001). Globally, chronic wounds are a growing public health burden (due to increased incidence of diabetes, obesity and cancer) and drain healthcare budgets (Harding *et al.* 2002).

Chronic wounds that do not heal without specialised treatment affect people who are already sick. In the 1990s in the United Kingdom around 24 000 people were admitted for treatment of diabetic foot ulcers (DFU) at a cost of £17m (Harding, Morris *et al.* 2002). In the USA 6.5 million people suffer from chronic wounds and the global wound care products market was estimated to be worth US\$15.3 billion by 2010 (Sen, Gordillo *et al.* 2009). In Africa, where infectious diseases and non-communicable diseases (NCDs) co-occur, acute and chronic wounds are a major cause of morbidity and mortality.



FIGURE 1: The rat as marked and treated in the experiment.

Wound healing is a complex process which is affected by various factors including bacterial colonisation and infection which tend to delay the healing process (Edwards & Harding 2004). The genus *Terminalia* contains about 250 species worldwide, of which 11 occur in southern Africa (Carr 1988). *T. sericea* Burch. Ex DC is dominant in southern Africa. Known as Vaalboom (Afrikaans), Silver cluster-leaf (English), and Mokonono (Shona), it is used in traditional medicine for the treatment of various conditions including abscesses and wounds, abdominal pain, intestinal worms, diarrhoea, sore throat and dilatation of the birth canal (Arnold & Gulumian 1984; Tshikalange, Meyer *et al.* 2005). In this study, extracts and a pure compound, terminoic acid isolated from *T. sericea*, were applied to wounds infected with *Staphylococcus aureus*. *T. sericea* was selected because preliminary *in vitro* antimicrobial assays had shown the activity of its acetone leaf extract was better than that of *T. prunoides*, *T. phanerophlebia*, *T. gazensis*, *T. sambesiaca*, *T. mollis* and *T. brachystema* (Kruger, 2004).

Material and methods

Ethics approval was given by the Research and Animal Use and Care Committee of the Onderstepoort Veterinary Institute (OVI). The study was designed as a true experimental design with self-control. This experiment was carried out in 2004 (Kruger, 2004) and the same approach and methods with some improvements were used by Masoko *et al.* (2010) on fungal wound infection. The reason for the delay in publication was because one of us (JPK) developed a formulation of the crude extract and it has successfully been patented, registered and marketed as Phytobiotic.

Eleven *Rattus norvegicus* Wistar rats were kept at the Department of Toxicology, Onderstepoort Veterinary Institute of the Agricultural Research Council (ARC). The hair on the back of each of the test rats was removed with scissors and the skin shaved with a surgical blade. The area was sterilised with 70% alcohol and allowing the alcohol to evaporate. The rats were sedated with a benzodiazepine and left for 15 minutes for the drug to take effect. Lesions were made by cutting four roughly circular areas with a pair of scissors and the test organism (*Staphylococcus aureus* ATCC 29213) was introduced onto the test area. The area was covered with an occlusive wrapping (TransporeR) and left to incubate for 48 hours after which the two treatments, gentamicin (positive control) and negative control, were applied. Each animal therefore served as a control in itself by having two test sites for the crude and isolated compound, one for a positive control with gentamicin and one site as a negative control (Figure 1).

The treatments were an acetone extract from *T. sericea* leaves formulated into a 20% (w/w) cream with emulsifying cream (A) and terminoic acid, a pure compound (Figure 2) isolated from the leaves of *T. sericea* in a previous study (Kruger 2004), formulated into a 1% (w/w) cream with emulsifying cream (TA). A 1% (w/w) gentamicin cream (Garamycin®) was used as the positive control. The negative control was the emulsifying cream. Each animal was only treated once at the beginning of the experiment

The effects of the treatments were evaluated by assessing the reduction in erythema (red discolouration of wound), exudate (puss) formation and physical size of the lesion, on a daily basis for five days. For erythema and exudate, a

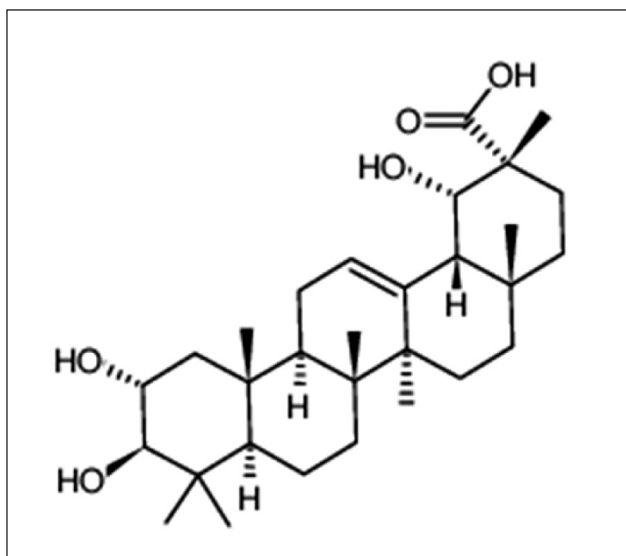


FIGURE 2: Chemical structure of terminoic acid.

scale from 0 to 5 was used, with 0 being the lowest degree of erythema or exudate formed and 5 the highest degree of erythema or exudate. At the end of the experiments the rats were euthanased and the histology was examined.

Results and discussion

The artificial colonisation of the wounds with *S. aureus* was deemed to have led to infection after 48 hours. Infected wounds display classic signs which include increasing pain in the ulcer area, erythema, oedema, exudate (both purulent and serous) and delayed healing, among others (Cutting 2004). In this study we assessed exudate production, erythema and wound size reduction as indicators for treatment efficacy.

The TSE and TA treatments showed similar effects on exudate production leading to a steep reduction over the treatment period (Table I, Figure 3). The treatments were more effective than the positive control and negative controls. On Day 5 of treatment the plant extract (TSE) value was 0,18 and the TA (0,36) compared to 0.91 for gentamicin and 1.00 for the negative control. The exudate production also decreased in the negative control over time (1,00 on Day 5) and was only slightly higher than the gentamicin (0,91) treatment. The reduction with no treatment indicated the possible innate ability of the immune system to resolve the infection. Exudate formation is important for providing a moist environment to allow a wound to heal; excessive exudate formation can however delay the healing process and consequently negatively affect the quality of life of patients (Dowsett 2011).

TABLE I: Effects of treatments on exudate production average of 10 values

	Day 1	Day 2	Day 3	Day 4	Day 5
Leaf extract 20%	2,27	2,09	1,09	0,82	0,18
1% terminoic acid	2,64	1,91	1,36	0,73	0,36
Negative control	2,73	2,91	2,18	1,73	1,00
1% gentamicin	2,82	2,27	1,82	1,27	0,91

In the production of erythema the extract and isolated compound had similar effects and were superior to the positive control (Table II, Figure 4). However, unlike with the exudate, the gentamicin-treated wounds already had higher erythema scores on Day 1 (3) which decreased to 1 by Day 5, while all the other wounds started off with an average score of 2.10. On Day 5, the TSE value was 0.18 compared to 0.36 for TA. The increase in erythema on Day 2 may be due to inflammation due to the infection.

There were no major differences in the size of the wounds by Day 5 between the treatments and the controls (Table III). The terminoic acid treatment, however, led to better wound reduction from Day 1 to Day 4. The wound

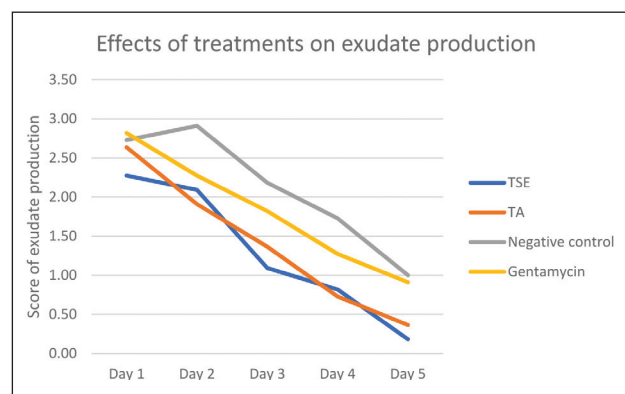


FIGURE 3: Effects of treatments on exudate production (TSE = *T. sericea* acetone leaf extract (20% w/w), TA = terminoic acid 1% w/w, Gentamicin 1% w/w, negative control only emulsifying cream).

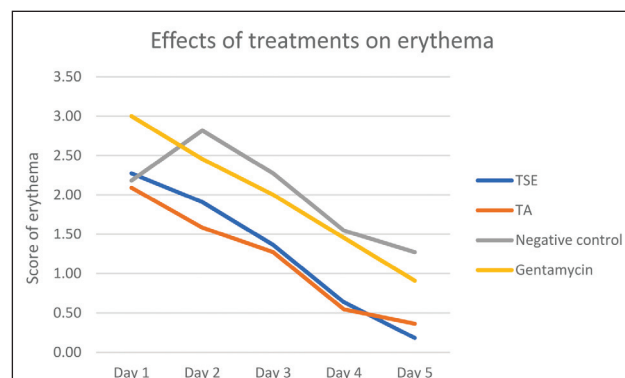


FIGURE 4: Effects of treatment on erythema.

TABLE II: Effects of treatment on erythema.

	Day 1	Day 2	Day 3	Day 4	Day 5
Leaf extract 20%	2,27	1,91	1,36	0,64	0,18
Terminoic acid 1%	2,09	1,58	1,27	0,55	0,36
Negative control	2,18	2,82	2,27	1,55	1,27
Gentamicin 1%	3,00	2,45	2,00	1,45	0,91

TABLE III: Effects of treatment on wound size.

	Day 1	Day 2	Day 3	Day 4	Day 5
Leaf extract 20%	3,36	4,09	3,00	2,82	2,50
Terminoic acid 1%	3,73	3,55	2,82	2,27	2,45
Negative control	4,18	4,36	3,55	2,73	2,64
Gentamicin 1%	4,36	3,91	3,45	3,18	2,82

reduction was practically the same in the negative control as in the positive control and the two treatments. This may be due to the high immune surveillance in the rats, which were relatively healthy apart from the wounds. In subsequent experiments (Masoko *et al.* 2010) in the same laboratory, rodents were treated with an immune suppressant to simulate the situation when immune-compromised patients have wounds.

A *post mortem* examination on the rats indicated no lesions or abscesses subcutaneously in the vicinity of the treated areas. This indicates that treatments were not systemically toxic.

Wound healing is a complex physiological process involving the interaction of epidermal and dermal cells, the extracellular matrix, angiogenic and plasma-derived proteins modulated by various cytokines and growth factors (Harding, Morris *et al.* 2002). This process is delineated into overlapping phases i.e. inflammation, angiogenesis, collagen deposition, re-epithelialisation, and tissue remodeling (Martin 1997).

In this study both the isolated compound and the crude extract had better activity than gentamicin cream. The isolated compound and the acetone extract may have influenced other stages in the wound healing process. They may, for example, have anti-inflammatory activity or induce cell proliferation or angiogenesis. The specific mode of action in addition to antimicrobial activity should be investigated in future.

Terminalia sericea is rich in polyphenolic compounds and tannins, but these compounds may not have been extracted by acetone. Polyphenolic compounds facilitate wound healing by modulating inducible vascular endothelial growth factor (VEGF) expression which stimulates wound angiogenesis. Sen, Khanna *et al.* (2002) found that tannin extracts from the related species *T. chebula* have antibacterial activity against *Klebsiella pneumonia* and *Staphylococcus aureus* and strong angiogenic effects ultimately resulting in wound contraction at the granulation formation and scar remodeling phases (Li, Diao *et al.* 2011).

We found that the terminoic acid formulation was as active as the crude extract. This indicates that it is not necessarily tannins or polyphenolics that are responsible for the wound healing effect. Terminoic acid is an oleanolic triterpenoid and a related compound lupeol causes epithelialisation faster with a high rate of wound contraction and collagenisation (Harish, Krishna *et al.* 2008). Triterpenoids from *Calendula officinalis* stimulated proliferation and migration of fibroblasts at low concentrations indicating that triterpenoids exert their wound healing effects in a different way from polyphenolic compounds (Fronza, Heinzmann *et al.* 2009).

A number of difficulties were identified in this experiment that were improved in work done subsequently on several extracts on rats in our laboratory (Masoko *et al.* 2010).

These were immunosuppressing the rats by subcutaneous injection of 500 µg of estradiol valerate at the start, using 0.5% chlorhexidine in ethanol to surface sterilise the skin, handling animals in a biosafety cabinet, inserting a temperature probe to monitor the temperature of the animals, measuring crust formation, treating animals three times a week, expanding the experimental period to 17 days, determining the mass of animals during the experiment, more exhaustive pathological examination at the end and determining the *in vitro* cellular toxicity of the extracts and isolated compounds. Masoko *et al.* (2010) also found that there were no differences in wound healing at the end of the experiment although there were differences in the rate of healing.

It appears that the main function of the extract is its antibacterial activity that was similar to that of the terminoic acid. The results obtained in exudate production and especially in erythema were better than those obtained by the positive control gentamicin.

Conclusion

Although traditional healers do not have acetone available as an extractant and water may not extract the antimicrobial compounds in *Combretum microphyllum* (Kotze & Eloff 2004), the results validate the use of *T. sericea* extracts in treating infections. Because *T. sericea* occurs very widely all over southern Africa in sandy soils, and only leaves are used, provision of plant material is not a problem should commercial supply for the pharmaceutical value chain be required. Acetone is also a low-cost extractant so the potential of developing a topical herbal product from this species to treat infections and wounds is a strong possibility. Future work could investigate anti-biofilm activity of the extracts as bacteria within chronic wounds live in a biofilm enabling the development of resistance to antibiotic treatment (Edwards & Harding, 2004).

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Authors' contributions

JPK conducted the experiment, DRK advised on the phytochemical extraction and was co-supervisor of JPK, JNE conceived the project and was the project leader. All authors were involved in drafting the paper.

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