Wound Medicine 25 (2019) 100152

Contents lists available at ScienceDirect

Wound Medicine

journal homepage: www.elsevier.com/locate/wndm

In vivo wound healing and antimicrobial activity of *Alkanna strigose*

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ARTICLE INFO

Keywords:
Alkanna strigose
Wound healing

ABSTRACT

Several plant preparations and extracts are used in folk medicine for treatment of topical wounds. Yet the validity of their use as well as the mechanism has not been ascertained in well-designed experimental settings. The wound healing activity of a topical hexane extract of *Alkanna strigosa* roots (HEASR) in excision and incision wound models in rats was evaluated. The pro-healing activity was assessed by the rate of wound contraction & wet granulation tissue weight and tensile strength in the excision and incision models, respectively.

The active components of HEASR were isolated. Finally, the antimicrobial activity against five microorganisms; namely *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Candida albicans* were assessed for both crude extract and pure compounds.

The HEASR increased the weight of wet granulation tissue per wound area and tensile strength of wounds, and showed antimicrobial activity against Gram-positive bacteria and *C. albicans*. The pure compounds were alkanin & shikonin and showed remarkable activity against *S. aureus*, *E. coli* and *B. subtilis*. The results document the beneficial effect of *A. strigosa* extract and its pure compounds for treating the supurative excision as well as the incision wounds.

1. Introduction

Herbal medicine has its origins in ancient cultures. There are over 750,000 plants on earth. Herbalists use the leaves, flowers, stems, berries, and roots of plants to prevent, and treat illnesses. Extracts and pure components of medicinal plants were evaluated for wound-healing properties in various animal models [1,2].

The primary role of skin is to serve as a defensive barrier against the environment. Loss of the integrity of large portions of the skin due to injury or illness may result in a significant disability or even death [3,4]. It was estimated in 1992 that there were 35.2 million cases of significant skin loss requiring major therapeutic intervention in the world. Of these, approximately 7 million wounds became chronic [5].

Wound healing is a dynamic process of biochemical and physiological changes that progresses orderly to re-establish the integrity of the damaged tissue. It involves inflammatory, proliferative and remodeling stages. In many cases, wounds are complicated by infection [6]. Earlier studies have shown that antimicrobial activity of various plants supports wound healing [7]. Therefore, plant extracts that have both antimicrobial and wound healing activities are highly sought for.

Alkanna tinctorium is well- known in folk medicine and used for its anti-inflammatory, anti-bacterial and wound healing properties. The plant is also used in cosmetic and textile industries [8].

A relevant species, *A. strigosa*, locally known as Hawa Jawi, is widely used in Jordan and surrounding area for the treatment of different ailments [9]. A few studies have been conducted on this species, showing unremarkable properties. The plant was shown to be deprived of inhibitory activity on alpha-amylase of female rats [10]. The aqueous and methanolic extracts of the plant revealed no significant antioxidant activity [11]. However, Oran reported an antitumor activity of the plant in Potato Disc Bioassay [12].

In fact, the most popular use of *A. srtigosa* among Jordanians in rural and urban areas is for the treatment of wounds and burns. The laymen and herbalists prepare the plant in ointment base to be applied directly on the wound or burn. Usually honey and bees wax are added to the formula. The users of this formula believe that it is more effective than some of the hospital procedures [13,14]. However, the activity and chemical composition of *A. strigosa* have not been experimentally explored yet.

This study was aimed at evaluating the wound-healing and antimicrobial properties of the hexane root extract of *A. strigosa*. Moreover, purified compounds of the extract were isolated and their antimicrobial activity evaluated.

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https://doi.org/10.1016/j.wndm.2019.100152
Received 8 January 2019; Accepted 27 March 2019
Available online 28 March 2019
2213-9095/

2. Materials and methods

2.1. Plant collection and identification

A. strigosa was collected locally in Jordan in March, 2008. The plant was taxonomically identified by Prof. Dawoud Al-Eisawi, a plant taxonomist, the Department of Biological Sciences, University of Jordan. Voucher specimen (*A. stigosa*, 008) has been deposited at the Department of Pharmaceutical Sciences, Faculty of pharmacy, University of Jordan. The air-dried plant was ground to a fine texture and stored in a well closed container for further use.

2.2. Extraction and purification

Dried, powdered root (250 g) was extracted in hexane (1 l) using Soxhlet apparatus for 3 h. The organic phase was evaporated to dryness using vacuum rotary evaporator at 50 °C to obtain a dry residue (6.2 g). One gram of the residue was dissolved in petroleum ether and ethyl acetate (9.5:0.5, v/v), and chromatographed over silica gel column (60 × 3.2 cm, 60–120 mesh, Merck). Different proportions of petroleum ether and ethyl acetate (100% to 50%) were used for elution. Fractions obtained were collected (20 ml) and combined according to TLC analysis (Kieselgel 60-F254, Merck) using ($CHCl_3$: MeOH: H_2O 5:4:1) as solvent system. Two partially purified fractions were recovered. These fractions were further purified with preparative TLC (silica gel 60, 20 × 20 cm, 0.25 mm layer thickness, Barcelona, Spain) with two resulting pure compounds, namely alkannin and shikonin.

2.3. Animal studies

2.3.1. Animals

A total of 88 adult Albino Wistar rats *Rattus norvgecis* UJ-1 weighing 140–170 g were used in the wound healing experiments. They were bred at the animal house of Applied Sciences University, Amman. The rats were fed with pelleted dried feed (Hammoda Company, Amman, Jordan) and were kept at conventional conditions of humidity, temperature, and light. Food and water were provided *ad libitum*. All animal procedures were conducted in accordance with Jordanian regulations for animal experimentation and care, and approved by the Committee of Institutional Animal Care and Use.

2.3.2. Wound creation

Healthy rats that did not have any observable healing impairment were first anaesthetized with 3.5% chloral hydrate solution, at dose of 0.35 mg/g body weight intraperitoneally [15]. Hair was removed by shaving the back of the rats. The rats were distributed in groups randomly and then each rat was placed in a separate cage. Two types of wounds, namely full thickness excision and incision wounds were created in the shaved areas of the skin, extending down to the subcutaneous tissue.

2.3.3. Excision wound

Excision wound was made by cutting out a 4 cm^2 piece of the skin from a pre-determined shaved area on the back of each animal. The wounds were left undressed and no local or systemic antimicrobial agent was used. This model was employed to study the effect of the extract on the rate of wound contraction and wet granulation tissue weight.

2.3.4. Incision wound

One three cm dorso-lumbar skin incision was made with scalpel blade on the medial of the shaved back of each rat in the incision wound model. The two borders of the wound were stitched together at its center by polypropylene blue monofilament non-absorbable suture (Ethicon, PC-1, 3\8, 13 mm, 7-0). This model was employed to study the effect of the extract on the tensile (breaking) strength of incision wounds.

2.3.5. Animal grouping

Rats with incision wounds were divided into two groups of untreated (control) and extract-treated ones, each consists of 20 rats. Five rats of each group were sacrificed on days 4, 8, 12, and 16 for measurement of tensile strength.

On the other hand, rats with excision wounds were divided into two groups of untreated (control) and extract-treated ones, each consists of 24 rats. Six rats of each group were sacrificed on days 4, 8, 12, and 16 for determination of wound area and wet granulation tissue weight.

2.3.6. Extract application

Fifty milligrams of the extract was applied topically on the wound of each rat in the treatment groups once a day for the first seven days of the experimental period.

2.3.7. Calculation of wound contraction rate and wet granulation tissue weight

For the rats of the excision wound model, wound margins were traced on transparent graph paper having a millimeter scale, immediately after surgery and on days 4, 8, 12, and 16 to determine the wound area. Contraction rate was calculated by comparing the wound area at the specified day with the original area at time zero (after surgery). The rats were then sacrificed and the wound granulation tissue excised and weighed. Wet granulation tissue weight per area of the wound was then determined by dividing the weight over wound area on each day of measurement.

2.3.8. Determination of tensile strength of incision wounds

A rectangular section (a length of 3.4 cm and a width of 3 cm) of the skin, including the healing incision wound was excised. The tensile strength of each section was measured using a tensiometer designed according to the method of Vaisberg et al. [16]. In brief, one edge of the rectangle parallel to the wound was fixed while applying incremental loads to the other edge. The tensile strength was then taken to be the load in grams required to disrupt the wound.

2.3.9. Safety of the extract

Four groups of mice, 6 mice each, were subjected to four different doses of the extract (500, 1000, 1500, 2000 mg/kg, given p.o). The survivals were recorded up to one week.

3. Determination of the antimicrobial activity of *A. strigosa* and its pure compounds

3.1. Microorganisms and culture conditions

In the present study overnight cultures of five microorganisms were used. These are *Staphylococcus aureus* ATCC 6538 P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231. Bacteria were grown in nutrient broth medium (Oxoid, UK) while the yeast was grown in Sabaroud media (Hi Media, India). Batches of medium (20 ml) were inoculated from fresh culture slopes and incubated overnight at 37 °C. Long-term maintenance was on nutrient agar plates at 4 °C.

3.2. Broth microdilution method (MIC)

MIC of *A. strigosa* and its pure compound was determined using microdilution method in 96 well plates (Cellstar®, Greinerbio-one, Germany) [17,18]. Mueller Hinton broth media (180 µl) of bacterial culture and Sabaroud media (yeast culture) were used to fill the first experimental well. The other wells were filled with 100 µl media. A volume of 20 µl of *A. strigosa* plant extract was added to the first well. Double fold serial dilution was then carried out across the plate. Similar dilution was performed in different rows of the plate for alkannin and shikonin. Overnight batch culture of the microorganisms (10 µl) was used to inoculate each well to achieve an inoculum size of *ca.* 1×10^6 CFU ml $^{-1}$. The plates were incubated for 24 h at 37 °C. MIC was calculated according to Al Bakri *et al.* [19]. DMSO at the same tested concentration was used as a negative control while Nalidixic acid (Sigma) and Miconazole (a gift from Dar Al-Dawa Pharmaceutical Industries, Amman, Jordan) were used as positive controls to assess the accuracy of MIC method. Each MIC determination was carried out in triplicate.

4. Statistical analysis

Results were expressed as mean ± SD. The data obtained were subjected to statistical analysis using t-test, with the differences considered significant at $p < 0.05$.

5. Results

Extraction and purification of *A. strigosa* obtained two enantiomers; namely alkanin and shikonin. They were structurally elucidated using different spectroscopic techniques (NMR, IR, and UV). The amount of pure racemic mixture was sufficient to carry out antimicrobial, but not wound healing evaluation.

5.1. The effect of HEASR on contraction rate and wet granulation tissue weight of excision wounds

The HEASR did not increase the rate of wound contraction. However, it increased the wet granulation tissue weight per area of the wound, indicating increased protein content (Table 1).

5.2. The effect of HEASR on tensile strength on incision wounds

The tensile strength was significantly higher for the HEASR group throughout the experimental period compared with the control group, as shown in (Fig. 1).

5.3. Safety of the extract

There were no deaths observed in one week after oral doses of *A. strigosa* root extract up to 2000 mg/kg. It was difficult to exceed this dose as the plant extract was very viscous.

5.4. The antimicrobial activity of the HEASR and its pure compounds

As shown in (Table 2), the root extract of *A. strigosa* was active against Gram-positive bacteria (*S. aureus* and *B. subtilis*). In addition, an antifungal effect against *C. albicans* was detected, though the potency was very low. On the other hand, the chiral mixture of the two compounds (alkannin and shikonin) showed an improved potency against Gram-positive bacteria, and an excellent activity against *E. coli*.

6. Discussion

Up to our knowledge, the present work is the first to evaluate, in experimental settings, the wound healing and antimicrobial properties of *A. strigosa*.

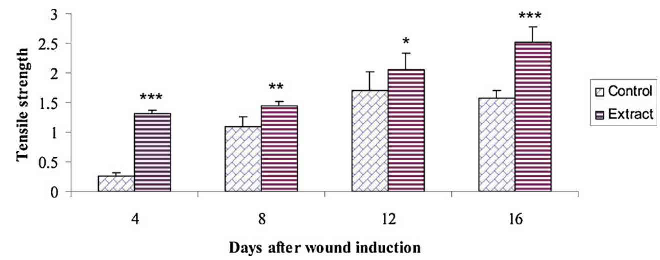

Fig. 1. The tensile strength (mean ± SD, n = 5) of incision wounds in control rats and rats treated topically with HEASR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Tensile strength reflects the maturity and strength of the collagen fibers formed in the area of incision wound. So, healing wounds with compromised tensile strength are more prone to rupture and subsequent complications such as wound contamination [20,21]. The HEASR improved tensile strength of incision wound significantly. The improvement was most prominent on day 4. This early improvement, although unexpectedly high, is paralleled by similar results for Manuka honey in burn wounds, where tensile strength was dramatically increased in rat model of the wounds as early as 3 days post wound induction [22,23]. One clinical implementation of tensile strength enhancement is in postoperative manipulation of surgical wounds, where impaired healing of the surgical incision represents a common complication [24].

Granulation tissue formed in the final part of the proliferative phase is predominantly composed of fibroblasts, collagen, edema, and new small blood vessels. The increase in wet granulation tissue weight per wound area suggests higher protein content and better healing profile [25]. Such an effect was observed for the HEASR-treated animals in excision wound model.

The inability of the HEASR to increase the rate of wound contraction is in accordance with other studies in which the interference did not increase contraction rate, but enhanced other healing parameters. Khalil et al showed that *Inula viscosa* formulated in Pluronic F127 improved histological wound healing without improving contraction rate [26]. Furthermore, phenytoin, applied topically to enhance wound healing did not increase wound contraction rate but significantly improved the acceptance of skin auto grafts, histological collagenization, tensile strength, protein and hydroxyproline contents [15,27].

In general, the antibacterial activity of the HEASR and its pure compound was more pronounced on Gram-positive than on Gram-negative bacteria. These findings correlate with previous screenings of medicinal plants for antimicrobial activity, where most of the active plants showed activity against Gram-positive strains only [28,29]. Interestingly, however, the current findings show a remarkable antimicrobial activity against the Gram-negative bacterium *E. coli*.

The MIC values of 0.125 and 0.185 mg/ml suggest a bacteriostatic activity of the hexane extract of *A. strigosa* root against the bacterial strains tested in this study. Although these values are high compared with those of the usual antibiotics, these results are of interest since they have been obtained with a crude extract. The fact that lower MIC values were obtained with the chiral mixture of alkannin & shikonin

Table 1
Wet granulation weight per area of the wound (g/cm2) for untreated control and HEASR-treated rats. Data are expressed as mean ± SD of six rats for each group at the specifificied day.

Group of rats	Day 4	Day 8	Day 12	Day 16
Untreated control	0.153 ± 0.039	0.116 ± 0.019	0.081 ± 0.016	0.039 ± 0.008
Extract-treated	0.204 ± 0.040*	0.196 ± 0.052*	0.101 ± 0.015*	0.054 ± 0.005*

* $p < 0.05$ when compared to control.

Table 2
Minimal inhibitory concentrations (MIC, μg/ml) of the HEASR, its pure compounds, and reference antimicrobials against some Gram-positive and Gram-negative bacteria and *C. albicans*.

Microorganisms	Plant Extract	Chiral mixture of alkannin/shikonin	Nalidixic acid	Miconazole
Pseudomonas aeruginosa ATCC 9027	> 500.0	> 200.0	375	–
Escherichia coli ATCC 8739	> 500.0	3.5	23.4	–
Staphylococcus aureus ATCC 6538	125.0	14.1	11.7	–
Bacillus subtilis ATCC 6633	185.0	37.5	4.3	–
Candida albicans ATCC 10231	250.0	> 200.0	–	4.7

suggests that these components are responsible for the antibacterial activity. While the crude extract showed a weak effect against *C. albicans,* the pure compounds didn't show this effect. This may be due to the low concentration of the pure compounds that we could apply (< 0.2 mg/ml) due to limitedness of its quantity.

7. Conclusion

The hexane extract of *A. strigosa* roots combines the two beneficial wound healing and antimicrobial properties, which supports its used in the treatment of suppurative wounds. Our current interest is in isolation of alkanin and shikonin in high amounts and evaluating them for pro-healing activity in wounds and burns.

Ethical statement

All animal procedures were conducted in accordance with Jordanian regulations for animal experimentation and care, and approved by the Committee of Institutional Animal Care and Use.

Financial disclosure

There are no financial conflicts of interest to disclose.

Conflict of interest

The authors declare no conflict of interest.

References

[1] D.Hema Nunkoo, M. Fawzi Mahomoodally, Ethnopharmacological survey of native remedies commonly used against infectious diseases in the tropical island of Mauritius, J. Ethnopharmacol. 143 (2) (2012) 548–564.
[2] Christian Agyare, Emelia Oppong Bekoe, Yaw Duah Boakye, Susanna Oteng Dapaah, Theresa Appiah, Samuel Oppong Bekoe, Medicinal Plants and Natural Products With Demonstrated Wound Healing Properties, (2016).
[3] Sabine A. Eming, Paul Martin, Marjana Tomic-Canic, Wound repair and regeneration: mechanisms, signaling, and translation, Sci. Transl. Med. 6 (265) (2014) 265sr6.
[4] James Q. Del Rosso, Jacqueline Levin, The clinical relevance of maintaining the functional integrity of the stratum corneum in both healthy and disease-affected skin, J. Clin. Aesthet. Dermatol. 4 (9) (2011) 22.
[5] Richard A.F. Clark, Kaustabh Ghosh, Marcia G. Tonnesen, Tissue engineering for cutaneous wounds, J. Invest. Dermatol. 127 (5) (2007) 1018–1029.
[6] Ana Cristina de Oliveira Gonzalez, Tila Fortuna Costa, Zilton de Araújo Andrade, Alena Ribeiro Alves Peixoto Medrado, Wound healing-a literature review, An. Bras. Dermatol. 91 (5) (2016) 614–620.
[7] Rupesh Thakur, Nitika Jain, Raghvendra Pathak, Sardul Singh Sandhu, Practices in wound healing studies of plants, Evid. Based Complement. Altern. Med. 2011 (2011).
[8] Usman Ali Khan, Hazir Rahman, Muhammad Qasim, Anwar Hussain, Azizullah Azizllah, Waheed Murad, Zakir Khan, Muhammad Anees, Muhammad Adnan, Alkanna tinctoria leaves extracts: a prospective remedy against multidrug resistant human pathogenic bacteria, BMC Complement. Altern. Med. 15 (1) (2015) 127.
[9] D.M. Al-Eisawi, I.A. Al-Khader, Checklist of Plants of the Hashemite Kingdom of Jordan, (2004).
[10] Imad I. Hamdan, Fatma U. Afifi, Screening of Jordanian flora for α-amylase inhibitory activity, Pharm. Biol. 46 (10-11) (2008) 746–750.
[11] Feras Q. Alali, Khaled Tawaha, Tamam El-Elimat, Maha Syouf, Mosa El-Fayad, Khaled Abulaila, Samara Joy Nielsen, William D. Wheaton, Joseph O. Falkinham III, Nicholas H. Oberlies, Antioxidant activity and total phenolic content of aqueous and methanolic extracts of Jordanian plants: an ICBG project, Nat. Prod. Res. 21 (12) (2007) 1121–1131.
[12] Sawsan A. Oran, Potato disc bioassay for some Jordanian medicinal plants, Pharm. Biol. 37 (4) (1999) 296–299.
[13] Zivile Pranskuniene, Jurga Bernatoniene, Zenona Simaitiene, Andrius Pranskunas, Tauras Mekas, Ethnomedicinal uses of honeybee products in Lithuania: the first analysis of archival sources, Evid. Based Complement. Altern. Med. 2016 (2016).
[14] Mohammad Hudaib, Mohammad Mohammad, Yasser Bustanji, Rabab Tayyem, Mohammed Yousef, Mustafa Abuirjeie, Talal Aburjai, Ethnopharmacological survey of medicinal plants in Jordan, Mujib Nature Reserve and surrounding area, J. Ethnopharmacol. 120 (1) (2008) 63–71.
[15] E.A. Qunaibi, A.M. Disi, M.O. Taha, Phenytoin enhances collagenization in excision wounds and tensile strength in incision wounds, Die Pharmazie-Int. J. Pharm. Sci. 64 (9) (2009) 584–586.
[16] Abraham J. Vaisberg, Marcos Milla, Mariadel Carmen Planas, Jose L. Cordova, Elva Rosas de Agusti, Ramon Ferreyra, Mariadel Carmen Mustiga, Lily Carlin, Gerald B. Hammond, Taspine is the cicatrizant principle in Sangre de Grado extracted from Croton lechleri, Planta Med. 55 (02) (1989) 140–143.
[17] Mohamed Elshikh, Syed Ahmed, Scott Funston, Paul Dunlop, Mark McGaw, Roger Marchant, Ibrahim M. Banat, Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants, Biotechnol. Lett. 38 (6) (2016) 1015–1019.
[18] Laura M. Koeth, Jeanna M. DiFranco-Fisher, Sandra McCurdy, A reference broth microdilution method for dalbavancin in vitro susceptibility testing of bacteria that grow aerobically, J. Visual. Exp.: JoVE 103 (2015).
[19] A.G. Al-Bakri, G. Othman, Y. Bustanji, The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin–EDTA combination and their effectiveness as antibiofilm agents, J. Appl. Microbiol. 107 (1) (2009) 280–286.
[20] Sal Guo, Luisa A. DiPietro, Factors affecting wound healing, J. Dent. Res. 89 (3) (2010) 219–229.
[21] V.N. Arantes, R.Y. Okawa, A.J. Barbosa, A. Petroianu, Effect of methylprednisolone on jejunal anastomotic tension, Arq. Gastroenterol. 31 (3) (1994) 97–102.
[22] Benjamin Minden-Birkenmaier, Gary Bowlin, Honey-based templates in wound healing and tissue engineering, Bioengineering 5 (2) (2018) 46.
[23] M.Z. Rozaini, A.B.Z. Zuki, M. Noordin, Y. Norimah, A. Nazrul Hakim, The effects of different types of honey on tensile strength evaluation of burn wound tissue healing, Int. J. Appl. Res. Vet. Med. 2 (4) (2004) 290–296.
[24] M. Santangelo, P. De Rosa, S. Spiezia, G. Spinosa, S. Grassia, M. Zuccaro, A. Renda, Healing of surgical incision in kidney transplantation: a single transplant center's experience, Transplant. Proc. 38 (4) (2006) 1044–1046 Elsevier.
[25] M.K. Gautam, V. Purohit, M. Agarwal, A. Singh, R.K. Goel, In vivo healing potential of Aegle marmelos in excision, incision, and dead space wound models, Sci. World J. 2014 (2014).
[26] EnamA. Khalil, Fatma U. Afifi, Maysa Al-Hussaini, Evaluation of the wound healing effect of some Jordanian traditional medicinal plants formulated in Pluronic F127 using mice (Mus musculus), J. Ethnopharmacol. 109 (1) (2007) 104–112.
[27] Abla Albsoul-Younes, Nidal A. Younes, Darwish H. Badran, Topical phenytoin ointment increases autograft acceptance in rats, Saudi Med. J. 27 (7) (2006) 962–966.
[28] Ashraf A. Mostafa, Abdulaziz A. Al-Askar, Khalid S. Almaary, Turki M. Dawoud, Essam N. Sholkamy, Marwah M. Bakri, Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases, Saudi J. Biol. Sci. 25 (2) (2018) 361–366.
[29] Jonathan E. Kelmanson, Anna K. Jäger, Johannesvan Staden, Zulu medicinal plants with antibacterial activity, J. Ethnopharmacol. 69 (3) (2000) 241–246.