Original Research Article

Wound healing property and phytochemical analysis of Zanthoxylum species from Uganda

ABSTRACT

Aim: The study evaluated the efficacy and possible mechanism of the stem bark of Zanthoxylum species used by communities and herbalists for wound healing in South Western Uganda.

Study design: Experimental controlled

Place and Duration: Departments of Pharmacy, Pharmacology and Pharmaceutical Sciences, Faculty of Medicine, Mbarara University of Science and Technology. The study done between August 2016 and February 2017.

Methodology: Excision wounds were humanely made on the bark of healthy albino rats and then randomly divided into four groups i.e Group I (Zanthoxylum spp (Zanthoxylum species) water extract) n=9, Group 2 (control herbal drug) n=6, Group 3 (distilled water) n=9 and Group 4 (neomcycine antibiotic) n=3. Treatments were applied twice a day for 15 days. The wound areas determined at baseline (day 1), then at day 6 and day 15 for each of the animals in groups 1, 2 and 3. Percentage reduction in wound areas was determined on day 6 and 15 and statistically compared. On day 7 the rats in group 4 and three rats randomly picked by a blinded laboratory technician from groups 1 and 3 were humanely sacrificed for histology examination of wound tissues. Phytochemical analysis of the water extract of Zanthoxylum spp and the effect of the solvent on extract efficacy were also evaluated.

Results: The Plant Zanthoxylum spp water extract was found to significantly reduce wound areas better than distilled water on day 6 and 15 , (55.93±2.845) Vs (35.06±3.508),p=0.0312 and (93.18±1.721) Vs (74.89±5.604), p=0.0097 , and marginally better than herbal control drug on day 6, (55.93±2.845) Vs (39.55 ± 6.524) , p=0.0799. Five previously known alkaloids were identified by HPLC and LC-MS methods in the plant species as possible active compounds in wound healing. No significant difference was observed in the effects of the solvent on the efficacy of Zanthoxylum species on wound healing.

Conclusion: Zanthoxylum species studied shows great potential for stimulation of collagen formation and promoting natural wound healing mechanisms and therefore offers an alternative for wound treatment.

Key words: Zanthoxylum species, Wound, Healing, Alternative.

1.0 INTRODUCTION

Injury is one of the leading causes of death in children and working adults in almost every country and there are more than five million injury-related deaths every year, as well as a tremendous burden of disability [1]. The injury healing process involves a complex series of interactions between different cell types, cytokine mediators, and the next extracellular matrix [2]. It also occurs naturally in four phases namely; hemostasis (coagulation), inflammation, proliferation and remodeling [3]. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelization and wound contraction. Alterations in any of these steps can lead to delay in healing or even the inability to heal completely [4]. Severe injuries lead to formation of visible wounds on the skin or other parts of the body most of which are difficult to heal and even where they are healed, significant scars are left on the affected part of the body.

Almost 25 to 40% of the active components of the synthetic allopathic medicine had origins from higher flowering plants of the world and the clues to discover them came from folklore medicines of various cultures [5, 6]. Some of these plants have immense potential in management of wounds especially for people living in resource limited nations [7]. Despite deliberate efforts to treat wounds, some specific ones due to influence of some disease processes like diabetes mellitus, HIV and varicose ulcers among others have been unhealable and have continued to be entry point of disease causing organisms that can eventually lead to amputation or death of the patients [8]. According to **S**asidharan et al., (2010) nearly 6 million people suffer from chronic wounds worldwide and the prevalence of chronic wounds in the community was reported as 4.5 per 1000 population, whereas that of acute wounds was nearly double, at 10.5 per 1,000 population [9]. In an effort to address this challenge, interventions like stem cell treatment have been considered but this is too expensive for the ordinary patients especially in developing countries like Uganda. Also administration of oral and topical antibiotics has been other options but is rarely successful in treating non-healing wounds [10]. In a bid to find a sustainable therapy, herbalists of

Budibugyo (South Western Uganda) have discovered the usefulness of Zanthoxylum species stem bark powder (a Zanthoxylum species of the family, Rutaceae) in management of both acute and chronic wounds. The young sample of the whole plant brought by the herbalist was a identified by a botanist and taoxonomist Dr. Olet and Voucher specimen Patrick 001 deposited at the pharmacy department for future reference. The identity of the species is concealed because the herbalists did not grant us permission to state the identity of the plant till the formula is patented and the experiments the plant was indicated as plant X This present research work aimed at validating the wound healing activity of this Ugandan medicinal plant so as to establish a scientific evidence for the observed community use of the plant to treat non healing wounds of various causes.

2.0 MATERIALS AND METHODS

2.1 Plant Material

The fresh stem barks of the Plant were supplied by the herbalist from Budibugyo attached to Medical Research Center, Wandegeya in Kampala and were received at Mbarara University Pharmaceutical Analysis Laboratory. The plant materials were washed, shade dried for 7 days and the dry material pulverised in to a fine powder using electric grinder. The fine powder was stored in air tight dry container at room temperature till extraction time.

2.2 Preparation of the Extracts

A portion (500g) of the dry fine powder was hot macerated using distilled water in the manner advised by the herbalist who supplied the material and allowed to cool to room temperature. The extract after cooling was filtered using a muslin cloth followed by Whatman's filter paper to obtain a clear filtrate. The filtrate was evaporated using rotary evaporator (RV 10 D S99) at 40℃, revolutions per minute (rpm) of 50 and low pressures of -500mmHg followed by oven drying at 50℃ for 24 hrs to a constant weight extract, a method previously described in a similar works [11, 12]. Another set of powder portion (500g) was serially extracted in solvents of varying polarities as follows: It was first extracted in Petroleum ether using Soxhlet apparatus at 40ºC. The petroleum ether extract obtained by soxhlet was filtered through a watmann filter and then concentrated by rotary evaporator and oven drying under conditions described above. The plant material residue from the petroleum ether extraction process was then dried at room temperature and macerated in ethanol (96%) at room temperature for 48 hours and then filtered through

a muslin cloth followed by whatmann filter. The filtrate was then concentrated in a rotary evaporator and oven dried as described for petroleum ether extract. Finally the residue from the ethanol extraction after drying at room temperatures was hot maceration in distilled water and allowed to extract with regular shaking over 48 hours. The filtrate of the water extraction process concentrated using rotar evaporator and also oven dried to obtain a dry residue under conditions described above. The four extraction processes i.e one using direct hot water as described by the herbalist and the three obtained by serial extraction process were used in the wound healing experiment.

2.3 Detection of phytochemicals in the water extract

A portion of the water extract as guided by the herbalist was used directly in the detection of phytochemicals as previously described in a similar study [12] and as detailed below;

Polyuronides: To a test tube containing (10ml) was added drops of water leading to formation of a thick precipitate. The precipitate obtained was placed on the filter paper and on staining with hematoxylin formed a blue precipitate for presence of polyuronides.

Reducing compounds:1ml of extract was diluted with water (2ml) in test tube. Fehling's solutions I (1ml) and Fehling's solution II (1ml) were added and heated in water bath at 90℃ forming a brick-red precipitate.

Saponins :A diluted solution of the extract (2 ml) was placed in a test tube and shaken for 15 minutes. A soapy like column of about 2cm formed above liquid level indicating presence of saponins.

Tannins :To the extract (1ml) was added water (2ml) and a 3 drops of ferric chloride. A blackish blue color formed indicating presence of tannins.

Amino acids : To the extract (1ml) was added *ninhydrin*, which was originally yellow, reacted with *amino acid* and turned deep purple indicating presence of amino acids

Alkaloid salts :The extract (15ml) was evaporated to dryness in an oven at 55℃ and residue dissolved in 10% v/v Hydrochloric acid (10 mL). 10 % v/v ammonia solution (10ml) was added to precipitate the alkaloids and then extracted with ether (15ml). The ether portion was evaporated to dryness and hydrochloric acid (1.5ml) added. To 0.5ml of the acidic solution was added 2–3 drops of Mayer's reagents forming opalescence precipitate.

To detect Steroid glycosides, Anthracenosides , coumarins and flavonosides, 25ml of the extract was mixed in 10% v/v hydrochloric acid (15ml), refluxed for 30minutes, cooled and extracted with diethyl ether (36ml) in portions of 12ml each.

Steroid glycosides:To a residue obtained by evaporating to dryness the Diethyl ether extract (10 ml), was added acetic anhydride (0.50 ml) and chloroform (0.50 ml) and transferred into a dry tube. Conc. Sulphuric acid (2 ml) was added by means of a pipette at the bottom of the tube forming reddish-brown ring at the contact zone of the two layers indicating presence of steroid glycosides.

Anthracenosides :To the diethyl ether extract (4 mL) was added to conc. Sulphuric acid (2 mL) and shaken with 25% v/v ammonia solution (2ml) forming cherished-red solution on the top layer indicating presence of anthracenosides.

Coumarin derivatives:To a residue obtained by evaporating diethylether extract (5 mL) was added hot water (2ml) to dissolve. 10% v/v ammonium solution (0.5 ml) was then added forming a blue fluorescence solution under UV indicating presence of coumarin derivatives.

Flavonosides :The residue obtained by evaporating diethyl ether extract (5ml) was heated in 50% methanol (2 mL). Metallic magnesium (0.5g) and conc. Hydrochloric acid (5 drops) was added forming a red solution indicating presence of flavonosides.

2.4 Preparation of the treatments and controls

A 5% extract solution of each extract was made by dissolving 5g in 100mls of the solvent used in the extraction of the plant powder i.e water, ethanol and petroleum ether. These solutions were the applied on the excision wounds in different study animal groups. A herbal drug for wound treatment on the Ugandan market and neomycine cream antibiotic were used as a positive control[12] while distilled water was used as a blank control.

2.5 Creation of wounds and application of treatments

Fifty seven (57) inbred Wistar albino rats (150 – 200g) of either sex and of approximately the same age were obtained from the same colony at the animal research facility of department of Pharmacology,

Mbarara University of Science and Technology. They were housed in clean cages with access to clean water and standard laboratory pellet diet *ad libtum* throughout the experimentation period as per National Institutes of Health (NIH) guidelines for animal handling in teaching and research.

Excision wounds were created on the backs of study rats after shaving and application of analgesia using diclofenac injection 100mg/kg and local anesthesia using lignocaine and adrenaline by injection into the site for wound excision [13]. After creation of the wounds on the backs, the study animals were then randomly picked without replacement by a blinded Laboratory Technician and placed into groups: group 1 (n=9) for Zanthoxylum species aqueous extract, group 2 (n=6) for the herbal wound healing drug, group 3 (n=9) for the distilled water, group 4 (n=3) for the neomycine group, group 5 (n=10) for the petroleum fraction, group 6 (n=10) for the ethanol fraction and group 7 (n=10) for the distilled water fraction. The fresh wounds were left for 24hours and the treatments in solution form were applied to cover the entire wound area on rat backs by dropping the medicine using plastic droppers. The treatments were applied on to the wounds twice a day (morning and evening) for 15 days. For each animal at baseline and then at day 6 and day 15, the wound diameters were measured using a digital Vernier caliper in diagonal way as 'a' and 'b' which were used to determine wound area (mm ²) using the formula (π a*b)/4. Wound contraction was calculated as percentage of the reduction in wound area from baseline value i.e. Percentage of wound contraction = [(Baseline wound area – day of measurement wound area) / Baseline wound area] x 100 equation [6].

2.6 Statistical Analysis of the wound reduction effect

The data obtained was analyzed using GraphPad Prism software version 7.03. One way ANOVA was used for determining the statistical significant difference in the group means. The inter group significance was analyzed using Turkey's multiple comparison test and a P value $< .05$ was considered to be statistically significant. All the values are presented as Mean ± SEM with their corresponding P values.

2.7 Histological Analysis of wound tissues

Three animals each from Zanthoxylum species treated, Neomycine treated and Water treated were sacrificed humanely under diethyl ether anaesthesia, their wound tissue excised and fixed in formalin (10%) for histological examination. Wound tissue Sections were stained with Trichome stain and

examined by microscope under power X40 and X100 for collagen formation (blue colouration) and inflammation (density of inflammatory cells). Comparison of tissue appearance was made between Zanthoxylum species and controls i.e neomycine treated and water treated Due to equipment limitation the hydroxyproline content in the wounds could not be determined.

2.8 Detection and Identification of the alkaloids in Zanthoxylum species

This was done one in Wits University, Johannesburg-Republic of South Africa. The received material in fine powder was processed using different solvent materials, until the obtained alkaloid fractions were run in confirmatory alkaloid tests using High performance liquid chromatography and liquid chromatograph-mass spectrometry, abbreviated as HPLC and LC – MS respectively as follows:

The extracts were centrifuged for 2 minutes at 10,000 rpm using a micropipette, the samples were transferred into small clean vials. From the solution already made, 1ml of it was put into HPLC vial, while one third of the solution placed into LC – MS vial for analysis.

The HPLC analysis consisted of subjecting the sample to a waters 600 pump with a 600 E controller, waters 717 plus auto sampler coupled to a waters 996 photodiode carry detector and the alkaloids were found by using 200 to 500mm scan per second at intervals of 10mm for 30mm using a program gradient solvent and the extracts were analyzed at a flow rate of 1cc per minute.

Accurate mass LC – MS analyses were carried out on a thermo scientific LTQ Orbit rap XL with an electrospray source operating on positive and negative mode with an Accela system.

The data was analyzed using X caliber software and chromatography achieved on a phenomenex Lune C18 column 150mm x 3mm.

The samples were run in both positive and negative mode in full MS scan mode.

Alkaloids from the extract were observed on the UV spectra and retention times (HPLC analysis) while LC – MS analyses was based on accurate mass, molecular formula and mass fragmentation and were compared with known compounds from the standard laboratory figures.

3.0 RESULTS AND DISCUSSION

The crude aqueous extract revealed the presence of various phytochemical groups with alkaloids being abundant as shown in table I.

Table I: Phytochemical groups identified in the crude aqueous stembark extract of Zanthoxylum species .

Phytochemical group	Presence	Phytochemical group	Presence
Terpenoids	+	Saponins	+
Tannins	+	Anthroquinone glycosides	+
Flavonoids	-	Alkaloids	++
Amino acids	+	Phenols	+
Glycosides	+	Steroids	+

Presence (+) Absent (-) Abundant (++)

Zanthoxylum species crude water extract demonstrated better wound size reduction effect than the control treatments (Table 2).

Table 2: Percentage wound reduction effect of Zanthoxylum species compared with controls.

	Percentage Mean ± SEM, n=6		
Time	**Distilled water**	**Zanthoxylum species . extract**	**P-values**
Day 6	35.06 ± 3.508	55.93 ± 2.845	0.0312*
Day 15	74.89 ± 5.604	93.18 ± 1.721	0.0097**
	Distilled water	**Control herbal drug**	
Day 6	35.06 ± 3.508	39.55 ± 6.524	0.7950
Day 15	74.89 ± 5.604	86.75 ± 2.498	0.0784
	Zanthoxylum species extract	**Control herbal drug**	
Day 6	55.93 ± 2.845	39.55 ± 6.524	0.0799
Day 15	93.18 ± 1.721	86.75 ± 2.498	0.4228

There was no statistically significant difference between the healing effects produced by ethanol, petroleum ether, ethanol and aqueous fractions (Table 3).

Table 3: Comparison of wound area reduction effect of Zanthoxylum species extracted in various solvents

	% wound reduction (mean ± SEM, N=10)		
	Petroleum ether fraction	**Ethanol fraction**	P-value
Day 6	13.03 ±1.988	1.675 ± 6.18	0.5434
Day 15	70.7 ± 5.579	56.09 ± 3.893	0.3274
	P. ether fraction	**Aq. Fraction**	
Day 6	13.03 ±1.988	11.76 ± 10.82	0.9926
Day 15	70.7 ± 5.579	57.03 ± 9.794	0.3742
	Ethanol fraction	**Aq. Fraction**	

Day 6	1.675 ± 6.18	11.76 ± 10.82	0.5974
Day 15	56.09 ± 3.893	57.03 ± 9.794	0.9948

Qualitative (blue coloration) histology analysis revealed that Zanthoxylum species stimulated collagen formation more than the control treatments (neomycin or water) and the wound tissue had fewer inflammatory cells indicative of better healing effects and possible anti-inflammatory effects of the Zanthoxylum species extract (Figure 1-3). Quantification of hydroxypropline as a good measure of collagen formation could not be done due to limitation of capacity.

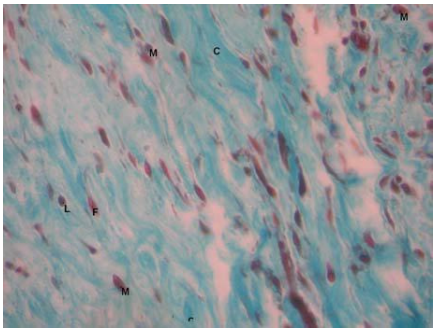

Figure 1: Zanthoxylum species treated

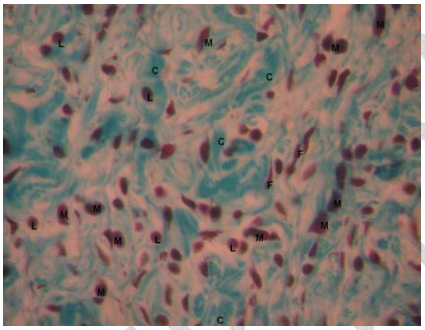

Figure 2: Neomycine treated

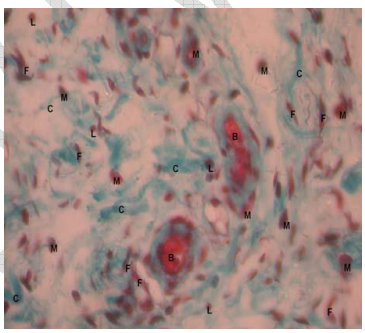

Figure 3: Water treated

M= Macrophages; L= Lymphocytes: C= Collagen fibers: F= Fibroblasts; B=Blood vessel

Five previously known alkaloids were detected in the Zanthoxylum species by HPLC and LC-MS (Figures 4 to 8).

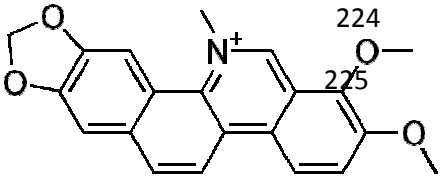

Figure 4: Chelerythrine

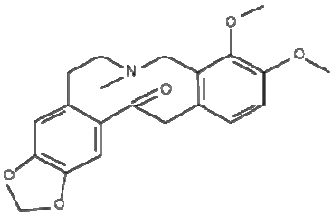

Figure 5: Fagarine

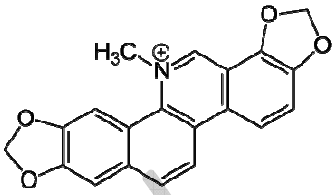

Figure 6: Sanquinarine

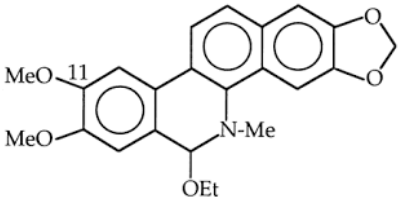

Figure 7: Dihydrionitidine

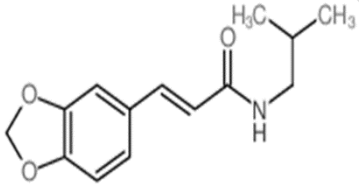

Figure 8: Trans fagaramide

4.0 DISCUSSION

Wound healing is a complex process involving a series of physiological and biochemical changes, but these steps can be shortened by herbs which possess antiseptic, antioxidant and anti-inflammatory activities [14]. Our findings indicate that the water extract of Zanthoxylum species obtained by hot maceration as guided by the herbalist compared to blank control (water treated) had better wound healing effect than the herbal drug on the market compared to blank control (water treated) i.e at Day 6 (***35.06 ± 3.508 Vs*55.93 ± 2.845, p=0.0312) *and* day 15 (**74.89 ± 5.604 Vs 93.18 ± 1.721, p=0.0097) for the Zanthoxylum species water extract and day 6 (35.06 ± 3.508 Vs 39.55 ± 6.524, p=0.795) and day 15 (74.89 ± 5.604 Vs86.75 ± 2.498, p=0.0784) for herbal drug on the market. The Zanthoxylum species water extract was also marginally significantly better than the herbal drug on the market at day 6 of treatment (55.93 ± 2.845 Vs 39.55 ± 6.524, p=0.0799) although not significantly different at 15 of treatment. Comparison of the wound healing effect of the serially extracted fractions of Zanthoxylum species showed no significant difference in the solvents used for extraction justifying the use of water for

its extraction by the herbalists since water is a cheap and safer solvent than ethanol and petroleum ether and also accessible by the communities and herbalists.

The observed wound healing effects of Zanthoxylum species could be attributed to the phytochemical groups detected in the water extract especially phenols and alkaloids which were previously implicated in wound healing reported in another study[15]. Also according to Sunita et al., (2017), the wound healing potential of natural phytomedicines can also be explained by the presence of saponins which are known to have anti-oxidant and antimicrobial activities, tannins which are known to have antimicrobial effects and triterpenoids which promote wound contraction and the rate of epithelization[16]. The above group of compounds were also detect in the Zanthoxylum species water extract (table 1) and may therefore explain the observed wound healing property of Zanthoxylum species .

Five previously known alkaloids (Figures 4-8) were also detected in the water extract Zanthoxylum species by High Performance Liquid Chromatography and Liquid Chromatography Mass Spectrometry methods. The alkaloids particularly Sanquinarine is a known tissue regenerator with anti-inflammatory effects [17, 18], and chelerythrine is known to have antimicrobial and antitumor properties [19].

The histology pictures (Figures 1, 2 and 3) indicate significant differences in collagen formation (blue color) with the Zanthoxylum species water extract showing the highest density of collagen (blue coloration) than the neomycin and blank control (water treated) groups. The marked collagen formation in Zanthoxylum species extract signify induction of collagen formation as possible mechanism by which the plant promotes wound healing. The low levels of infiltration by inflammation promoting cells in the histology picture of Zanthoxylum species treated wounds compared to neomycine and blank control (water treated) groups further indicates the possible anti-inflammatory effects of the Zanthoxylum species compounds such as sanquinarine which was detected in Zanthoxylum species . The histology picture of Zanthoxylum species also showed well-organized tissue building materials such as fibroblasts, grand substance and fibrous tissue usually associated with excessive angiogenesis and accelerated natural wound mechanism also reflected by the shorter wound healing time seen in Zanthoxylum species treated group compared to other group in the study (Table 2).

5.0 CONCLUSION

Zanthoxylum species shows great potential for use in stimulation of collagen formation, shortening wound healing time and promoting natural wound healing mechanisms. This mechanism offers great hope for a cheaper alternative for healing of difficult to heal wounds and needs further exploration for possible development into a drug for wider clinical application as a low cost alternative.

ETHICAL APPROVAL

The Ethical clearance TREC007/17 was obtained from THETA Uganda Research Ethics Committee, accredited by Uganda National Council for Science and Technology with a focus on traditional medicine research approval and the study was conducted in accordance with the national and international institutional rules concerning animal experiments and biodiversity rights. The experimental animals were humanely treated throughout the study and at the end of the study were sacrificed under general anesthesia and incinerated.

ACKNOWLEDGEMENT AND CONFLICT OF INTEREST

The authors declare no conflict of interest the plant material tested and results reported in this paper. The authors are grateful to Government of Uganda through the National Agricultural Research Organisation grant for funding this research on Zanthoxylum species Coded as plant X under the NARO/CGS/COHORT IV 2015/17.

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