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EVALUATION OF ETHANOLIC EXTRACT OF ZIZYPHUS XYLOPYRUS WILLD ON WOUND HEALING ACTIVITY IN RATS

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ABSTRACT

Ziziphus xylopyrus Willd. (*Z. xylopyrus*) is reported for widely use in diarrhoea, chest pain, analgesic, anti-inflammatory and healing of wounds in folk medicine. The *in vivo* wound healing activity of ethanol extract of *Z. xylopyrus* leaves was studied using excision and incision wound models in Swiss Albino rats. In order to achieve the aim, wound healing activity of ethanolic extract of *Z. xylopyrus* leaves were studied using excision and incision wound models in healthy Swiss Albino rats. The excision wound model was applied to measure breaking strength and hydroxyproline content and Incision wound model were applied to measure the bound area in mm. The extract administered topically at different dosages (50 mg/kg, 100 mg/kg and 200 mg/kilogram) per day for 10 days and evaluated for wound healing action. The wound healing potential of ethanolic extract of leaves at 200 mg/kg dose in both the model excision and incision was extremely significant and equivalent to standard. In the excision wound model, treated animals showed a significant reduction in the wound area, epithelization period and increase percent of wound contraction which was equivalent to standard. Results obtained from the present work, it can be inferred that the ethanolic extract of *Z. xylopyrus* leaves contributing to the stated activities. Further an exhaustive phytochemical work is needed in order to isolate, characterize and perform bioassays for the compound providing angiogenic as well as wound healing activity.

KEYWORDS: *Z. xylopyrus*, Wound healing activity, wound healing potential.

INTRODUCTION

Angiogenesis, a complex physiological process required for healing wounds and for restoring blood flow to tissues after injury. Angiogenesis is the establishment of new capillaries from pre-existing vascular network, plays an important role in physiological and pathological process such as embryonic development of coronary artery disease (Tonnesn *et al.*, 2000). Extension of the circulatory network also looks at one of the most important factors during cancer genesis. Inhibition of angiogenesis may lead to inhibition of tumor development, whereas stimulation may improve wound healing.

Z. xylopyrus (Family: Rhamnaceae) is distributed in North West India, Uttar Pradesh, Bihar and Central South India. In Hindi it is known as katber, in Tamil - kottei, and in Telugu- Gotte . The leaves of said plant are alternate, entire, with three prominent basal veins, and 2-7 cm long; some species are deciduous, others evergreen. The major chemical composition are tannins, flavonoids: quercetin, kaempferol-4-methylether and kaempferol, cyclopeptide, alkaloids: amphibine-H and nummularine-K. The root bark of this plant is reported to have antinociceptive, anticonvulsant and anti-inflammatory activity. Aside from that, the leaves show antidepressant activity, antimicrobial, antiulcer and anti-inflammatory activity (Singhal *et al.*, 2012; Sharma *et al.*, 2011; Sharma *et al.*, 2011; Modi *et al.*, 2014; Sharma *et al.*, 2013; Jena *et al.*, 2012; Washid *et al.*, 2011).

The purpose of our work is to offer scientific evidence concerned with the wound healing potential of this unexplored herb. Wound healing processes are easily-organized biochemical and cellular events leading to the outgrowth and regeneration of wounded tissue in a particular way. Healing of wounds is an important biological process involving tissue repairs and tissue regeneration. It implies the bodily function of an intricate network of blood cells, cytokines, and growth factors, which finally passes to the restoration to the normal shape of the injured skin or tissue. However, until now there is no scientific works reported that its leaves showing wound healing potential while stem bark showed the wound healing potential. Therefore, the present study aimed to explore this indigenous plant leaves for wound healing activity.

MATERIALS AND METHODS

Plant materials

The leaves of the selected plant were collected from the village Barodiya Kalan district Sagar, India. The plant material was identified and authenticated taxonomically at the Department of Botany, Dr. H. S. Gour University, Sagar M.P. India and Plant authentication reference number BOT/HER/B/1534 was obtained.

Preparation of leaves extracts

The plant leaves were cleaned, dried and powdered by a grinder. 100 g of the leaves were defatted with petroleum ether (60-80°C), to ensure complete defatting, few drops taken in from the thimble, which was set up to have no oily spot on evaporation. After the complete defatting material was filtered and the filtrate was subjected to evaporation under pressure up to dryness. The marc was dried in oven extracted with ethanol in soxhlet unit for 72 h periodically. It was filtered and evaporated under reduced pressure up to dryness, then solid dark brown extract was collected and stored in well closed container. The percentage yield of the ethanolic extract of the leaves of *Z. xylopyrus* was 14.5 % w/w (Strodbeck, 2001; Bailey *et al.,* 1975).

Preparation of hydrophilic ointment base

Water-soluble ointment base (Hydrophilic ointment USP) was prepared with the following composition: stearyl alcohol (25 % w/w), white petrolatum (25 % w/w), sodium lauryl sulfate (1 % w/w), propylene glycol (12 % w/w), methyl paraben (0.025 % w/w), propyl paraben (0.015 % w/w), and purified water (37 % w/w). Stearyl alcohol and white petrolatum were melted on a steam bath and warmed at 75°C. The assessed quantity of Sodium lauryl sulfate, propylene glycol, methyl paraben and propyl paraben were dissolved in 37 g of purified water and warmed to at 75°C. The aqueous solution was added slowly to the alcohol-petrolatum melt. The mix was stirred until congealed. To about 20 g, each of the two above preparation was taken and to them 1 g and 2 g of ethanol extract of *Z. xylopyrus* Stem leaves were added and stirred until mixed properly. Thus, all the negative control and test drugs were prepared. Formaldehyde solution, acetone, benzene and paraffin wax (58-60°C) were purchased from Ranbaxy Laboratories Ltd., India. All other chemicals and solvents were of analytical grade.

In vivo wound healing evaluation

Animal: Healthy Swiss Albino rats of either sex around of the same age, weighing 150-250g were used for the study. Animals were housed individually in polypropylene cages containing sterile paddy husk bedding under controlled conditions at 25 ± 5°C temperature and kept under 10/14 h light/dark cycles with free access to standard rodent pellets diet and water ad libitum. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee (CPSEA No. SIPS/EC/2012/17). Animals were acclimatized to laboratory conditions before experiments were held away. Except the drug under study, no topical, systemic or oral therapy of any other drug was given to the animals subjected to any of the wounds. Animals showing infection, deterioration of wounds were excluded from the study and replaced with new animals.

Dosing schedule: Ointments of ethanolic extract of leaves of *Z. xylopyrus* with water soluble ointment base USP were applied topically twice daily from day 1 to till day of complete healing or respective post operative day whichever was earlier. Dexamethasone cream and Povidone iodine ointment was used as standard for excision and incision wound model respectively (Jena *et al.,* 2012).

Animals

Rats were divided into 5 groups of 6 animals in each group.

Group 1: served as Negative control

Group 2: served as standard treated with Dexamethasone/povidone iodine ointment topically

Group 3: animal received ethanolic extract of *Zizyphus xylopyrus* 50mg/kg, topically

Group 4: animal received ethanolic extract of *Zizyphus xylopyrus* 100mg/kg, topically

Group 5: animal received ethanolic extract of *Zizyphus xylopyrus 20*0mg/kg, topically.

Excision wound model

The wound was created in the dorsal interscapular region of each rat excising a predetermined area of 7 mm skin under ether anesthesia. Wounds were left open and medicine was applied topically twice a day (once in the morning and evening) onto each rat and wound contraction was monitored by measuring wound area with a 3 day gap till 16 post operative day (Lee, 2009). (% wound contraction = [(Initial wound size - Specific day wound size) /Initial wound size] × 100).

Measurement of Wound Breaking Strength

Breaking strength measurement was carried out using CT3 Texture Analyzer (Brookfield Engineering Laboratories, USA) by breaking the skin joint under tension. Rats are anesthetized and each rat was placed on a stack of paper towels on the middle of the board. The amount of towels could be adjusted in such a way so that the wound was on the same level of the tip of the arms. The clamps are then carefully clamped on the skin of the opposite sides of the wound at a distance of 0.5 cm away from the wound. The longer pieces of the fishing line were placed on the pulley and finally to polyethylene bottle and the position of the board was adjusted so that the bottle receives a rapid and constant rate of water from a large reservoir, until the wound began to open. Amount of water in the polyethylene bag was weighed and considered as the tensile strength of the wound. The mean determination was made on both sides of the animals and was taken as a measure of the tensile strength of the wound. Thus, tensile strength of all the rats of all groups was measured. Increase of tensile

strength indicates better wound healing promotion of the applied drug.

Hydroxyproline Estimation

Excised tissues were dried in a hot air oven at 60–70°C to constant weight. Dried tissues were hydrolyzed by 6N HCl at 130°C for 4 h in sealed glass vials. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by the addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60°C. The absorbance was measured at 557 nm using a spectrophotometer (SpectraMax 190, Molecular Devices, USA). The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline (Yadav *et al.*, 2014).

Incision wound model

Two longitudinal para-vertebral incisions of 6 cm length were made through the full thickness of skin on either side of the vertebral column of the rat. Wound was close with interrupted sutures 1 cm apart. The sutures were taken out along the 7th post wounding day. Wound breaking strength was measured in anesthetized rats on the tenth day (Mortan *et al.* 1972)

RESULTS

Excision wound model: The effect of *Z. xylopyrus* at different dose (50mg/kg, 100mg/kg, 200mg/kg p.o.) on the excision wound healing model were used to measure tissue breaking strength and dry weight of granuloma, compared with the negative control group.

Tissue breaking strength was measured by CT3 Texture Analyzer in g (gram). In the treatment group, 200mg/kg dose shows extremely significant effect on tissue breaking strength (396.19±12.7425, P<0.001) compared with negative control group (279.04±12.7425). Extract with 100mg/kg dose shows significant in tissue breaking strength (286.9±10.8435, P<0.01) compared with the negative control group.

Hydroxyproline content were determined in mg/g of tissue by UV-spectroscopy (Shimadzu Ltd.). Hydroxyproline content were increased with increased of wound healing. In the treatment group, hydroxyproline were significantly increased in 200mg/kg dose (24.14±9.8934, P<0.001) compared with negative control group (16±6.5942).

In the excision wound model, dry granuloma weight was significantly increased with increased of wound healing activity. Dry granuloma weight was significantly increased at 200mg/kg dose (13.09±5.3659, P<0.001) compared with negative control group (7.69±3.1536) and results are shown in (Table 1 and Fig. 1).

Incision wound model: The effect of *Z. xylopyrus* at different dose(50mg/kg, 100mg/kg, 200mg/kg p.o.) for 16 days on wound healing activity in rats were measured and compared with the negative control group. The negative control group served to receive only normal saline (1ml/kg p.o.). Povidine iodine group served as standard group.

After applying the incision, wound area was measured from zero to 16th day in mm. Wound healing activity with 200mg/kg dose shows the extremely significant effect (6.97±0.18 to 1.08±0.09, P<0.001) compared with negative control group (7.96±0.11 to 4.78±0.25). Dose of 100mg/kg shows significant effect (6.98±0.18 to 1.08±0.09, P<0.01) compared with the negative control group and results are shown in (Table 2 and Fig. 2).

Table 1: It shows effect of ethanolic extract in wound healing in Excision wound and dead space model

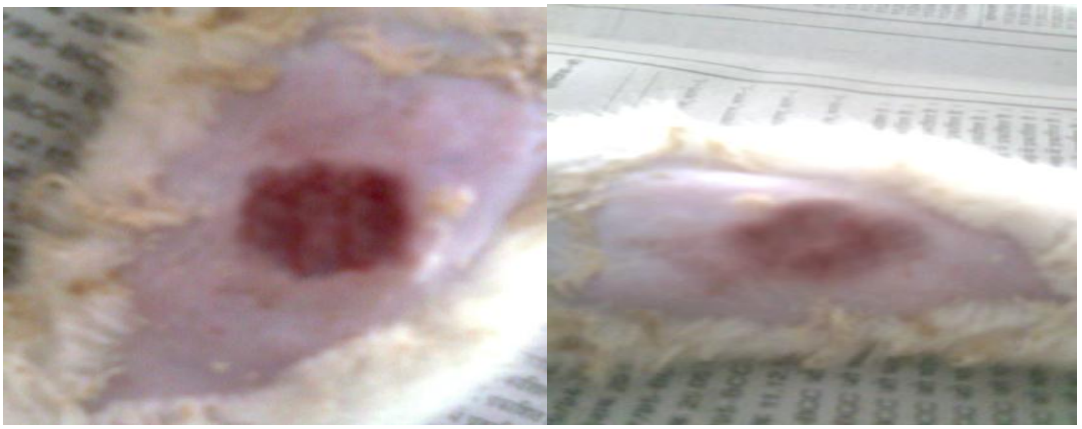
Treatment	Dose (mg/kg) & route of administration	Excision Wound	Dead space wound model	
		Breaking Strength (g)	Dry weight of granulation tissue (mg/100 g of rat)	Hydroxyproline content (mg/g of tissue)
Negative Control (Normal Saline)	1ml/kg p.o.	279.04±6.8461	7.69±3.1536	16±6.5942
Dexamethasone	0.34 (i.m.)	491.01±9.0819***	15.55±6.3729***	28.45±11.6598***
Extract 50mg/kg	50 (p.o.)	277.73±10.6686^{ns}	8.83±3.6188^{ns}	15.89±6.5123^{ns}
Extract 100mg/kg	100 (p.o.)	286.9±10.8435*	9.45±3.8729*	17.89±7.3319*
Extract 200mg/kg	200 (p.o.)	396.19±12.7425***	13.09±5.3659***	24.14±9.8934***

n=6; values are in Mean±SEM, Statistical significant test with negative control using one way ANOVA followed by Dennett Test. *** extremely significant P<0.001, * Partial significant P<0.05, ns=non-significant. p.o.=per oral, i.m.= Intramuscular

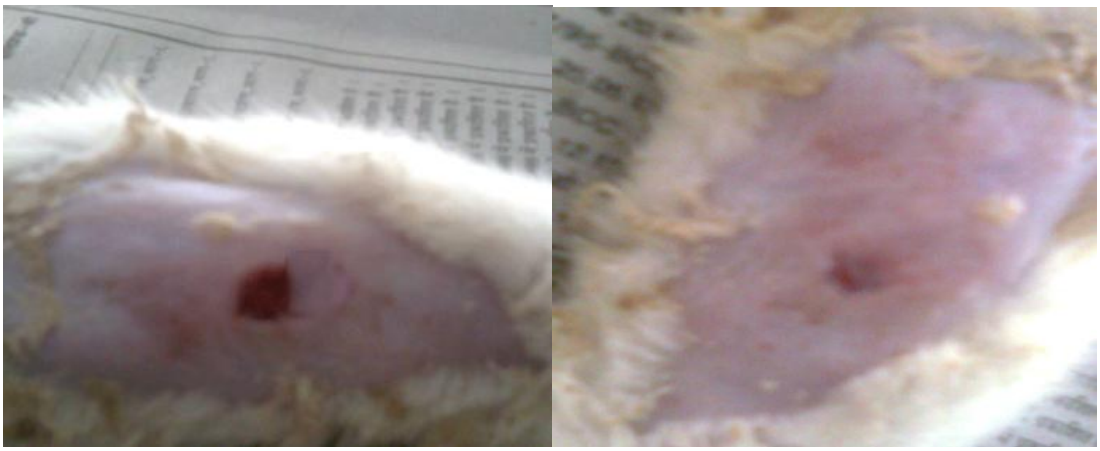
Table 2: It shows evaluation of wound healing activity on incision wound model

Group	Parameter wound area (mm)				
	0th day	4th day	8th day	12th day	16th day
Negative Control	7.96±0.11	6.82±0.34	6.38±0.09	5.43±0.14	4.78±0.25
Povidine iodine	6.95±0.23	5.44±0.12***	4.39±0.15***	2.67±0.17***	1.25±0.25***
Extract (50 mg/kg)	7.01±0.09	6.14±0.33ns	5.20±0.20ns	3.24±0.19ns	2.28±0.21ns
Extract (100 mg/kg)	6.98±0.17	5.89±0.34**	4.85±0.21**	2.96±0.24**	1.85±0.17**
Extract (200 mg/kg)	6.97±0.18	5.25±0.19***	4.31±0.14***	2.21±0.10***	1.08±0.09***

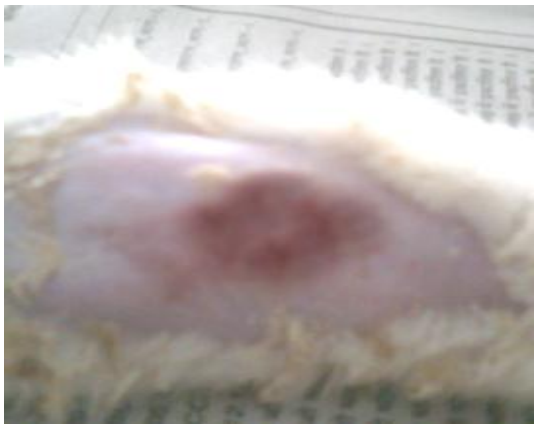
n=6; values are in Mean±SEM, Statistical significant test with negative control using one way ANOVA followed by Dennett Test. *** extremely significant P<0.001, ** significant ns=non significant. p.o.=per oral, i.m. Intramuscular



(a) Negative control **(b) Dexamethsone (Standard)**



(c) Extract (50mg) **(d) Extract (200mg)**



(e) Extract (100mg)

Fig. 1. It shows the effect of excision wound model on 16th day with different treatment group

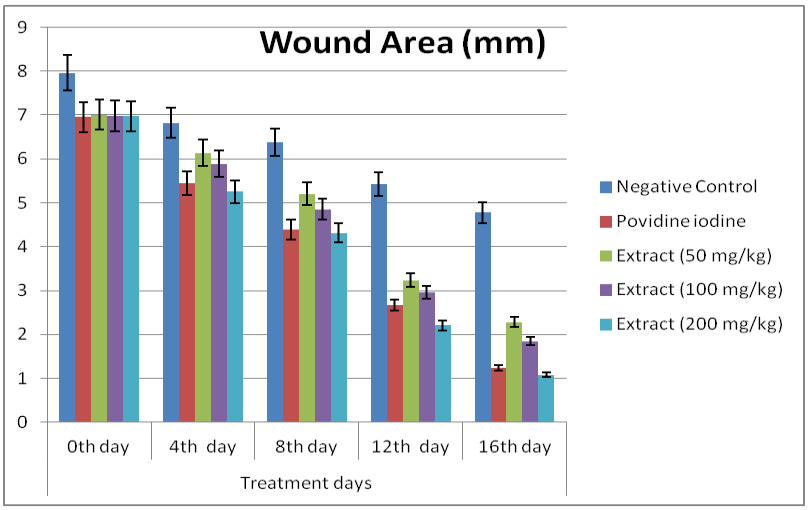


Fig. 2. It shows effect of ethanolic extract on Incision wound model

DISCUSSION

Granulation, collagen maturation, and scar formation are some of the many phases of wound healing, which run con-currently, but independent of each other. The use of a single model is inadequate which can collectively represent the various phases of wound healing. Hence, two different models, namely, excision and incision, have been used in the present study to assess the effect of *Z. xylopyrus* on the various phases of wound healing.

Contraction decreases healing time because it decreases the size of the wound and reduces the amount of extracellular matrix needed to repair the defect. Contraction also facilitates reepithelization by shortening the distance for migrating keratinocytes. Likewise, the wound will close at fast rate if the medication is more efficient. In excision wound model, the extract of *Z. xylopyrus* at the doses of 200mg/kg p.o. showed significantly increased healing by wound contraction, when compared to 100mg/kg p.o., 50mg/kg p.o. as well as negative control group.

Hence, it is clear that the ethanol extracts of *Z. xylopyrus* at a dose 200mg/kg p.o. are more efficacious than the other dose group, while the marketed formulation was found to be the most effective among all treatments.

Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength. The breakdown of collagen liberates free hydroxyproline and its peptide. Therefore, measurement of hydroxyproline could be used as an index for determining collagen turnover. The extract-treated groups (200mg/kg) showed significant increases in the level of hydroxyproline than other two dose group, which was a reflection of the increased collagen content. This was confirmed by histopathological examination of wound granulation tissue, which showed a well-developed matrix in extract-treated groups with the collagen was well-organized and bundles formed between the cells. There was also better neo-vascularisation in the extract-treated groups than in the wound control groups (Yadav *et al.*, 2014).

Progressive increase in biomechanical strength of the tissue is one of the most crucial phase in dermal wound healing. The mechanical properties of the skin are mainly ascribed to the function of the dermis in relation to the structure of collagen and elastic fiber networks. Breaking strength of the healed wound is measured as the minimum force required to break the incision apart. Measurement of wound strength provides highly quantifiable estimates of the efficacy of the aggregate healing process. In the present investigation, there was significant increase in breaking strength in all treated groups. These results suggest that *Z. xylopyrus* has strong wound healing potential.

CONCLUSION

The present study indicates the wound healing and antioxidant activities of *Z. xylopyrus* in experimental animal using incision, excision and dead space wound models. Further studies are going on in our laboratory to isolate and identify the active principles responsible for the wound healing activities of this extract.

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