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RESEARCH ARTICLE

WOUND HEALING POTENTIAL OF ZIZIPHUS XYLOPYRUS WILLD. (RHAMNACEAE) STEM BARK ETHANOL EXTRACT USING *IN VITRO* AND *IN VIVO* MODEL***Basanta Kumar Jena¹, Bhabagrahi Ratha², Subrat Kar¹, Satyaranjan Mohanta¹, Monali Tripathy¹, Amit Kumar Nayak¹**¹Seemanta Institute of Pharmaceutical Sciences, Jharpokharia, Mayurbhanj-757086, Odisha, India²V.S.S. Medical college, Burla, Sambalpur, Odisha, India*Corresponding Author's e-mail: pharma_jena@rediffmail.com

Received 01 Oct 2012; Review Completed 28 Oct 2012; Accepted 01 Nov 2012, Available online 15 Nov 2012

ABSTRACT:

Ziziphus xylopyrus Willd. is reported for widely use in diarrhoea, chest pain and as an analgesic, anti-inflammatory and healing of wounds in folk medicine. The angiogenic activity of ethanolic extract of *Z. xylopyrus* Willd. stem bark was studied using chorioallantoic membrane (CAM) model (*in-vitro*) in 9 days old fertilized chick eggs. The extract found to promote angiogenesis as evidenced in CAM model, presenting increasing number of capillaries on the treated CAM surfaces, which might be beneficial in the treatment of wound healing. The wound healing activity of the test extract was also investigated using excision and incision wound model (*in-vivo*) in Swiss Albino rats. In excision wound model, the percent contraction of wound was found significantly higher in the ointment containing ethanolic extract of *Z. xylopyrus* Willd. stem bark (10 %) treated group compared to the control group. Linear incision by using tensiometer and circular excision wound models were evaluated on rats. In incision wound model, tensile strength of the healing tissue after treatment with the ointment containing *Z. xylopyrus* Willd. stem bark ethanolic extract (10 %) was found significantly higher than the control group ($p < 0.05$), indicating the better wound healing activity. The results of histological examination supported the outcome of linear incision and excision wound model as well. The experimental data demonstrated that *Z. xylopyrus* Willd. stem bark extract displayed remarkable wound healing activity.

Key Words: *Ziziphus xylopyrus* Willd.; chorioallantoic membrane model; angiogenesis; wound healing**INTRODUCTION:**

The basic principle of wound healing minimizing tissue damage, debriding non-viable tissue perfusion and oxygenation, proper nutrition and a moist wound healing environment have been recognized for many years. Wound healing processes are well-organized biochemical and cellular events leading to the growth and regeneration of wounded tissue in a special manner. Healing of wounds is an important biological process involving tissue repairs and tissue regeneration. It involves the activity of an intricate network of blood cells, cytokines, and growth factors, which ultimately leads to the restoration to normal condition of the injured skin or tissue¹. The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration².

Angiogenesis, a complex physiological process required for healing wounds and for restoring blood flow to tissue after injury³. Angiogenesis is the formation of new capillaries from pre-existing vascular network, plays an important role in physiological and pathological process such as embryonic development of atherosclerosis. Extension of circulatory network is also considered one of the most important factors during cancer genesis. Inhibition of angiogenesis may lead to inhibition of tumor growth whereas stimulation may improve wound healing⁴. The chorioallantoic membrane (CAM) is a vascular extra embryonic membrane found in eggs of some amniotes, such as birds, and is formed on day 4 of

incubation⁵. It is formed by the fusion of the allantoises and chorionic⁶. Blood capillaries and sinuses form between epithelial cells of the chorionic layer, allowing close contact (within 0.2 μ m) with air found in pores of the cell membrane of the eggs⁷. CAM from developing eggs is routinely used in biological and biomedical research to investigate development,⁸⁻⁹ pathogenesis,¹⁰ tumors,¹¹ and to propagate and investigate viruses or helminthes¹²⁻¹³. The membrane is used for testing biomaterials also¹⁴. The CAM model has been used to evaluate the wound healing potential of natural substances *in vitro*^{15,16}. Research achievements suggest the use of plants and their extracts as potential therapeutic agents with pro- or anti-angiogenic activity. Since the anticancer and anti-angiogenic properties of many phytomedicins have been amply reviewed as elsewhere this paper will focus on the treatment of vascular insufficiency in wound healing. Globally accepted herbal drugs are thought to be safe and effective, however, there is a need for more evidence best confirmation in controlled and validated trials.

Ziziphus xylopyrus Willd. (Family: Rhamnaceae) is found throughout North-Western India, Pakistan and China. A large, straggling shrub or a small tree, armed with spines, up to 4-7 m. in height. Its local name in Sanskrit: Ghoti, Gotika; Bengali: Kulphal; English: Jujab; Gujrati: Gatbadar, Gatabordi; Hindi: Ghunta, Kakora, Kaathabera; Kannada: Yeranu; Marathi: Ghoti, Bhorghoti; Tamil: Kottai, Mulkottai; Telugu: Gotti, Got, Gotiki¹⁷. This plant is widely used in Turkish folk

medicines as a potent sedative. The leaves are chewed for 15 days as well as fruit is used in urinary troubles¹⁸. Fifty grams of the fresh stem bark of this species is soaked in two hundred ml of water for twelve hours and filtered. This filtrate is taken orally on an empty stomach for a period of three days in a single dose to relieve stomachache¹⁹. The roasted seed powder paste is applied over the chest for relieving pain after cough and colds²⁰. The methanol extract shows the analgesic and anti-inflammatory activity in animal model²¹. The major chemical composition of *Z. xylopyrus* are Quercetin, Kempferol-4'-methylether and Kempferol, Cyclopeptide alkaloids Amphibine-H and Nummularine-K²²⁻²³. Although local traditional healers know the wound healing value of *Z. xylopyrus* Willd. stem bark, there have no reports of biological nor pharmacological investigation. Hence, the present study was undertaken to evaluate the angiogenic potential of *Z. xylopyrus* Willd. Stem bark ethanolic extract using CAM model (*in vitro*) and the wound healing activity in Swiss Albino rats using excision and incision model (*in vivo*).

MATERIAL AND METHODS:

Plant materials: The stem bark of the selected plant was collected from the forest of Simlipal Biosphere Reserve, Mayurbhanj, Odisha, India in August 2006. The plant material was identified and authenticated taxonomically at the Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah-711103, West Bengal, India (Ref. no. CNH/I(59)/2006/Tech-II, Dated 27-10-2006). A voucher specimen of the collected sample was deposited in the institutional herbarium for future reference.

Preparation of extracts: The said plant parts were cleaned, dried under shade and powdered by a mechanical grinder. 100 grams of the pulverized stem bark was extracted with the solvent, petroleum ether, chloroform, and ethanol in increasing polarity successively in a Soxhlet apparatus. For defatting the plant materials, petroleum ether was used in initial step of extraction followed by chloroform and ethanol. The successive extracts were separately filtered while hot and concentrated at reduced temperature on rotary evaporator. The condensed extract was weighed and kept at 4°C prior to testing. The percentage yield of the ethanolic extract of the stem bark of *Z. xylopyrus* Willd. was 7.79 % w/w.

Phytochemical screening: The extracts of *Z. xylopyrus* Willd. stem bark were subjected to some phytochemical tests to determine the presence of alkaloids (Dragendorff's test), glycosides (Keller-Killiani, Borntrager's, and modified Borntrager's tests), carbohydrates (Fehling's and Molisch's tests), steroids and sterols (Liebermann-Burchard test, Salkowski test), tannins (ferric chloride test), proteins and amino acids (Ninhydrin test), tri-terpenoids (tin and thionyl chloride test), saponin (foam test), and flavonoids (NaOH and H₂SO₄ test)²⁴⁻²⁵.

In vitro chick CAM model for screening of angiogenic potential: The chick CAM model^{5,15-16,26} was used as an *in vitro* model to assess the angiogenic activity of ethanolic extract of *Z. xylopyrus* Willd. stem bark. 9 days

old fertilized chick eggs were selected and a window in the eggshells was opened carefully that there should not be punctured. Then, sterile discs of methylcellulose loaded with the extracts (10 µg/disc and 50 µg/disc) and blank methylcellulose disc (as control) were placed in the windows of each eggshell used in the investigation. The windows were resealed with adhesive tape and the eggs were incubated at 37 ± 1°C in a well-humidifier chamber. After 72 hours, the tapes were opened and CAMs treated with the methylcellulose discs were observed for new blood vessels formation and compared with the control CAM treated with the bland methylcellulose disc (without extract).

In vivo wound healing evaluation:

Animals: Healthy Swiss Albino rats of either sex approximately of same age, weighing 150-250 grams were used for the study. They were housed under controlled conditions at 25 ± 5% and kept under 10/14 hours light/dark cycles with free access to food and water *ad libitum*. Animals were housed individually in polypropylene cages containing sterile paddy husk bedding. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee. Animals were acclimatized to laboratory conditions before experiments were carried out. Except the drug under study, no topical, systemic or oral therapy of any other drug was given to the animals subjected with any of the wounds. Animals showing infection, deterioration of wounds were excluded from the study and replaced with new animals.

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 laurylsulphate (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 measured amount of Sodium laurylsulphate, propylene glycol, methyl paraben and propyl paraben were dissolved in 37 grams of purified water and warmed to at 75°C. The aqueous solution was added slowly to the alcohol-petrolatum melt. The mixture was stirred until congealed. To about 20 grams, each of the two above preparation was taken and to them 1 gram and 2 grams of ethanol extract of *Z. xylopyrus* Willd. stem bark was added and stirred until mixed properly. Thus, all the 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 used were of analytical grade.

Dosing schedule: Ointments of ethanolic extract of *Z. xylopyrus* Willd. stem bark with water soluble ointment base USP were applied topically twice daily from day 1 till day of complete healing or 18 post operative day whichever was earlier. Framycetin sulphate cream 1 %

w/w (Sufnamycin, Avantis Pharmaceuticals Ltd., B.No-5107, Mfg.date-06/2010) was used as standard. Animals with hydrophilic ointment base topically application served as control.

Animal grouping for tests: Rats were divided into 5 groups of 4 animals in each group.

Group 1: Untreated control;

Group 2: Treated topically with hydrophilic ointment base USP;

Group 3: Treated topically with framycetin sulphate cream (1 % w/w);

Group 4: Treated topically with 5 % w/w of ethanol extract of *Z. xylopyrus* Willd. stem bark in hydrophilic ointment base USP; and

Group 5: Treated topically with 10 % w/w of ethanol extract of *Z. xylopyrus* Willd. stem bark in hydrophilic ointment base USP.

Excision wound model: The excision wounds were made by excising the full thickness circular skin (approximately 300 mm²) on the back of the animal under ether anaesthesia²⁷. Wound contraction was assessed by tracing the wound area on polythene paper first and subsequently transferred to 1 mm² graph sheet from which the wound surface area was evaluated on 0, 3, 6, 9, 12, 15 and 18 days until wounds were completely healed. The evaluated surface area was then employed to calculate the percentage of wound contraction (taking the initial size of the wound, 300 mm² as 100 %) by using the formula:

$$\% \text{ wound contraction} = \frac{[(\text{Initial wound size} - \text{Specific day wound size}) / \text{Initial wound size}] \times 100}{}$$

Incision wound model: Two longitudinal para-vertebral incisions of 6 cm length were made through the skin and cutaneous tissue at a distance of 1.5 cm from the midline on either side of the vertebral column with the help of sharp blade on depilated area of anaesthetized rats²⁸.

After the incision made, the parted skin was kept together and stitched with black silk by 0.5 cm apart. Surgical thread (No. 000) and curved needle (No. 11) are used for stitching. The continuous threads on both wound edges are tightened for good adoption of wound. All the test, standard & control drugs are applied topically to their relevant groups once daily. On day 8 post wounding, sutures were removed and breaking strength was determined on day 10.

Breaking strength or tensile strength represents the promotion of wound healing. Usually wound-healing

agents promote the gaining of tensile strength. Tensile strength (the force required to open the healing skin) was used to measure the amount of healing. The instrument used for the purpose is called the tensiometer. This was designated on the same principle as the thread tested in textile industry. Rats are anaesthetized and each rat is 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 is on the same level of the tips 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 are placed on the pulley and finally to polyethylene bottle and the position of the board is adjusted so that the bottle receive a rapid and constant rate of water from a large reservoir, until the wound began to open. Amount of water in the polyethylene bag is weighed and considered as tensile strength of the wound. The mean determination is made on both sides of the animals and is taken as a measure of the tensile strength of the wound. Thus, tensile strength of all the rats of all groups is measured. Increase of tensile strength indicates better wound healing promotion of the applied drug.

Statistical analysis: The incision data was tested for significant differences ($p < 0.05$) by paired samples t-test. All other data was analyzed with simple statistics. The simple statistical analysis and paired samples t-test were conducted using MedCalc software version 11.6.1.0.

RESULTS AND DISCUSSION:

The qualitative phytochemical analysis of *Z. xylopyrus* Willd. bark ethanolic extract was performed and the result of this study is presented in Table 1. In ethanolic extract of *Z. xylopyrus* Willd. bark confirmed the presence of glycosides, carbohydrates, steroids, tannins and flavonoids.

In the chick CAM model, the ethanolic extract of *Z. xylopyrus* Willd. bark showed an increase in density of blood capillaries on the treated membrane surfaces when compared with the control (without extract) (Fig. 1). However, the angiogenic activity of the test extract found dose dependent. Increase in blood vessel density at a dose of 50 µg/disc was observed than that of a dose of 10 µg/disc (Fig. 2). Angiogenesis is important in normal processes like the development of embryo, formation of corpus luteum, and wound healing^{5,15}. Again, angiogenesis during wound repair serves the dual function of providing the nutrients demanded by the healing tissues and contributing structural repair through the formation of granulation tissue¹⁵.



Figure 1: Chick CAM after the treatment with ethanolic extract of *Z. xylopyrus* Willd. bark: control (a); 10 µg/disc (b), and 50 µg/disc.

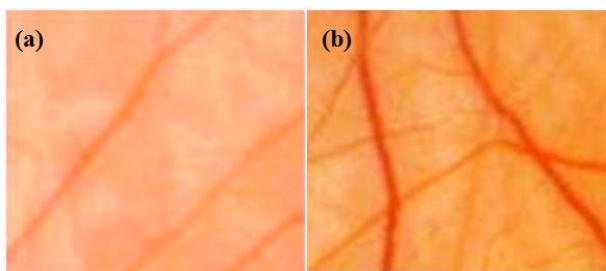


Figure 2: Chick CAM showing blood vessels after the treatment with ethanolic extract of *Z. xylopyrus* Willd. Bark: 10 µg/disc (a), and 50 µg/disc (b).

Excision, dead space and incision wound models were used to study wound contraction, skin braking strength, which are the parameters of tissue cell regeneration, collagenation capacity, and mechanical strength of the skin, respectively²⁹. In this study, an enquiry on wound healing activity of the ethanolic extract of *Z. xylopyrus* Willd. bark, for the use in the treatment of wounds was

evaluated on rats by excision and linear incision wound models to verify the claimed traditional use of the plant material on a scientific base.

The measurements of the progress of wound healing induced by the ointments containing 10 % ethanolic extract of *Z. xylopyrus* Willd. bark of in the excision wound model are shown in Fig. 3 and Fig 4, indicating a remarkable wound healing activity of the test extract. In the excision wound model, the group of rats treated with ointment containing 10 % ethanolic extract of *Z. xylopyrus* Willd. bark showed a complete healing of excision wound on the day 18; whereas 96.10 % of wound healing was measured on the same day for the treatment with ointment containing 5 % ethanolic extract of *Z. xylopyrus* Willd. bark. The reference, framycetin sulphate cream (1 % w/w) showed complete wound healing on day 15. On the same day (day 15), the test ointments containing 5 % and 10 % extract showed 85.20 % and 91.60 % of wound healing, respectively (Fig. 4b).

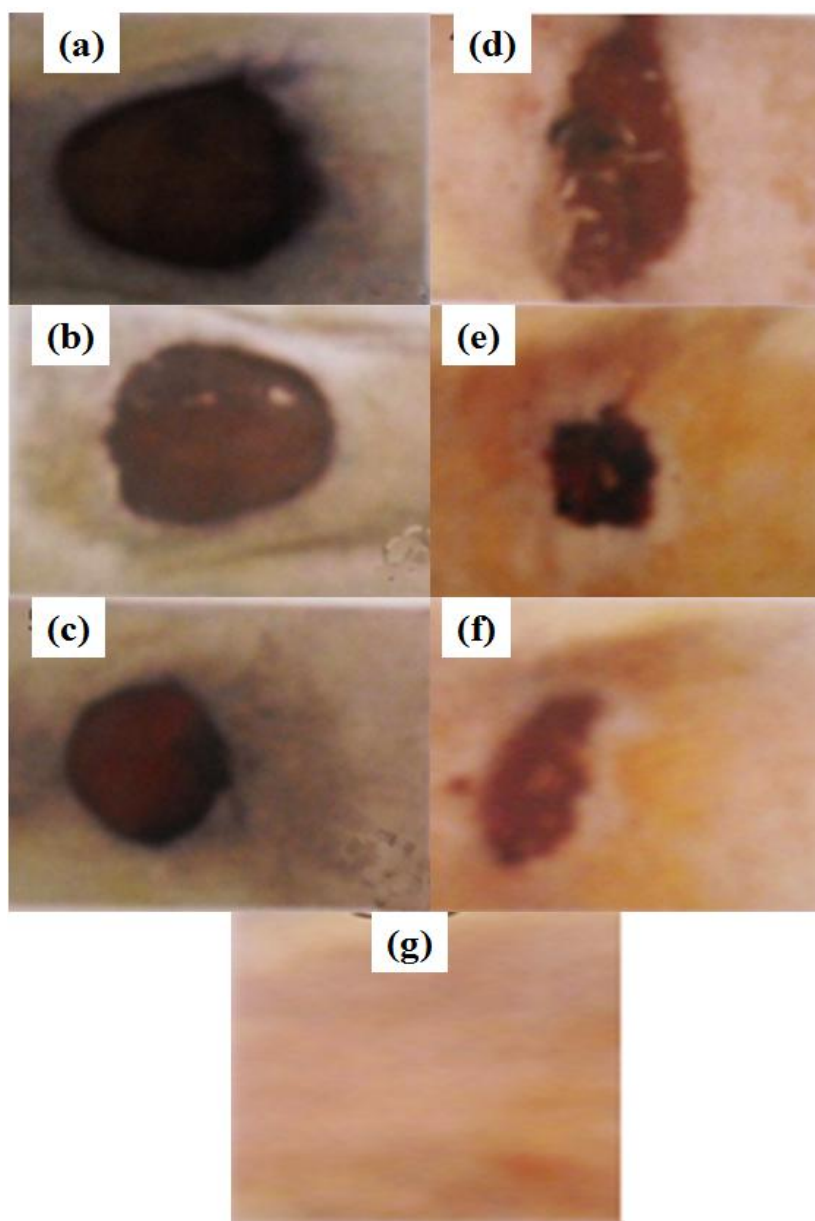


Figure 3: Healing of excision wound on day 0 (a), 3 (b), 6 (c), 9 (d), 12 (e), 15 (f), and 18 (g) of 10 % ointment containing ethanolic extract of *Z. xylopyrus* Willd. bark.

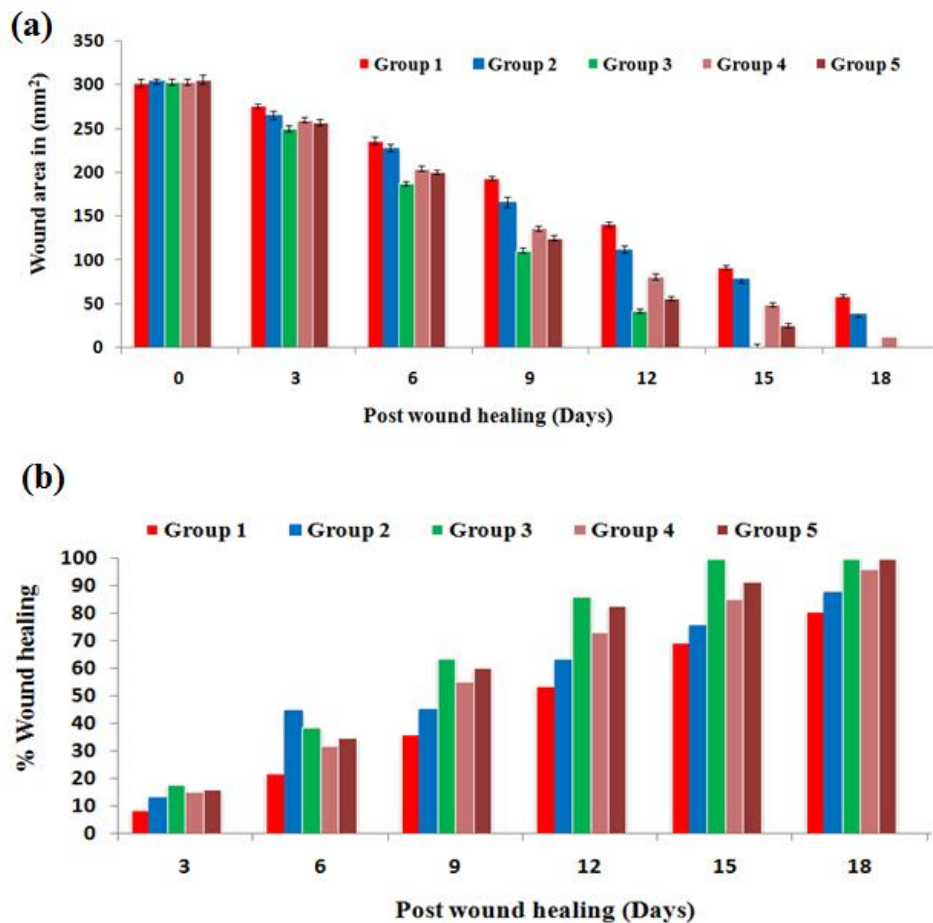


Figure 4: (a) Excision wound area healing (mm², mean ± S.D., n = 4) by topical application of samples; and (b) Percent wound healing of excision wounds by topical application of samples on various animal groups (Group 1: Untreated control; Group 2: Treated topically with hydrophilic ointment base USP; Group 3: Treated topically with 1 % w/w framycetin sulphate ointment; Group 4 and Group 5: Treated topically with ointment base USP containing 5 % w/w and 10 % w/w of ethanol extract of *Z. xylopyrus* Willd. stem bark, respectively).

The ethanol extract of *Z. xylopyrus* Willd. stem bark on the incision wound model demonstrated a significant increase in tensile strength on day 10 for the ointments containing 5 and 10 % w/w test extract, compared to

untreated incision wound, control (Treated with ointment base USP) and standard (1 % w/w Framycetin sulphate cream). The evaluation results of tensile strengths (in Newton/meter²) are shown in Figure 2.

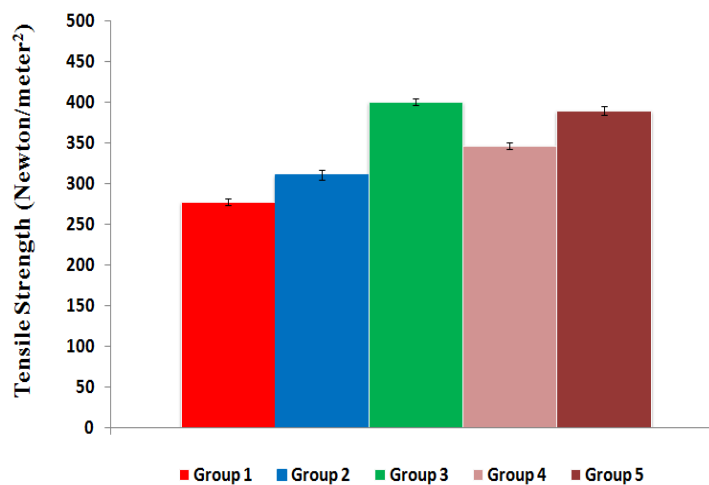


Figure 5: Measured tensile strength (Newton/meter², mean ± S.D., n = 4) for incision wound healing by topical application of samples on various animal groups (Group 1: Untreated control; Group 2: Treated topically with hydrophilic ointment base USP; Group 3: Treated topically with 1 % w/w framycetin sulphate ointment; Group 4 and Group 5: Treated topically with ointment base USP containing 5 % w/w and 10 % w/w of ethanol extract of *Z. xylopyrus* Willd. stem bark, respectively).

From phytochemical identification tests, the nature of the compound responsible for angiogenesis as well as wound healing present in the tested extract and the possible mechanism responsible for these phenomena was not identified. The possible phytoconstituent(s) may be any or a combination of glycosides, carbohydrates, steroids, tannins and flavonoids.

CONCLUSION

According to the results reported here, it can be concluded that the ethanolic extract of *Z. xylopyrus* Willd. Stem bark has a positive angiogenic as well as wound

healing potential. Studies to isolate the active ingredients of the extract that promote both the angiogenesis and wound healing are recommended before proposing its potential application for therapeutic use. However, it needs further evaluation in clinical settings before consideration for the treatment of wounds.

ACKNOWLEDGEMENT

Sincere thanks to Principal and management of Seemanta Institute of Pharmaceutical Sciences, Jharpokharia, India for providing necessary research facilities throughout this study.

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