WOUND HEALING POTENTIAL OF ZIZIPHUS XYLOPYRUS WILLD. (RHAMNACEAE) STEM BARK ETHANOL EXTRACT USING IN VITRO AND IN VIVO MODEL

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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 µm) with air found in pores of the cell membrane of the eggs⁷. CAM from developing eggs in 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 vitro¹⁵,¹⁶. 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 phytomedicines 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.

Zizyphus xylopyrus Willd. (Family: Rhamnaceae) is found throughout North-Western India, Pakistan and China. A large, straggling shrub or a small three, armed with spines, up to 4-7 m. in height. Its local name in Sanskrit: Ghoti, Gotika; Bengali: Kulphal; English: Jujab; Gujrati: Gatabad, Gatabordi; Hindi: Ghunta, Kakora, Kaathabera; Kannada: Yeranu; Marathi: Ghoti, Bhorgoti; 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 troubles. 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 stomachache. The roasted seed powder paste is applied over the chest for relieving pain after cough and colds.

The methanol extract shows the analgesic and anti-inflammatory activity in animal model. The major chemical composition of Z. xylopyrus are Quercetin, Kemperol-4'-methylene and Kemperol, Cyclopeptide alkaloids Amphibine-H and Nummularine-K. 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), carbohydrates (Keller-Killiani, Bornträger’s, and modified Bornträger’s tests), carboxydrates (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 H$_2$SO$_4$ test).

In vitro chick CAM model for screening of angiogenic potential: The chick CAM model 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 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 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%
Suframycin, Avantis

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

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.
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.
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 phytocomponent(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.

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