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Evaluation of wound healing potential of crude extracts of *zyziphus oenolpia* l. Mill (indian jujuba) in wistar rats.

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Abstract

Zyziphus oenoplia is a perennial shrub used in traditional medicine for treating hyper acidity, *Ascaris* infection, stomachalgia and healing of wounds. The objective of this study was to investigate the root extracts of this plant, to evaluate their wound healing potential in rats.

Excision, Incision and Dead space wound modals were used to evaluate the wound healing activity of the extracts on wister rats. In each modal, animals were divided into six groups of six animals. Each model, group I & II served as control and reference standard. In wound models, group III, IV, V and VI animals were treated with plant root extracts (300 and 400mg/kg/day) for 18 days and 10days respectively. The vehicles effect on the rate of wound healing were assessed by the wound closure rate, epithelialisation period, tensile strength, granulation tissue weight, hydroxyproline content and histopathology of granulation tissue. The ethanol extract of plant root promoted wound healing activity significantly in all the wound modals. High rate wound contraction ($P<0.001$), decrease in the epithelialisation period (14.67±0.33), high skin breaking strength (501.53±2.18g), significant increase in granulation tissue weight ($P<0.001$) and hydroxyproline ($P<0.001$) content were observed in ethanol root extract. Histological studies of the granulation tissues of the ethanolic extract treated group showed the lesser number of inflammatory cells, and increase collagen formation than the control and also compared with other groups. Phytoconstituents like flavanoids, alkaloids, steroids, triterpenoids, saponins etc were also confirmed by phytochemical test. The data obtained in this investigation indicated that the ethanolic root extract possesses better wound healing activity and it can treat different types of wounds in human being too.

Keywords: Zyziphus oenoplia, wound models, Drug formulation.

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Introduction

Wound may often possess in problems in clinical practices. Healing of wounds is one of the most complex biological process after birth, as a result of interplay of different tissue structure and a large no of resident and infiltrating cell type constituted [1]. Even no synthetic drug has been attributed in market for direct healing of wounds. WHO has been promoting traditional medicine as a source of less expensive, especially in developing countries and also recognized the traditional medicine.

Zyziphus oenoplia (L.) Mill.(Family: Rhamnaceae), commonly known as Jakal jujube or Small fruited jujube is an erect, straggling or climbing shrub grows up to 3 meter. Branches are fasciculate, often densely rusty tomentose. Nodes slightly enlarged around the leaf scars. This plant can be found throughout India in dry forests and open bushy places. Traditionally the roots are used as astringent bitter, anthelmintic, digestive, antiseptic, hyperacidity, *Ascaris* infection, stomachalgia and healing of wounds. Fruits are found to have medicinal properties like blood purifier, febrifuge, abdominal pain. In Siddha medicine the fruits and the seeds are used in fever, retention of urine, poisoning, aphrodisiac, tonic etc [2]. Chemically the root bark contains cyclopeptide alkaloids (zizyphine-A and zizpyhine-B), betulinic acid, d-glucose, d-fructose, sucrose and unidentified polysaccharides. Stem bark contains cyclopeptide alkaloids Zyziphine (A-G) and abyssinine A and B [3,4,5]. Antimicrobial activity has been reported on methanol extracts of plant *Zyziphus oenoplia* (L.) Mill [6] and also bioassay-guided fractionation of the ethanol extract of the roots resulted four new 13-membered cyclopeptide alkaloids (zizipine N-Q). Zizipine N&Q, which also exhibited significant antiplasmodial activity against the parasite *Plasmodium falciparum*. Ziziphine N and Q also found weak anti mycobacterial activity against *Mycobacterium tuberculosis* [7]. Malamalasar tribe of Parambikulam wild life Sanctuary of Kerala used this plant traditionally to promote the healing of fresh wounds, [8] eventually this plant parts also used by the Chakma tribe in Bangladesh for gastrointestinal disorder [9] As per Ayurveda and ancient Indian medical treaties, this plant has

different types of therapeutic activities like antibacterial, immunomodulatory, antioxidant and wound healing. However, information about wound-healing activity of *Zyziphus oenoplia* is sparse. Keeping above in view, attempts were made to evaluate the wound-healing potential of different extracts of *Zyziphus oenoplia* roots.

Materials and Methods

Preparation of extracts

The shade dried root powder of *Zyziphus oenoplia* was subjected to successive solvent extraction. The powder material was refluxed successively with pet-ether (60-80^{o}C), chloroform, and ethanol (90%) in a Soxhlet extractor for 18 hrs in batches of 50g each cycle. The marc was gently pressed and dried before extracting with the next solvent.

For preparation of aqueous extract 400 gm of powdered material was macerated with 1000ml of distilled water for three days with intermittent stirring and filtered to obtain the aqueous extract. The extracts obtained by the above techniques were concentrated in vacuum under reduced pressure using a rotary flash evaporator.

Preliminary phytochemical screening

The preliminary phytochemical screening was carried out according to the recommended standard procedures [10,11,12] by performing different qualitative chemical tests.

Acute Toxicity Study

Zyziphus oenoplia (L.) Mill root extracts (petroleum ether, chloroform, ethanol and aqueous) were subjected for acute toxicity study according to the recommended method [13] as follows:

Healthy albino mice of either sex weighing about 20-25gm were used to determine the safer dose. The animals were fasted overnight prior to the acute experimental procedure. The extracts were suspended in Tween 80 (1%w/v) and administered at a dose of 1000-4000 mg/kg b.w. orally via gastric catheter. The dose which caused no mortality and was well tolerated was determined in a step wise manner and the effective dose was calculated.

Animals for wound healing activity

Swiss wistar strain rats of either sex weighing 150-200 g were procured from NCP, Shimoga, Karnataka and were maintained at standard housing conditions. The animals were fed with commercial diet (Hindustan Ltd. Bangalore, Karnataka, India) and water *ad libitum* during the experiment. The animal study was permitted by the Institutional Animal Ethical Committee, NCP, Shimoga (Reg No.NCP/IAEC/CLEAR/P.COL/05/07/2007-08).

Evaluation of wound Healing Activity

The wound healing efficiency of *Zyziphus oenoplia* root extracts were evaluated employing three animal models viz., excision wound model, incision wound model and dead space wound model.

Standard reference

Framycetin sulphate skin cream I.P. (FSC) sold as 'Soframycin skin cream (1%w/w)' was obtained from Aventis Pharma Ltd. and used as standard reference for the wound healing activity. All other chemicals used were of analytical grade, purchased from S.D. Fine, Pvt. Ltd. Mumbai.

Drug formulations

Two types of drug formulations were prepared for evaluation of wound healing activity. For topical application 5g of each extract was separately incorporated with 100g of simple ointment IP [14]. The formula for simple ointment I.P. is:

Sl no	Ingredients	Quantity(gm)
1	White bees wax	20
2	Hard paraffin	30
3	Cetosteryl alcohol	50
4	White soft paraffin	900

For oral administration, suspensions of 300mg/ml chloroform extract and 400mg/ml of all other extracts were incorporated with Tween 80 (1%w/v).

Excision wound model

The rats were inflicted with excision wounds as described by Morton and Malone (1972) [15]. Under light ether anaesthesia, a circular wound of about 500sq.mm.was made on depilated ethane sterilized dorsal thoracic region of rats. The wounds were divided into six groups of six each. The animals of group I were left untreated (control gp.), group II served as reference standard and treated with 1% (w/w) Framycetin sulphate cream (FSC) IP, animals of group III, group IV, group V and group VI were treated with 50mg of 5% ointment prepared from pet-ether, chloroform, ethanol and aqueous root extracts of *Zyziphus oenoplia*. The ointment was topically applied once a day, starting from the day of operation, till complete epithelialization. The animals were housed individually. The wounds were traced on mm^2 graph paper on the day of 4, 8, 12 and 16 post wounding days and thereafter on alternate days until healing was complete. The percentage of wound closure (% contraction), and period of epithelialization (number of days required for falling of the dead tissue remnants of the wound without any residual raw wound) were calculated.

Determination of Percentage Wound Contraction [16]

Percentage wound contraction was calculated as:

$$\text{Percentage wound contraction on N}^{th}\text{ day} = 100 - \frac{\text{Wound area on N}^{th}\text{ day}}{\text{Wound area on 1}^{st}\text{ day}} \times 100$$

Incision wound model

The method of Enrlich and Hunt [17] was adapted for incision wound model. Under light ether anaesthesia, 6cm long Para vertebral incisions were made through the full thickness of the skin on either side of the vertebral column. The wounds were closed with interrupted sutures of 1cm apart. The animals were divided into six groups of six animals each. The animals were left undressed and housed separately. The animals of group I left untreated, the group II served as reference standard and received 1% (w/w) FSC. The animals of group III, group IV, group V, and group VI were treated with pet-ether, chloroform, ethanol and aqueous root extracts of *Zyziphus oenoplia*. The ointments were applied to the wound topically once a day from the day of operation till complete healing. The sutures were removed on 8th post wounding day and the skin breaking strength of the wounds were measured on the 10th day according to the continuous constant water flow technique of Lee et al [18] as follows:

The Allis forceps were firmly applied on the lines, facing each other. The forceps on one side was hooked to a metal rod, fixed firmly to the operation table, while the other to a light polythene container through a string runs over a pulley. Water was allowed to flow at a constant rate into the polythene container so as to build a gradual pulling force necessary to disrupt the wound. The flow of water was regulated by means of an occlusion clamp on rubber tubing connected to a reservoir, kept at a suitable height. As soon as the gaping of the wound was observed, the water flow was stopped. The volume of water in the polythene container was measured and converted to the corresponding weight assuming the density to be equal to 'one'. The tensile strength was expressed as the minimum weight of water necessary to bring about the gaping of the wound.

Dead space wound model

Under light ether anesthesia, the dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5cm x 0.3cm) in the region of groin on both the sides and then the wounds were sutured [19]. The animals were divided into five groups of six each. The group I animals were left untreated and served as control, group II, group III, group IV and group V animals received oral suspensions of pet-ether, chloroform, ethanol and aqueous root extracts (300 and 400mg/kg. b.w., *p.o.*) of *Zyziphus oenoplia* respectively. The granulation tissues formed around the piths were carefully harvested on the 10th post wounding day. The wet weight of the granulation tissue was noted. The breaking strength of the granulation tissue was measured by the method of Lee et al [18], as described already under incision wound model. The granulation tissue was dried at 60ºC for 24 hrs and weighed and the dry weight of the granulation tissue was noted. The dried tissue was added 5 ml of 6N Hydrochloric acid and kept at 110ºC for 24hrs the acid hydrolysate of dry tissue was used for estimation of hydroxyproline content [20].

Histopathological studies

A section of wet granulation tissue was subjected to histopathological examination so as to determine the pattern of lay down of collagen using haematoxylin and eosin stains.

For histopathological studies, the granulation tissue was fixed in 10% neutral formalin solution for 24 h and dehydrated with a sequence of ethanol-xylene series of solutions step wise [20, 21]. The tissue was then impregnated in molten paraffin and xylene (1:3) and incubated at 60ºC for 1hr. Then transferred to a vessel containing molten paraffin and xylene (1:1) and then to another vessel containing molten paraffin and xylene (3:1) and incubated at 60ºC. Microtone sections were taken at 5-10 µ thickness. The sections were then hydrated by passing through decreasing grades of alcohol and finally distilled water. The hydrated tissue sections were stained with haematoxylin for a few minutes and washed with ammonia water and then with distilled water. The sections were carefully dehydrated with ascending grades of alcohol-xylene mixtures (1:3, 1:1 and 3:1) and counter stained with eosin and then dehydrated with alcohol. The sections were observed for histopathological changes under a microscope and photographs of the same were taken for interpretation of results.

Collagen estimation (Hydroxyproline content)

For the preparation of protein hydrolysate, 50 mg of tissue sample in 1.0 ml of 6.0 N Hydrochloric acid was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 15 1.056 kilograms per cm^2 for 3 hrs. The hydrolysate was neutralized to pH 7.0 and brought to the appropriate volume. Test tubes marked as sample, standard and blank were taken. One ml of test sample was added to test tubes marked as sample, 1.0 ml of de-mineralized water to test tubes marked as blank and 1.0 ml standard solutions to test tubes marked as standard. One ml of 0.01 M Copper sulphate solution was added to all the test tubes followed by the addition of 1.0 ml of 2.5 N Sodium hydroxide and 1.0 ml of 6% Hydrogen peroxide. The solutions were occasionally mixed for 5 min and then kept for 5 min in a water bath at 80ºC. Tubes were chilled in ice-cold water bath and 4.0 ml of 3.0 N Sulphuric acid was added with agitation. Two ml of *p*-(dimethylamino) benzaldehyde was then added and heated in water bath at temperature 70ºC for 15 min. The absorbance was measured at 540 nm using UV spectrophotometer.

Statistical analysis

The data obtained from each experiment were subjected to one way ANOVA followed by Turkey's Multiple Comparison test. The F

values, dF values and P values were analysed and recorded in respective tables.

Results and Discussions

The phytochemical tests shown the presence of proteins, carbohydrates, alkaloids, saponins, tannins, flavonoids, steroids, triterpenoids etc. as mentioned in Table 1. Petroleum ether extract gave positive tests for steroids and triterpenoids; chloroform extract shown positive tests for alkaloids and triterpenoids; ethanol and aqueous extracts were found to contain all the constituents except glycosides.

Acute toxicity studies showed that the maximum tolerated dose of the root extracts of *Zyziphus oenoplia* were 4000mg/kg b.w. for pet-ether, ethanol, and aqueous extracts while 3000mg/kg b.w. for the chloroform extract. Animals were observed for behavioral change and death. No animal was found dead after 14 days. Hence 1/10th of these doses were selected for the evaluation of wound healing activity. The result indicates that the extracts were safe and non toxic.

The wound healing activities of crude root extracts of *Zyziphus oenoplia* was evaluated employing three different animal models viz., excision, incision and dead space wound models. In all the models studied, significant wound healing activity was observed with the ethanol root extract of *Zyziphus oenoplia* followed by aqueous extract when compared to reference standard framycetin sulphate.

In excision wound model the parameters studied were percentage wound closure and mean epithelialization time. Significant wound healing activity was observed in the animals treated with ethanol extract (Fig.1 d, e, & f) compared to standard reference group (Fig.1 a, b, & c). The percentage of wound closure and percentage wound contraction as observed on 16th day in framycetin treated group were 8.33±0.88 Sq.mm and 98.35±0.16% respectively. The percentage of wound closure and wound contraction were found to be more significant with ethanol extract (14.67±0.33 Sq.mm and 97.09±0.05%) followed by aqueous root extract (25.67±0.88 Sq.mm and 94.91±0.20%) compared to the standard reference. The mean epithelialization time was also comparatively less in these groups when compared to control (Table 2. & Fig. 2). The data obtained from chloroform extract treated group was not appreciable and that of pet-ether extract treated group was very poor and hence less significant compared to standard reference.

In the incision wound model the parameter studied was wound breaking or tensile strength of wounds treated with different extracts of *Zyziphus oenoplia*. The data obtained from the incision wound model are shown in Table 3. The animals treated with ethanol extract exhibited significant tensile strength (501.53±2.18g) on the post wounding day 10 compared to the reference standard treated group (544.00±2.08g) followed by aqueous extract treated group (465.00±1.36g). However the tensile strength of animals treated with the chloroform extract was less significant and that of pet-ether extract treated group and control group were not appreciable.

The results of the various parameters studied under dead space wound model (Fig. 3) such as tensile strength/breaking strength of granulation tissue, wet and dry weight of granulation tissue and hydroxyproline content of the granulation tissue are shown in Table 4. The tensile strength of the granulation tissue in ethanol extract treated animals was significant (396±1.53g), followed by aqueous extract treated animals (363±2.03) when compared to that of control group (282±1.15g), while that of chloroform and pet-ether extract treated groups were not significant. The weight of the dry granulation tissue was also more significant in the animals treated with alcoholic root extract (74±1.73mg/100g rat) and aqueous root extract (56±2.08 mg/100g rat) when compared to control (34.67±1.45 mg/100g rat).

The hydroxyproline content of the granulation tissue followed the same pattern as that of tensile strength. Hydroxyproline content of granulation tissue obtained from animals treated with alcoholic extract was comparatively more significant (56.00±2.08mg/g tissue), followed by animals treated with aqueous root extract (41.67±1.20 mg/g tissue) when compared to that of control (20.33±0.88 mg/g tissue). The chloroform and ether extract treated groups yielded less significant results (Fig. 3).

The alcoholic root extract of *Zyziphus oenoplia* was found to be more efficient as evidenced by the histopathological studies of the granulation tissue. The histopathological section of control group showed more macrophages and fibroblasts, insignificant collagenation and vascularization. The sections of ethanol extract treated group indicated significant collagenation, less macrophages and well formed capillaries, while the histopathological sections of aqueous extract treated group showed moderate collagenation and capillary formation. The collagenation in chloroform extract treated group was insignificant while that of pet-ether extract treated group, the wound healing was found to be improper as insignificant collagenation was observed (Fig. 4).

The results of the present study revealed that the ethanol root extract of *Zyziphus oenoplia* possesses a definite pro-healing action, which was demonstrated by a significant increase in the rate of wound contraction and by enhanced epithelialization. Significant increase was also observed in skin breaking strength and hydroxyproline content of granulation tissue in ethanol root extract treated group which was a reflection of increased collagen levels that was further supported by histopathological evidence and gain in granulation tissue breaking strength. This indicated improved collagen maturation by increased cross linking while an increase in dry granulation tissue weight indicated higher protein content. The animals treated with aqueous root extract also shown

Table 1. Preliminary phytochemical analysis of various root extracts of *Zyziphus oenoplia*.

Sl no.	Chemical constituents	Pet. ether extract	Chloroform extract	Ethanol extract	Aqueous extract
01.	Carbohydrates	--	--	+	+
02.	Protein	--	--	+	+
03.	Alkaloids	--	++	++	+
04.	Glycoside	--	--	--	--
05.	Saponins	--	--	+	+
06.	Tannins	--	--	+	+
07.	Flavonoids	--	--	++	++
08.	Steroids	++	+	++	++
09.	Tritepenoids	+	+	+	+

+ = Present, - = Absent.

Table 2. Effect of topical application of crude root extract of *Zyziphus oenoplia* on excision wound area (Sq.mm ± S.E).

Group (N=6)	0day	4th day	8th day	12th day	16th day	Period of epithelialization
Control	508.33±1.18	472.67±2.0	366.33±1.48	284.0±1.73	137.0±1.15	23.0±0.58
Std (FSC)	506.34±1.76	363.67±2.19	257.0±1.15	145.33±1.20	8.33±0.88*	17.33±0.33*
Pet.Ether	506.68±0.98	421.33±1.4	333.67±1.45	238.0±2.08	119.67±1.2	23.33±0.33
Chloroform	505.67±1.93	410.33±2.0	315.0±1.75	180.33±0.88	57.0±2.08	22.53±.088
Ethanol	503.0±1.46	378.67±1.6	264.67±1.33	155.33±2.03	14.67±0.33*	18.67±0.37*
Aqueous	505.0±2.06	385.67±2.60	289.67±1.76	167.67±2.10	25.67±0.88*	19.27±0.34*
		F	283.90	391.60	867.10	2105.0
Df	5	5	5	5	P	0.001	0.001	0.001	0.001

[N=6 animal in each group. *P<0.001 indicates significant compared to control. Values are expressed as mean±S.E.]

Table 3. Effect of topical application of crude root extract of *Zyziphus oenoplia* on Incision wound model.

Group (n=6)	Wound breaking strength (g)
Control	316.33±1.48
Std (FSC)	544.0±2.08*
Pet. Ether ext.	231.33±1.20
Chloroform ext.	347.34±1.53
Ethanol ext.	501.53±0.18*
Aqueous ext.	465.00±1.36*
F	1483.00
Df	5
P	0.0001

[n=6 animal in each group. *significance difference at P<0.0001 when compared to control. Values are expressed as mean ±S.E]

Table 4. Effect of oral administration of crude extracts of *Zyziphus oenoplia* on Dead space wound model.

Group (N=6)	wet wt. of granulation tissue (mg/100g rat)	Dry wt. of granulation tissue (mg/100g rat)	Wound breaking strength (g)	Hydroxyproline (mg/g tissue)
Control	190.67±2.0	34.67±1.45	282±1.15	20.33±0.88
Pet. Ether	227.33±1.98	29.87±1.76	263±1.20	17.33±0.88
Chloroform	246.00±1.74	44.67±1.45	300.67±1.20	30.0±1.15
Ethanol	359.00±1.81	74.0±1.73**	396.0±1.53**	56.0±2.08**
Aqueous	309.0±1.65	56.0±1.73*	363.0±2.03	41.67±1.20*
F	404.10	118.60	1296	147.0
Df	4	4	4	4
P	0.001	0.001	0.001	0.001

[n=6 animals in each group. *significance difference at P<0.001 when compared to control. Values are expressed as mean ± S.E]

Figure 1. Excision wound model.

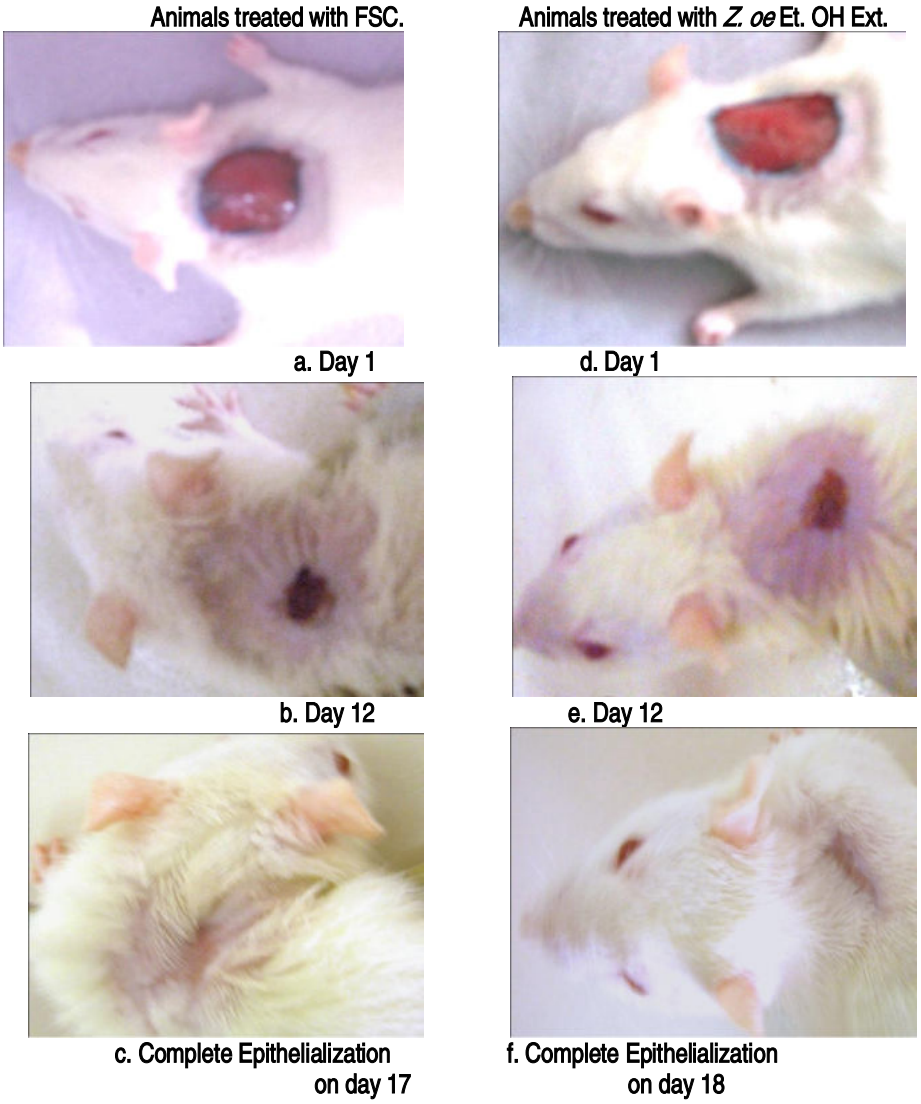


Fig.2. Effect of various root extracts of *Zyziphus oenoplia* on percentage wound contraction in Excision wound model.

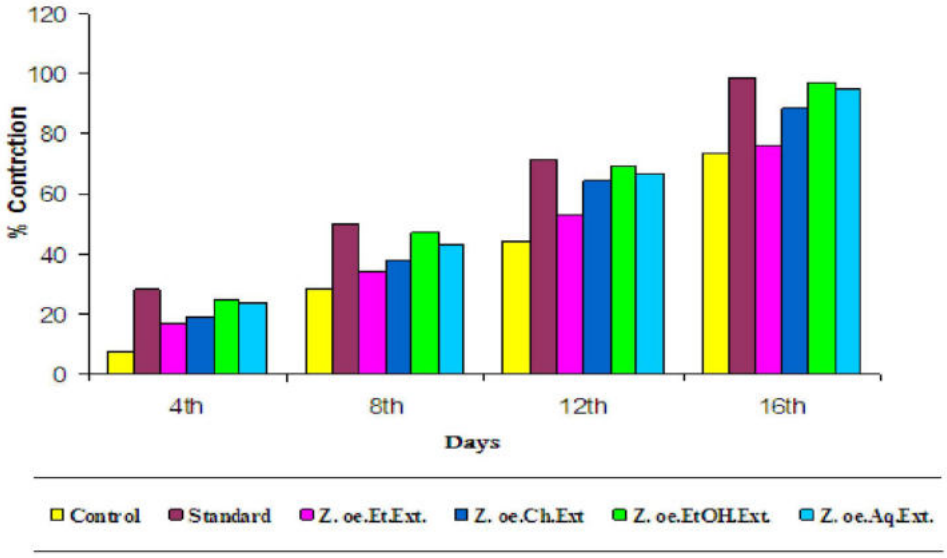


Fig. 3. Effect of oral addministration of crude root extract of *Zyziphus oenoplia* on dead space wound model.

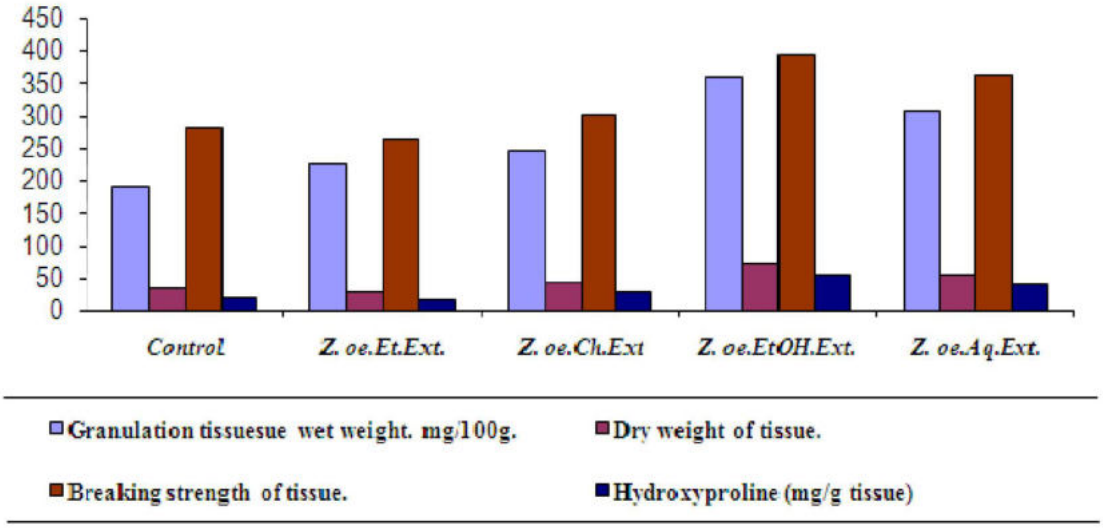
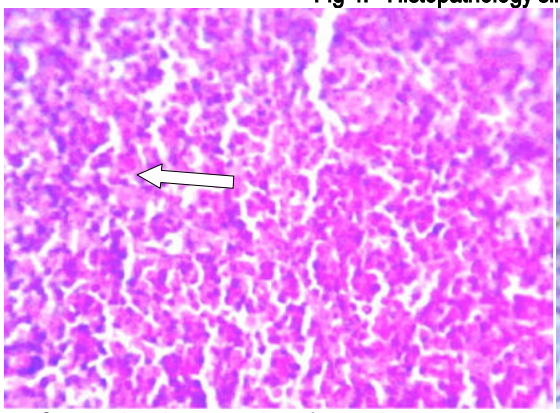
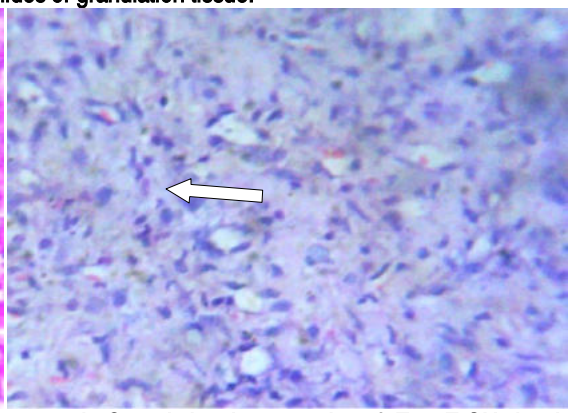


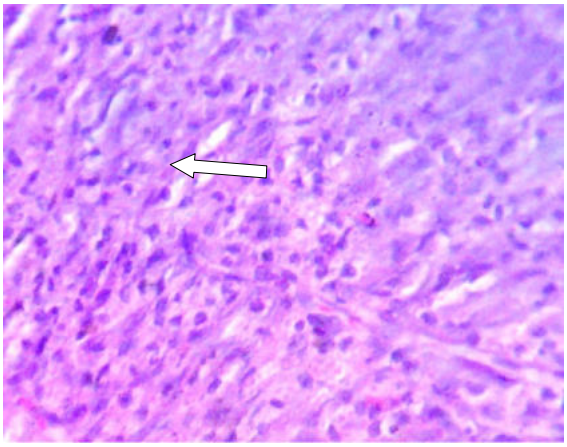
Fig 4. Histopathology slides of granulation tissue.



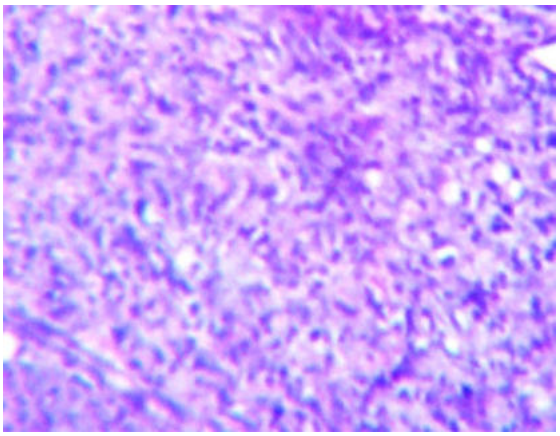
a. Granulation tissue section of control group



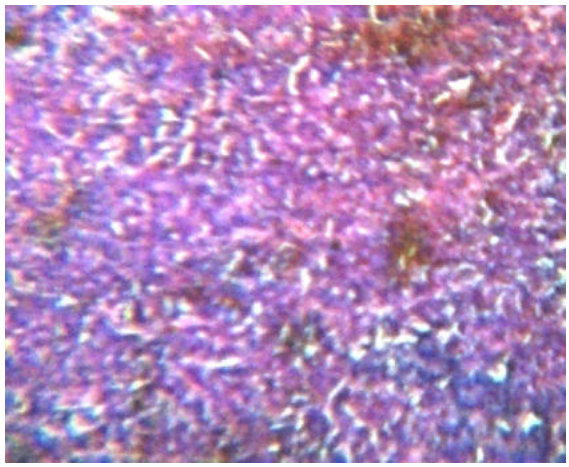
b. Granulation tissue section of *Z. oe* EtOH showing more macrophagesand less collagenation. Ext. treated group showingsignificantcollagenation and less macrophages.



c. Granulation tissue section of *Z. oe* Aq Ext. significant collagenation.



d. Granulation tissue section of *Z. oe* Ch. Ext. treated group showing treated group showing insignificant collagenation.



e. Granulation tissue section of *Z. oe* Et. Ext. treated animal group showing less collagenation.

significant wound healing activity in all the three models, while the results were insignificant with pet-ether and chloroform extracts

The wound healing property of *Zyziphus oenoplia* roots may be attributed to the phytochemicals present in the plant, and the quicker process of wound healing would be a function of either the individual or the additive effect of the phytoconstituents. Earlier works on evaluation of wound healing activity of various medicinal plants using animal models have suggested that plant constituents such as triterpenoids, flavonoids, saponins and tannins are responsible for wound healing effect [21,22,23,24]. The preliminary phytochemical analysis of root extracts of *Zyziphus oenoplia* revealed the presence of flavonoids in the alcoholic and aqueous extracts, where flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis, but also by improving vascularity. Hence any drug that inhibits lipid peroxidation is believed to increase viability of collagen fibrils by increasing the strength of collagen fibres; increasing circulation; preventing cell damage and by promoting the DNA synthesis [26]. Flavonoids [25], triterpenoids [27] are also known to promote the wound healing process chiefly due to their astringent and antimicrobial properties, which seems to be responsible for wound contraction and increase rate of epithelialization.

Hence the present investigation of the wound healing activity of alcoholic and aqueous root extracts of *Zyziphus oenoplia* may be attributed to chiefly flavonoids, tannins and triterpenoids. The results of this investigation provide pharmacologic evidence on the folkore use of *Zyziphus oenoplia* for wound healing activity.

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