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Evaluation of wound healing and antimicrobial potentials of *Ixora coccinea* root extract

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ABSTRACT

Objective: To evaluate the wound healing and antimicrobial activity of root extracts of *Ixora coccinea (I. coccinea)*. **Methods:** To investigate the wound healing efficacy of root extract of *I. coccinea* Linn, five groups of animals were divided each containing six animals. Two wound models including incision and excision wound models were used in this study. The parameters studied were tensile strength on incision wound model and in terms of wound contraction for excision wound model were compared with standard Nitrofurazone (NFZ) ointment (0.2% w/w). Six extracts (ethanol, aqueous, petroleum ether, benzene, chloroform and ethyl acetate) of *I. coccinea* were screened for *in vitro* growth inhibiting activity against different bacterial strains *viz*, *Staphylococcus aureus, Bacillus pumilius, Enterococcus faecalis, Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa* and fungi *Candida albicans* and *Aspergillus niger* were compared with the standard drugs ciprofloxacin and chloramphenicol for antibacterial and griseofulvin for antifungal screening. The serial dilution and cup (or) well plate methods were used for the antimicrobial study and MIC was determined. **Results:** The ethanolic extract showed significant ($P<0.001$) wound healing activity when compared to standard drug NFZ with respect to normal control group. Amongst all, ethanolic extract showed highly significant antibacterial activity against all bacterial strains used in this study when compared to standard. The aqueous extract showed moderate significant inhibition against all bacterial strains when compared to standard. All the extracts were shown negligible activity against the fungal strains used in this study. **Conclusions:** The ethanolic root extract of *I. coccinea* showed pronounced wound healing and antibacterial activity. The probable reason to heal the wound was that the external application of the extract prevented the microbes to invade through the wound thus the protection of wound occurs against the infection of the various organisms.

1. Introduction

Ixora coccinea (*I. coccinea*) is a well known medicinal plant widely distributed in East Indies and is in tropics and variously in sub tropics at altitudes of 1 300 m above sea level like Caribbean India and Srilanka[1]. Root in the form of tinctureis is used as a folk remedy in diarrhea and dysentery. The pharmacognostical study of the earlier reports revealed that the leaf extract of *I. coccinea* acquire antimicrobial[2], antiinflammatory[3], anti mitotic[4], astringent, sedative in hiccups, sedative in nausea, sedative in loss of appetite[5], antinociceptive[3] and other activities. Also the flowering part of *I. coccinea* possess anti mutagenic[6] activity with wide ranging biological action. Thus in the present revision the investigation of wound healing and antimicrobial studies of *I. coccinea* root extract against bacterial and fungal strains may led to expanding the *I. coccinea* as a versatile medicine in the current herbal scenario.

Wound infections are most common in developing countries because of poor hygienic conditions. *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeroginosa, Salmonella thphi* and *Klebsiella pneumoniae* are the principal pathogens of wound infection[7]. A wide range of antimicrobials can be used to treat wound infections, but the adverse effects and drug resistance developed due to indiscriminate use of commercial antimicrobial drugs commonly exist in the treatment of infectious diseases. In order to overcome this problem, recent attention turns towards herbal field for the new findings in particular, biologically active compounds isolated from plant species[8].

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This situation forced us to search a medicinal plant with dual activity (wound healing and antimicrobial activity) possibly an incredible and much useful in the treatment of antimicrobials. The present study deals with the evaluation of wound healing activity of ethanolic root extract of *I. coccinea* and various root extracts such as ethanol, aqueous, petroleum ether, benzene, chloroform and ethyl acetate of *I. coccinea* were carried out for antimicrobial studies for opportunistic wound infection.

2. Materials and methods

2.1. Preparation and phytochemical screening of various root extracts of I. coccinea

The roots of *I. coccinea* (Rubiaceae) were from Quilon and Kottayam districts of Kerala State. The root was collected, shade dried and powdered to get a coarse powder and subjected to solvent extraction successively with ethanol, water, petroleum ether, benzene, chloroform and ethyl acetate. All the extracts were filtered and lyophilized thus the pooled extracts were concentrated and dried under vacuum.

To observe the phytochemical constituents present in different extracts, standard methods *viz*. Dragendorff's test (alkaloids), foam formation (saponins), Liebermann-Burchard's test (terpenes and steroids), Molisch's reagent (glycosides), by using magnesium and dil. HCl (flavonoids), spot test (fixed oil), ninhydrine test (proteins) and 5% ferric chloride test (tannins) were employed[9].

2.2. Animal care and handling

Healthy, inbred Wistar albino rats (150-180 g) of either sex were used in this study. The animals were housed under standard conditions of temperature at (26±2) ℃, relative humidity of 44%-55% and light dark cycles of 10 and 14 h respectively. Animals were acclimatized to laboratory condition before commencement of experiment. Animals were housed individually in different polypropylene cages (U. N. Shah manufacturers, Mumbai, India) containing sterile paddy husk (procured locally) as bedding and were provided with rodent diet (Amrut lab animal feed, Pranav agro industry Ltd, Sangli, Maharashtra) and water *ad libitum*. The animal experiment protocols were approved by institute animal ethical committee.

2.3. Study design

Incision and excision wound models were used to assess the wound healing profile of ethanolic extract of *I. coccinea* in rats. Five groups of animals were used each containing six animals were used. The drug treatment was as follows:

Group I: Received simple ointment base and served as normal control group.

Group II: Received alcoholic extract ointment 0.5% w/w topically on wound created on the dorsal back of rats daily till the wounds completely healed[10].

Group III: Received alcoholic extract ointment 1% w/w topically on wound created on the dorsal back of rats daily till the wounds completely healed.

Group IV: Received alcoholic extract ointment 1.5% w/w topically on wound created on the dorsal back of rats daily till the wounds completely healed.

Group V: Received reference standard 0.2% w/w nitrofurazone ointment.

The antimicrobial studies were carried out by using serial dilution method and cup or well plate method[11] on all six extracts of *I. coccinea*.

2.4. Micro organism

The bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Bacillus pumilius* ATCC 6633, *Enterococcus faecalis* ATCC 29212 for gram positive and *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 26731 and *Pseudomonas aeruginosa* ATCC 27853 for gram negative. Fungal strain *viz*, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 were collected from National Chemical Laboratory, Pune and institute of microbial technology (IMTECH), Chandigarh. All the extracts were tested at a concentration of 100 μg/mL were prepared in dimethyl sulphoxide (DMSO).

2.5. Extracts and standards used

Three types of ointment formulations with different concentrations of the ethanolic extract were prepared *viz*. 0.5% (w/w) ointment where, 0.5 g of extract was incorporated in 100 g of simple ointment base B.P[12], 1% (w/w) ointment where, 1 g of extract was incorporated in 100 g of simple ointment base B.P and 1.5 % (w/w) ointment where, 1.5 g of extract was incorporated in 100 g of simple ointment base B.P. Nitrofurazone was obtained from Smith Kline-Beecham Pharmaceuticals, Bangalore, India; Ciprofloxacin from Ibis Chemie International Mumbai, India; Chloramphenicol from Mehta Pharmaceutical Industries, Mumbai, India; and griseofulvin from Simrone Pharma Industries Ltd, Mumbai, India were used as standard drugs. The solvents includes ethanol, petroleum ether, benzene, chloroform ethyl acetate and DMSO were procured from Rankem, Newdelhi, India.

2. 6. Evaluation of wound healing model

Wound healing activity was assessed by incision and excision wound healing models. All wounding were carried out under ketamine anaesthesia 50 mg/kg body weight of the rat, intraperitoneally (i.p). In the present study no animals showed visible signs of infection.

2.6.1. Incision wound model

In this model tensile strength of the wound was assessed. Two paravertebral-long incisions were made through the skin and cutaneous muscles at a distance of about 1 cm from the midline on each side of the depilated back of the rat about 6 cm. After the incision was made, the parted skin was kept together and stitched with black silk (Ethicon, Johnson & Johnson, Himachal Pradesh, India) at 0.5 cm intervals; surgical thread (No. 000) and a curved needle (No. 11) were used for stitching. The continuous threads on both wound edges were tightened for good closure of the wound. The wound was left undressed. Sample extract along with simple ointment (control) and standard drug were topically administered once daily for 9 days; when wounds were cured thoroughly the sutures were removed on the 9th day and on 10th day tensile strength was measured with a tensiometer. The mean tensile strength on the two paravertebral incisions on both sides of the animals was taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength increment indicates better wound healing stimulation by the applied drug[13].

2.6.2. Excision wound model

This model was utilized to study the rate of wound contraction. Five groups with six animals in each group were anaesthetised by the open mask method with anaesthetic ether. The rats were depilated on the back. One excision wound was inflicted by cutting away a 500 mm^2 full thickness of skin from a predetermined area. The wound was left undressed to the open environment. Then the drugs, i.e., simple ointment, ethanolic extract ointments (0.5% w/w, 1% w/w and 1.5% w/w), the reference standard (0.2%, w/w nitrofurazone ointment) were administered topically till the wounds completely healed. Wound contraction rate was monitored by planimetric measurement of wound area on alternative days. This was done by tracing the wound area on butter paper on every alternative days from the day of the treatment till the wound was completely healed. These readings were transferred to the standard graph sheet. The wound contraction was calculated as the percentage of original wound size 500 mm^2 taken as 100% for each animal of the group. From the healed wound, a specimen sample of tissue was isolated from each group of animals for histopathological examination. These tissues were stained with haematoxylin-eosin stain and viewed under microscope[14] for histological examination.

$$\%\ \text{of wound contraction} = \frac{\text{Initial wound size} - \text{specific day wound size}}{\text{Initial wound size}} \times 100$$

2.7. Statistical analysis

Pharmacological data were analysed by one-way analysis of variance (ANOVA) followed by Dunnet's t test. The difference was considered significant when P value <0.05. All the values were expressed as mean ± SEM.

2.8. Evaluation of antimicrobial activity

2.8.1. Cup or well plate method

For cup or well plate diffusion method[11] nutrient agar (Hi-Media) media was used for bacterial strains and Sabouraud dextrose agar (Hi-Media) media was used for fungal strains by measuring the zone of inhibition in millimetres

Nutrient agar plates were prepared aseptically to get a thickness of 5-6 mm. The plates were allowed to solidify and inverted to prevent condensate falling on the agar surface. The plates were dried at 37 ℃ just before inoculation.

The standard inoculum was inoculated in the plates prepared earlier (aseptically) by dipping a sterile swab in the inoculum, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium for three times, rotating the plate at an angle of 60° after each application. Finally press the swab around the edge of the agar surface. Leave the inoculum to dry at room temperature with lid closed. The wells are prepared in the petridishes aseptically. 0.2 mL (100 μg/mL) of the test solution of the extracts as well as standard was poured in it using a dropping pipette under aseptic condition and labelled accordingly.

Incubate the petridishes at (37±0.2) ℃ for about 18-24 h after placing them in the refrigerator for one hour to facilitate uniform diffusion. The average zone diameter of the plates were measured and recorded. All root extracts of *I. coccinea* were tested for antibacterial and antifungal activity. While those used for antifungal activity were incubated at 28 ℃ for 48-72 h. The results were compared to chloramphenicol (gram positive, 10 μg/mL), ciprofloxacin (gram negative, 10 μg/mL) and griseofulvin (10 μg/mL) as standard for antibacterial and antifungal activity respectively. DMSO was used as a control.

2.8.2. Serial dilution method

In this 2-fold serial broth dilution method[15, 16] double strength nutrient broth was used. This method depends upon the inhibition of growth of a microbial culture in a uniform solution of liquid medium that is favourable to its rapid growth in the absence of the antimicrobial agents. All root extracts were dissolved in dimethyl Sulfoxide (DMSO) to give a concentration of 2 000 μg/mL as stock solution.

All of the following steps were carried out using aseptic technique. A solution of 0.4 mL of 2 000 μg/mL test stock solutions in DMSO was transferred to a first sterile test tube containing 3.6 mL of broth to arrive 200 μg/mL as starting dose. These test tubes were serially diluted to give a concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 μg/mL. One test tube with no test compound but with equal volume of solvent DMSO (10%) served as the vehicle control. One test tube with no test compound and no vehicle but only with nutrient media served as the positive control to ensure the growth property of media. To all the test tubes 0.1 mL of suspension of bacteria and fungi (working inoculum) was added. The test tubes were incubated as given above in cup (or) well plate method. The highest dilution of the test extract that completely inhibited the growth of test organism was considered as MIC value and was expressed as μg/mL.

3. Results

3.1. Phytochemical screening

The preliminary phytochemical screening of all the root extracts obtained from alcohol, aqueous, petroleum ether, benzene, chloroform and ethyl acetate were evaluated for the presence of alkaloids, glycosides, steroids, fixed oils, fats, terpenes, tannins and flavonoids. The results revealed that the presence of flavonoids, fixed oils, tannins, fats in all the extracts, whereas steroids present additionally in ethyl acetate and aqueous extracts.

3.2. Measurement of tensile strength for incision wound model

The parameter tensile strength was used for the measurement of incision wound model. The wound which was untreated (Control group) had the minimum strength (426.0±8.2 g). The mean tensile strength of the group treated with ethanolic root extract of *I. coccinea* was significantly increased in a dose dependent manner when compared to control group. The mean tensile strength of wound treated with 1% w/w (524.0±6.4 g) and 1.5% w/w (557.0±8.4 g) ethanolic extracts showed highly significant (P<0.001) when compared to control group, which was almost equipotent to that of 0.2% nitrofurazone's (standard drug) mean tensile strength (570.0±7.5 g). It may be inferred that, 0.2% w/w nitrofurazone, 1% w/w and 1.5% w/w of ethanolic extract ointments exert more or less same results on the tensile strength of the healing tissue. This observation confirms that the ethanolic extract of *I. coccinea* possesses a very good wound healing property with regards to tensile strength of wound healing tissue was concerned.

3.3. Wound contraction studies for excision wound model

The parameter wound contraction used for the measurement of excision wound model. It was seen that the faster healing of wound took place in case of animals, which received 1.5% w/w ethanolic extract ointment than others such as 0.5% w/w, 1% w/w of plant extract ointment group. The least rate of wound healing was seen in the normal control group, which received simple ointment. Wound failed to heal completely even after 18th day for 0.5% w/w and 1% w/w groups; however the unhealed area was smaller than the control group. Treatment with standard group heals the wound in a faster rate than other group, but complete healing was obtained on 18th day (Table 1). The upper layer of wound was surgically removed and subjected to histological studies. Histological examination of the haematoxylin and eosin stained tissue of the rat wounds treated with plant extract and nitrofurazone ointment have led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinisation and epithelialisation as compared to control group.

3.4. Anti microbial activity

The antimicrobial activities of *I. coccinea* extracts (ethanol, aqueous, petroleum ether, benzene, chloroform and ethyl

Table 1
Evaluation of wound contraction of ethanolic extract of *I. coccinea* for excision wound model in rats.

Post wounding(days)	Wound area (mm^2)				
	Simple ointment (control)	Extract ointment (0.5% w/w)	Extract ointment (1% w/w)	Extract ointment (1.5% w/w)	Standard:nitrofurazoneointment (0.2% w/w)
0	485.0±1.2	461.0±4.1	478.0±2.4	459.0±2.8	464.0±1.4
2	433.0±0.8	422.0±1.8[a]	420.0±3.4[a]	401.0±1.9[a]	397.0±2.1[b]
4	386.0±3.2	357.0±2.5[a]	343.0±1.5[a]	321.0±1.1[a]	315.0±0.8[b]
6	352.0±1.3	285.0±2.7[a]	287.0±1.6[a]	267.0±2.5[b]	256.0±2.6[b]
8	331.0±1.5	220.0±2.4[b]	211.0±2.2[b]	194.0±0.9[b]	198.0±1.7[b]
10	292.0±2.8	179.0±1.9[b]	162.0±0.9[b]	125.0±1.8[b]	118.0±3.3[b]
12	265.0±1.8	145.0±1.6[b]	118.0±2.3[b]	74.0±0.9[b]	71.0±1.8[b]
14	238.0±0.9	108.0±2.4[b]	75.0±2.8[b]	43.0±2.0[b]	37.0±0.9[b]
16	201.0±2.0	71.0±1.0[b]	49.0±0.3[b]	11.0±0.6[b]	9.0±0.6[b]
18	177.0±0.9	43.0±0.9[b]	20.0±2.2[b]	0.0±0.0[b]	0.0±0.0[b]

Results are expressed as mean ± SEM. (n= 6). a P<0.05 and b P<0.001 when compared to control group (one-way ANOVA followed by Dunnett's t-test).

Table 2
Evaluation of antimicrobial activity of different root extracts of *I. coccinea* by disc diffusion method.

Plant Extracts	Zone of inhibition (mm)															
	Bacterial strains (μg/disc)												Fungal strains			
	Staphylococcus aureus		*Bacillus pumilius*		*Enterococcus faecalis*		*Escherichia coli*		*Salmonella typhi*		*Pseudomonas aeruginosa*		*Candida albicans*		*Aspergillus niger*	
	50	100	50	100	50	100	50	100	50	100	50	100	50	100	50	100
Ethanol	21	41	22	43	22	42	20	42	20	41	22	41	1	2	0	1
Aqueous	22	41	21	40	21	39	19	40	20	39	18	40	0	2	0	1
Petroleum ether	16	37	17	37	15	35	15	36	18	35	17	36	0	0	0	0
Benzene	14	35	15	36	14	34	14	33	15	34	15	36	0	0	0	0
Chloroform	18	39	17	38	17	40	19	39	18	39	19	38	1	2	0	1
Ethyl acetate	17	41	18	40	20	39	16	38	18	37	18	38	0	1	0	0
Ciprofloxacin[b]	45		44		44		42		43		40		–		–	
Chloramphenicol[b]	39		37		38		42		43		42		–		–	
Griseofulvinb	–		–		–		–		–		–		23		24	

b Ciprofloxacin (10 μg/disc); Chloramphenicol (10 μg/disc) were used as positive reference standards antibiotic discs.

Table 3
Evaluation of MIC values of different root extracts of *I. coccinea* against microorganisms.

Micro organism		Minimum inhibitory concentration (MIC)					
		Ethanol	Aqueous	Petroleum ether	Benzene	Chloroform	Ethyl acetate
Bacterial strains	*Staphylococcus aureus*	50	50	200	>200	100	>200
	Bacillus pumilius	25	50	200	>200	100	100
	Enterococcus faecalis	25	50	200	>200	100	200
	Escherichia coli	50	100	>200	>200	200	200
	Salmonella typhi	12.5	50	200	200	100	100
	Pseudomonas aeruginosa	25	100	200	>200	100	100
Fungal strains	*Candida albicans*	>200	>200	>200	>200	>200	>200
	Aspergillus niger	>200	>200	>200	>200	>200	>200

acetate) against various bacterial and fungal strains were examined in the present study and their potency were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter values. The inhibitory effect of all extracts were studied against 8 pathogens by disc diffusion method (Table 2). Amongst all, the ethanolic extract exhibited highly significant effect towards all bacterial strains than other extracts. But all extracts are inactive towards fungal strains. Among all the test organisms used *Salmonella typhi* was found to be most sensitive to ethanolic extract followed by *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeroginosa*. The MIC of all the extracts against every bacterial and fungal strain was summarized in Table 3. The ethanolic extract of *I. coccinea* shown least MIC value against all bacterial strains and inhibited the growth of *Salmonella typhi* at 12.5 μg/mL, while *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeroginosa* at 25 μg/mL. On the other hand all the extracts were inactive towards both the fungal strains used in this study. From the preliminary phytochemical screening it may be concluded that the antimicrobial activity was due to the presence of fixed oil and flavonoids[17] but marked activity is due to flavonoids.

4. Discussion

Wound healing process consists of acute inflammatory response, proliferation, remodeling of connective tissue, synthesis of extra cellular matrix proteins, acquisition of tensile strength, contraction and epithelization. Though healing process takes place by itself and does not require much help, but various risk factors such as infection and delay in healing brought attention to promote this process. Topical application of *I. coccinea* at the wound site produced significant wound healing activity, which may be due to the help for the process of angiogenic and mitogenic potential. A healing tissue synthesizes collagen, which is a constituent of growing cell. Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in homeostasis and in epithelialisation at the latter phase of healing[18].

The results of the present findings reveal that the title plant possess potent wound healing capacity as evident from the wound contraction, increased tensile strength and increased biochemical parameters in healing tissue thus validated the ethno therapeutic claim.

When a wound occurs and is exposed to external environment. It is more prone to attack by microbes, which invade through the skin and delay the natural wound healing process. The significant antibacterial effect of the different extracts against all the six pathogens confirmed that the compounds present in the extract are responsible for the effective antibacterial activity. But there is no efficient antifungal activity as compared to antibacterial activity. The use of *I. coccinea* for various skin infections is justified by this work, as it showed commendable activity against all the test organisms. The external application of the extract on wound prevented the microbes to invade through the wound, thus the protection of wound occurs against the infection of the various organisms.

Conflict of interest statement

We declare that we have no conflict of interest.

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