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Wound healing potential of flowers extracts of *Woodfordia fruticosa* Kurz.

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Wound healing or repair is the body's natural process of regenerating dermal and epidermal tissue. *Woodfordia fruticosa* Kurz (Family: Lythraceae) is used traditionally in wound healing by the tribals of Chhattisgarh district. However, there is a paucity of scientific data in support. In this study, we evaluated antimicrobial activity of petroleum ether, chloroform, ethanolic and aqueous extracts against a diverse range of gram +ve and gram -ve bacteria along with pathogenic fungi. The wound healing activity of ethanolic extract was also evaluated at dose levels of 250 and 500 mg/kg body wt in rats by excision, incision and dead space wound healing models along with histopathology of wound area of skin. The ethanolic extract showed potent wound healing activity, as evident from the increase in the wound contraction and breaking strength in dose-dependent manner. Treatment with ethanolic extract (250 and 500 mg/kg body wt) showed significant dose-dependently decrease in epithelization period and scar area. Hydroxyproline, hexuronic acid and hexosamine contents, the important constituents of extracellular matrix of healing were also correlated with the observed healing pattern. During early wound healing phase, pro-inflammatory cytokines TNF-α, IL-6 and anti-inflammatory cytokine IL-10 levels were found to be upregulated by the ethanolic extract treatment. The ethanolic extract exhibited a strong and broad spectrum antimicrobial activity, as compared to other extracts. It showed very low Minimum inhibitory concentration (MIC) values and inhibited the growth of *E. coli*, *Staphylococcus aureus* and *Candida albicans* in concentration of 2.5 µg/disc. Thus, the results of the present study demonstrated the strong wound healing potential and antimicrobial activities of *W. fruticosa*, flowers, supporting the folklore use of the plant by the tribal people of Chhattisgarh district.

Keywords: Anti-inflammatory cytokines, Antimicrobial, Dead space, Excision, Incision, Pro-inflammatory cytokines, *Woodfordia fruticosa*, Wound healing.

Wound healing is a dynamic process of tissue restoration and re-establishing the integrity of the injured skin and underlying tissues. It involves a systemic progression of events i.e. inflammation, angiogenesis, proliferation and collagen synthesis for final healing[1]. To restore the integrity and to avoid severe damage to the body, rapid wound healing is required. The present system of the treatment of using cortisone and other anti-inflammatory drugs may impair the healing process. Alternate method of treatment by using medicinal plants has been focused by many workers who have found the therapeutic benefits of traditional system of medicine in wound repair[2,3].

Woodfordia fruticosa Kurz. (Family: Lythraceae) locally known as Dhai is about 3.5 m in height, occurring throughout North India[4]. The flowers are brilliant red in colour and reported for immunomodulatory[5], anti-tumor[6], hepatoprotective[7], anti-ulcer[8] activities and also useful in diarrhoea[9] urinary disorders, burning sensation, wounds, bleeding injuries and headache[10]. Ayurvedic preparation containing *W. fruticosa* has the anti-leucorrhoeic property[11]. From dried flowers oenothein B, isochimacoalin-A, oligomers, namely woodfordins A, B, C, E, F, G, H and I along with quercetin-3-*O*-(6''-galloyl)-β-D-galactopyranoside, quercetin-3-*O*-α-l-arabinoside, quercetin-3-*O*-oxylopyranoside, myricetin-3-O-(6''-*O*-galloyl)-β-D-galactopyranoside and myricetin-3-*O*-arabinopyranoside have been isolated[5,12].

Earlier, we have reported anti-hyperglycemic, anti-inflammatory and anti-nociceptive activities from the flowers[13,14]. Traditionally, fresh and dried flowers are used by tribals in Chhatisgarh district to stop bleeding in emergency cuts and for healing of wounds[15]. The present study has been undertaken to

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investigate the *in vivo* wound healing activity of *W. fruticosa* (WF) in excision wound, incision wound and dead space models in rats to elucidate the folklore use of the plant in wound healing. Antimicrobial activity of petroleum ether, chloroform, ethanolic and aqueous extracts of flowers has also been evaluated against a diverse range of gram +ve and gram -ve bacteria along with pathogenic fungi.

Materials and Methods

Plant material and preparation of extracts

Fresh flowers of *W. fruticosa* were obtained as gift from M/s Aroma Chemicals, Saharanpur, UP, India. The flowers were identified and authenticated at source by Dr. A K S Rawat (Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (CSIR), Lucknow). A voucher specimen (No. NBRI/CIF/174/2010) was deposited in the herbarium of the institute for future reference.

Flowers were shade-dried and powdered by mechanical grinder. The powdered flowers were extracted successively with petroleum ether, chloroform, ethanol (95%) and finally with water by solvent-solvent extraction method. The petroleum ether, chloroform and ethanol from the pooled extracts were removed using Buchi Rotavapour under reduced pressure at 40-55°C to obtain their respective extracts, whereas aqueous extract was obtained by lyophilizer[13].

Microorganisms

The test microorganisms used for the antimicrobial activity screening were 7 bacteria — *Bacillus subtilis* MTCC (121), *Enterobacter aerogenes* MTCC (111), *E. coli* MTCC (443), *Pseudomonas aeruginosa* MTCC (424), *Salmonella typhimurium* MTCC (98), *Staphylococcus aureus* MTCC (96), *Streptococcus pneumoniae* MTCC (2672) and 2 fungi — *Aspergillus niger* MTCC (404) and *Candida albicans* MTCC (183). These organisms were identified and procured from Institute of Microbial Technology (IMTECH-CSIR), Chandigarh, India.

Experimental animals

The male Wistar albino rats (180-220 g) used in the study were procured from the animal house of Central Drug Research Institute, Lucknow and kept in departmental animal house in an environmentally controlled room (25 ± 2°C), relative humidity 50 ± 5% and on a 12 h light/dark cycle at National Botanical Research Institute (NBRI), Lucknow. They were allowed free access to standard rat feed (Dayal, India) and water *ad libitum*. Experiments were performed after one week of acclimatization. All the studies were performed in accordance with the guidelines for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 222/2000/CPCSEA).

Antimicrobial activity

The agar diffusion method[16] was used to evaluate the antimicrobial activity. Bacteria were cultured overnight at 37°C in Mueller-Hinton Broth (MHB) and fungi at 28°C for 72 h in Potato Dextrose Broth (PDB) and used as inoculum. A final inoculum using 100 µl of suspension containing 108 CFU/ml of bacteria and 104 spores/ml of fungi was spread on Mueller-Hinton Agar (MHA) and Potato Dextrose Agar (PDA) medium, respectively. The disc (6 mm in diameter) was impregnated with 10 µl of 100 mg/ml (1 mg/disc) extracts placed on seeded agar. Gentamicin (10 µg/disc), streptomycin (10 µg/disc) and tetracycline (10 µg /disc) were used as positive controls for bacteria and fluconazole (10 µg/disc), ketoconzole (10 µg/disc) and metronidazole (5 µg/disc) for fungi. The test plates were incubated at 37°C for 24 h for bacteria and at 28°C for 72 h for fungi, depending upon the incubation time required for a visible growth.

Minimum inhibitory concentration (MIC) values were determined as sensitive to the extract in disc diffusion assay. Sterile filter paper discs (6 mm in diameter) containing 2.5-500 µg/disc of plant extracts were placed on the surface of a medium. MIC was defined as the lowest concentration of extract that inhibited visible growth on agar. The extract showing low MIC and good antimicrobial activity were selected for wound healing activity.

In vivo wound healing activity

Excision wound model

The hair of the dorsal neck region was shaved with an electrical shaver, disinfected with 70% alcohol and anesthetized with 1 ml of lignocaine. HCl (2%, 100 mg/5 ml). A circular piece of skin of full thickness (~500 mm^2) was cut-off from the nape of dorsal neck of all rats aseptically. Wounds were traced on 1 mm^2 graph paper on the day of wounding and subsequently at a gap period of 4 days till day 12 and then on the alternate days, until healing was complete. Change in wound area was calculated, giving an indicator of

wound contraction. Number of days required for falling of scar without any residual raw wound indicated the period of epithelization[17].

Animals were divided into four groups of six animals (n = 6) each. Group I or control was treated with 0.5% carboxy methyl cellulose (CMC) in distilled water and groups II and III were treated with ethanolic extract at dose levels of 250 and 500 mg/kg body wt, respectively. Group IV was treated with standard drug vitamin E at a dose of 200 mg/kg body wt for the comparison of wound healing action. The animals received all drugs with the help of orogastric cannula.

Incision wound model

The method of Ehrich and Hunt[18] was adopted for incision wound model. Under light ether anesthesia, two para vertebral incisions of 6 cm were made through the entire thickness of the skin on either side of the vertebral column with the help of a sharp blade. The incisions were sutured using 4-0 silk threads with the help of straight round-bodies needle (no. 11). On 8th post-wounding day, sutures were removed and the breaking strength was determined on 10th post-wounding day by continuous water flow technique[19]. For wound healing study in incision wound and dead space wound models, the animals received all the treatments up to 10 days with the help of orogastric cannula.

Dead space model

Wounds were created by implanting two polypropylene tubes (0.5 × 2.5 cm^2 each), one on either side in the lumber region on the dorsal surface of each rat. On the 10th post-wounding day, the animals were sacrificed and granulation tissues formed on the implanted tubes were carefully dissected out[19,20]. The granulation tissue from one of the tubes was collected and dried at 60^{o}C for 24 h, weighed and kept in glass stoppered test tubes. 6 *N* HCl was added in each tube, so that it contained 40 mg of the dried granulation tissue per ml of acid. The tubes were kept on boiling water bath for 24 h for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralized by 10 *N* NaOH using phenolphthalein. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline[21], hexouric acid[22] and hexosamine[23], following the standard procedures.

Estimation of pro-inflammatory (IL-6, TNF-α) and anti-inflammatory cytokines (IL-10) induction

Blood samples were collected from all the animals of each group at different time intervals i.e. 24 h and 8th day after wound formation. Pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10) were estimated by performing enzyme-linked immunosorbent assay using IL-6 and TNF-α and IL-10 ELISA kit. Concentrations of IL-6, TNF-α and IL-10 were determined in pg/ml by plotting the graph for standard. All the experiments were performed in triplicate to ensure the observations.

Histopathology

Tissues were fixed in 10% formalin and after routine fixation procedures were embedded in paraffin wax. Serial sections of paraffin-embedded tissues of 5 µm thickness were cut. Haematoxylin and eosin stained preparations were examined under light microscope.

Statistical analysis

Results were expressed as mean ± S.E.M. (standard error of mean) and analyzed using Prism–3.0 version. The statistical differences between the groups in terms of wound healing were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. P value < 0.05 was considered statistically significant.

Results

Antimicrobial activity

The disc diffusion method was used to determine the inhibition zones of *W. fruticosa* flowers extracts. The antimicrobial activities and MIC of these extracts are summarized in Table 1. The extracts showed significant anti-bacterial and anti-fungal activities against all tested organisms, but their inhibition efficiency varied from one organism to another. A strong and broad spectrum antimicrobial activity was observed in the ethanolic extract compared to water and chloroform extracts, while petroleum ether extract did not show any activity. *E. aerogenes* had shown inhibition diameter 19.88 ± 1.11 mm with ethanolic extract. *S. typhimurium* was most sensitive to the ethanolic extract as revealed by inhibition zone diameter, followed by *E. coli*, *E. aerogenes, B. subtilis*, *A. niger*, *S. aureus*, *S. pneumoniae* and *C. albicans.* Increased inhibition was observed at higher levels of extract concentration. The ethanolic extract showed very low MIC values and inhibited the

Table 1—Antimicrobial activity (1 mg/disc) and MIC values (µg/disc) of *W. fruticosa* extracts against different microorganisms.

[Values are mean ± S.E.M. n = 3]

Microorganisms	Inhibition zone in diameter (mm)				Standard antibiotics						MIC values (µg/disc)			
	Petroleum ether	Chloroform	Ethanol	Water	Bactericide			Fungicide			Petroleum ether	Chloroform	Ethanol	Water
Bacterial strains					Str	Ttc	Gen	Ket	Flu	Met				
Bacillus subtilis	Ab	17.22 ± 1.21	19.43 ± 0.78	16.23 ± 0.79	23	29	24				Ab	100	10	10
Enterobacter aerogenes	Ab	13.67 ± 0.92	19.88 ± 1.11	17.21 ± 0.55	17	32	28				Ab	200	100	100
E. coli	Ab	14.66 ± 1.13	22.56 ± 0.98	14.78 ± 0.23	23	34	32				Ab	100	2.5	100
Pseudomonas aeruginosa	Ab	15.34 ± 0.84	17.89 ± 0.78	15.67 ± 0.34	22	28	29				Ab	100	200	200
Salmonella typhimurium	Ab	16.12 ± 0.52	23.56 ± 0.68	16.45 ± 0.67	24	27	31				Ab	100	100	100
Staphylococcus aureus	Ab	13.34 ± 0.81	18.79 ± 0.81	15.45 ± 0.65	32	32	28				Ab	200	2.5	100
Streptococcus pneumoniae	Ab	14.56 ± 0.62	18.32 ± 0.56	15.87 ± 0.45	15	33	27				Ab	100	100	100
Fungal strains														
Aspergillus niger	Ab	16.17 ± 0	19 ± 0.91	16 ± 0.23				28	32	29	Ab	200	10	100
Candida albicans	Ab	18.12 ± 0	18 ± 1.12	14 ± 0.21				24	22	30	Ab	200	2.5	10

Ab- denotes no inhibition shown by petroleum ether extract; Str, streptomycin (10 µg/disc); Ttc, tetracycline (10 µg/disc); Gen, gentamicin (10 µg/disc); Ket, ketoconazole (10 µg/disc); Flu, fluconazole (30 µg/disc); Met, metronidazole (30 µg/disc); zone of inhibition of solvents were the following: petroleum ether, 8-10 mm, chloroform, 7-9 mm and no inhibition in ethanol and water. The values of negative control were subtracted from those of samples; the corrected values are given.

Table 2—Effect of ethanolic extract of *W. fruticosa* flowers and vit E on wound contraction, epithelization period and scar area in rats

[Values are mean of ± S.E.M. n = 6]

Groups	Oral treatment (mg/kg)	Wound contraction area (mm^2/rat) Day									Epithelization period (days)	Scar area (mm^2)
		0	4^{th}	8^{th}	12^{th}	14^{th}	16^{th}	18^{th}	20^{th}	22^{nd}		
I	Control 0.5% CMC	410.2 ± 3.4	365.4 ± 9.7	247.6 ±7.8	121.7 ± 4.9	65.9 ± 2.5	39.2 ± 2.7	17.1± 1.4	0.9 ±0.2	0±0	20.6 ± 2.3	51.2 ± 3.1
II	Ethanolic extract 250	415.4 ± 5.6	359.3 ± 5.8[a]	206.8 ± 6.1[a]	88.3 ± 4.2[a]	36.2 ± 2.1[a]	2.6 ± 0.6[a]	0.5 ± 0.2[a]	0±0	0±0	19.0 ± 3.4[a]	43.2 ± 2.2[a]
III	Ethanolic extract 500	404.3 ± 7.8	331.7 ± 8.2[c]	203.5 ± 4.9[c]	43.6 ± 3.7[c]	2.9 ± 0.81[c]	0.7 ± 0.6[c]	0±0	0±0	0±0	17.0 ± 2.7[c]	36.4 ± 1.3[c]
IV	Vit E 200	422.8 ± 10.3	340.2 ±14.9[b]	131.1 ± 4.2[b]	61.5 ± 5.8[b]	32.0 ± 1.6[b]	13.6 ± 2.1[b]	1.2 ± 0.36[b]	0±0	0±0	19.0 ± 2.3[b]	38.5 ± 1.4[b]

Statistically significant differences in comparison with control group.
[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$

growth of *E. coli*, *S. aureus* and *C. albicans* in concentration of 2.5 µg/disc.

Wound healing activity

Excision wound model

Treatment with ethanolic extract of flowers at dose levels of 250 and 500 mg/kg body wt showed significant ($p<0.05$ and $p<0.001$) dose-dependently decrease in epithelization period, as compared to control group. Faster wound healing was observed at day 18 and 16 day in rats treated with 250 and 500 mg/kg of extract, respectively. Scar area also decreased dose-dependently, as compared to control group. Standard drug vitamin E at a dose of 200 mg/kg body wt showed significant ($p<0.01$) epithelization at day 20 with scar area of 38.5 ± 1.4 mm^2. The extract at dose of 500 mg/kg body wt showed decrease in the epithelization period along with reduction in scar area as compared to standard drug (Table 2).

Incision wound model

Breaking strength of incision wounds was significantly ($p<0.05$ and $p<0.001$) increased in the ethanolic extract-treated group at a dose of 250 and 500 mg/kg body wt, respectively, as compared to control group. Standard drug vitamin E at a dose of 200 mg/kg body wt also significantly ($p<0.01$) enhanced breaking strength (Table 3).

Table 3—Effect of ethanolic extract of *W. fruticosa* flowers and vit E on incision and dead space wound parameters in rats

[Values are mean of ± S.E.M. n = 6]

Groups	Oral treatment (mg/kg)	Incision wound parameter	Dead space wound parameters				
		Wound breaking strength (g)	Wet weight (mg/100 g bw)	Dry weight (mg/100 g bw)	Dry tissue study (mg/g)		
					Hydroxyproline	Hexuronic acid	Hexosamine
I	Control 0.5% CMC	256.1 ± 7.4	190.5 ± 4.3	37.2 ± 1.6	21.4 ± 0.73	11.8 ± 1.3	10.8 ± 1.1
II	Ethanolic extract 250	357.3 ± 5.2[a]	337.5 ± 2.9[a]	61.9 ± 2.3[a]	27.5 ± 1.8[a]	24.6 ± 2.7[a]	20.7 ± 0.79[a]
III	Ethanolic extract 500	462.7 ± 4.6[c]	389.1 ± 5.1[c]	69.5 ± 1.6[c]	34.4 ± 3.7[c]	29.5 ± 1.4[c]	24.6 ± 1.9[c]
IV	Vit E 200	405.9 ± 5.7[b]	301.7 ± 4.6[b]	53.7 ± 2.7[b]	32.9 ± 2.1[b]	28.7 ± 1.3[b]	20.8 ± 2.2[b]

Statistically significant difference in comparison with control group.
[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$.

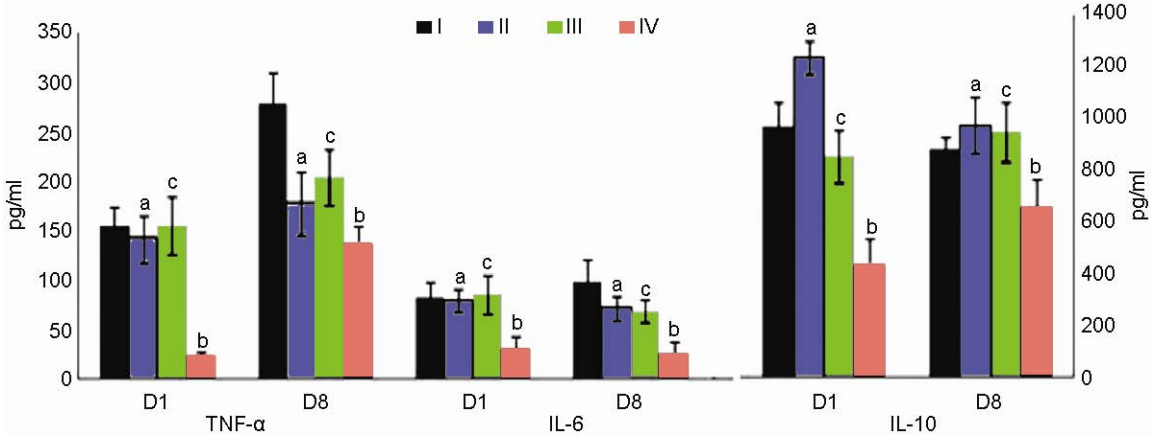

Fig. 1—Effect of ethanolic extract of *W. fruticosa* flowers and vitamin E on pro-inflammatory (TNF-α and IL-6) and anti-inflammatory cytokine (IL-10) at D1, day 1 post-surgery, D8, day 8 postsurgery [Group I: normal rats; Group II: rats treated with extract at a dose level of 250 mg/kg body wt; Group III: rats treated with extract at a dose level of 500 mg/kg body wt; Group IV: rats treated with Vit E at a dose level of 200 mg/kg body wt. Values are mean of ± S.E.M. for six rats; [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$]

Dead space model

The effect of oral administration of ethanolic extract was assessed by the increase in wet and dry weight of granulation tissue. The data are depicted in Table 3. A significant ($p<0.05$ and $p<0.001$) increase in the hydroxyproline, hexuronic acid and hexosamine contents was observed in ethanolic extract-treated groups at 250 and 500 mg/kg body wt, respectively as compared to control group. During the course of healing, hydroxyproline, hexuronic acid and hexosamine contents, the important constituents of extracellular matrix of healing were found to be higher in all the treated groups than control group. Maximum increase in content of hydroxyproline, hexuronic acid and hexosamine was observed rats treated at 500 mg/kg body wt. Standard drug vit. E also showed significant ($p<0.01$) results, as compared to control.

Pro-healing modulation of ethanolic extract on pro-inflammatory cytokine (TNF-α and IL-6) and anti-inflammatory cytokine (IL-10) production

The TNF-α, IL-6 and IL-10 levels on different time intervals are given in Fig. 1. The results indicated that TNF-α production was augmented in the ethanolic extract fed rats as compared to control rats and its level increased during 12-48 h. After induction of wound, the TNF-α level in control (0.5% CMC-treated) group (D1: 154.7 ± 19.3 pg/ml; D8: 279.6 ± 31.5 pg/ml) was significantly higher than that in the standard (vit. E) group (D1: 24.5 ± 3.2 pg/ml; D8: 137.5 ± 18.4 pg/ml). The TNF-α level in the ethanolic extract treated group at 500 mg/kg body wt (156.1 ± 28.4 pg/ml) was higher than that in standard group (24.5 ± 3.2 pg/ml) on day 1. On day 8, the TNF-α level in the same group (D8: 204.8 ± 28.3 pg/ml) was significantly ($p<0.05$) lower than that in the group received 0.5% CMC (D8: 279.6 ± 31.5 pg/ml)

and was different from that in standard group (137.5 ± 18.4 pg/ml).

The IL-6 level was found to be increased after 24 h post-surgery, while on 8th day post-surgery, IL-6 level decreased significantly in the ethanolic extract-treated rats (D1: 86.6 ± 19.7 pg/ml; D8: 68.7 ± 11.5 pg/ml). On 8th day post-surgery in control group, IL-6 level increased (D1: 82.3 ± 16.7 pg/ml; D8: 98.2 ± 23.1 pg/ml), while the value in the standard group was considerably low (D1: 31.6 ± 12.4 pg/ml; D8:26.8 ± 11.5 pg/ml).

The IL-10 level after induction of wound in 0.5% CMC-treated group (D1: 431.4 ± 21.6 pg/ml; D8: 654.7 ± 98.7 pg/ml) was significantly lower than that of standard (D1:967.7 ± 202.9 pg/ml; D8: 878.9 ± 89.7 pg/ml) treated group. The IL-10 level in the ethanolic extract-treated group at a dose of 500 mg/kg body wt was found to be higher (D1: 1234.7 ± 102.4 pg/ml; D8: 976.8 ± 112.6 pg/ml) than vit E-treated group.

Histopathological examination

The control group exhibited wide area of ulcerations containing fibrinous exudates and inflammatory cells, mild degree of inflammation and vascularizations with congestions in dermis, indicating the healing was not completed (Fig. 2A).

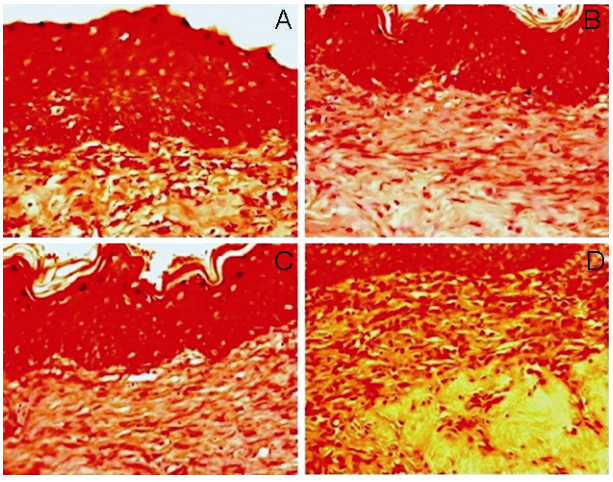

Fig. 2—Histopathology of granulation tissue on day 18 obtained from: (A) Control group ulcerations containing fibrinous exudates and inflammatory cells, mild degree of inflammation, vascularizations with congestions; (B) Ethanolic extract 250 mg/kg body wt treated group (mild inflammation, intact epidermis and scarless tissue on the dermis; (C) Ethanolic extract 500 mg/kg body wt treated group, restoration of collagen fibers, fibroblast and intact epidermis and scarless tissues; (D) Vit E 200 mg/kg body wt treated group also showed complete healing with mature epidermis, mature hair follicles and proliferation of fibroblast in dermis (H & E × 40)

Group treated with 250 mg/kg body wt, ethanolic extract showed mild inflammation, intact epidermis and scarless tissue on the dermis, also indication of healing (Fig. 2B). The treatment with 500 mg/kg body wt, ethanolic extract showed restoration of collagen fibers, fibroblast and intact epidermis and scarless tissues were obviously distinguished in the representing figure (Fig. 2C). The treatment with standard drug vit. E also showed complete healing with mature epidermis, mature hair follicles and proliferation of fibroblast in dermis (Fig. 2D).

Discussion

Higher plants produce diverse chemical compounds with different biological activities[24]. Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, in recent year much attention has been paid to extracts and biologically active compounds isolated from the plant[25,26]. Plants-based antimicrobials represent a vast untapped source for medicines and have enormous therapeutic potential, thus require further exploration and. They are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects often associated with synthetic anti-microbials[27].

Earlier, *in vitro* antibacterial activity in flowers (methanolic extract)[28] and leaves[29] and antimicrobial activity in leaf and flowers of *W. fruticosa* is reported[30]. In the present study, antimicrobial activity of different extracts of *W. fruticosa* flowers was evaluated against a diverse range of gram -ve and gram +ve bacteria along with pathogenic fungi. The ethanolic extract showed very low MIC values and inhibited the growth of *E. coli*, *S. aureus* and *C. albicans* in concentration of 2.5 µg/disc. The wound healing activity was studied, as the ethanolic extract showed strong and broad spectrum antibacterial and antifungal activities.

The complex process of healing involves various phenomena like wound contraction, granuloma formation and collagenation etc. The contribution for healing by these events depends upon the type of wounds. Wound contraction plays a significant role in healing of excision wound, while an increase in tensile strength and granuloma formation contributes in healing of dead space[3]. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage, whereby the area of wound undergoes shrinkage.

It has three phases — inflammatory, proliferative and maturation and is dependent upon the type and extent of damage, the general state of the host's health and the ability of the tissue to repair[31]. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis and collagen deposition in the proliferative phase. In maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound and there is a rapid increase in the synthesis of this protein in the wound area soon after an injury, which provides strength and integrity to tissue matrix[32]. Measurement of the hydroxyproline, which comes from the breakdown of collagen, has been used as an index of collagen turn over[33]. In the present study, ethanolic extract of flowers showed dose-dependent wound healing and increase in tensile strength of healed incision wounds. It also increased the wet and dry weight and the hydroxyproline content of granulation tissue, indicating the presence of higher collagen content and its turnover, leading to rapid healing. In wound healing process, collagen formation peaks at day 7 and epithelialization occurs in 48 h under optimal conditions[34]. The present results also indicated significant decrease in wound area from day 8 onwards, indicating early healing in excision wound model. In incision wound, an increase in tensile strength of treated wounds was observed and this might be due to increase in collagen concentration and stabilization of fibers. Hexosamine and hexuronic acid, synthesized by fibroblasts in the wound area are matrix molecules, which act as ground substratum for the synthesis of new extra-cellular matrix. These substances form a highly hydrated gel-like ground substance, a provisional matrix on which collagen fibres are embedded. They are known to stabilize the collagen fibres by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characterstic size[33]. In our study, hexuronic acid and hexosamine content was significantly increased in ethanolic extract-treated group dose-dependently.

The macrophage derived pro-inflammatory cytokines TNF-α and IL-6 are also known to play a major role in the inflammatory phase of wound healing by enhancing angiogenesis[35]. Our study revealed that during wound healing TNF-α and IL-6 level was detected after 12 h of wound infliction and reached at maximum after 24 h in ethanolic extract treated animals. The TNF-α and IL-6 levels declined after 24 h of wounding in untreated animals, while remained static up to 48 h in ethanolic extract-treated animals. It was thus apparent that ethanolic extract during the first stage of healing increased the TNF-α production from polymorphonuclear leukocytes and macrophages. Earlier, a strong early induction of TNF-α, IL-1α and β has been observed after cutaneous injury and highest level of these cytokines are reported as early as 12-24 h after wounding[36]. This supported the present findings that TNF-α and IL-6 increased up to 24 h of wounding and down-regulated after 48 h. However, it is also reported that TNF-α inhibits collagen formation and hydroxyproline production which are essential for the final part of proliferative phase in wound healing, but the low level of TNF-α and IL-6 after 48 h till 8th day does not interfere with collagen formation and hydroxyproline production[37].

In the present study, the ethanolic extract elevated IL-10 level on day 1 (24 h) and day 8 after induction of wound. IL-10 is an anti-inflammatory cytokine produced by various cells, including macrophages and T-lymphocyte. Wound healing has been associated with a decrease in pro-inflammatory cytokines. IL-10 can inhibit cytokine synthesis by macrophages[38]. It appears to influence the wound-healing environment by decreasing the expression of pro-inflammatory /profibrotic mediators, resulting in decreased recruitment of inflammatory cells to the wound[39]. In addition, the mild anti-inflammatory effects of IL-10 may be due to the suppression of TNF-α production. Earlier, it is shown that mucosal secretion of IL-10 and TNF-α is increased during wound healing[40]. IL-10 may be protective and can limit tissue damage caused by inflammation. Therefore, elevation of IL-10 could down-regulate TNF-α production in macrophage, leading to increased wound healing. Treatment with the ethanolic extract increased the serum IL-10 concentration and at same time down-regulated TNF-α and IL-6.

The findings suggested that the ethanolic extract regulated anti-inflammatory and pro-inflammatory cytokines and ultimately the systemic immune pathways associated with them, thus leading to cellular proliferation. It is reported previously that IL-10 has inhibitory effect on COX-2 synthesis

and hence the further action of prostaglandin which is a potent inflammatory mediator[41]. Howsoever, IL-10 has potent role in macrophage deactivation, blocking the induced synthesis of TNF-α, IL-1, IL-6, IL-8 and GMCSF by human monocytes[42]. Histopathological analysis corroborated these findings, showing less infiltration of neutrophils in wound area in the group orally administered with ethanolic extract. The mechanism of wound healing possibly involved the accumulation of anti-inflammatory and pro-inflammatory cytokines with cells of immune system (monocytes and macrophages) and antimicrobial system to the site of tissue injury.

Conclusion

The present study demonstrated that oral administration of the ethanolic extract of *W. fruticosa* flowers was effective in wound healing, supported the folklore use by the tribals of Chhattisgarh district. In the process of wound healing, the extract increased the wound contraction and breaking strength, decreased epithelization period and scar area, increased hydroxyproline, hexuronic acid and hexosamine contents and modulated pro-inflammatory and anti-inflammatory cytokines. The strong antimicrobial activity and histopathological analysis also confirmed the wound healing activity. Further studied are warranted to explore the detailed mechanism and the active constituents responsible for wound healing.

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