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Research Article

Comparison of Wound Healing Potential of Curcumin, Alcoholic Extract and Powder of *Curcuma longa* L. in Rats

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Abstract

Wound healing is an integral part of reestablishment of damaged structure. Many studies have reported that turmeric/*Curcuma longa* L. (*C. longa*) has significant anti-inflammatory, antioxidant, anti-infective and wound healing properties. Curcumin, is a one of the main active constituent of *C. longa* which acts on various stages of the natural wound healing process to hasten healing. In a previous study from our laboratory, it was found out that individual fractions or components and pure phytochemicals are sometimes less bioactive than crude, multicomponent extracts, i.e., most of the bioactive constituents possessing synergistic activity are lost during fractionation, isolation or processing. So, in the present study, we have compared the wound healing potential of *C. longa* preparations such as curcumin, *C. longa* alcoholic extract (CLAE) and crude *C. longa* rhizome powder (CLRP) in cutaneous wound model in normal rats by creating an open excision-type wound. The ointments were applied topically on the wound area twice daily for 14 days. The wound photography and contraction measurement were done on days 0, 3, 7, 11 and 14 post-wounding. Portion of granulation tissues were harvested, after euthanizing rats on 14th day, for assessment of pro-healing parameters level, oxidative parameters level and histopathological changes. The gross wound assessment revealed that among all groups, CLRP-treated groups showed greater wound contraction and faster wound closure which was followed by CLAE-treated group, curcumin and control groups. The levels of pro-healing and anti-oxidative parameters were high in treatment groups compared to control group. In histopathological study, CLRP-treated groups showed good thickness of epithelialization, marked fibroblast proliferation, angiogenesis, enhanced collagen deposition and low level of polymorph nuclear lymphocytes (PMNL) in granulation tissue compared to other groups. All treatment groups have shown faster wound healing property when compared to control group. Moreover, CLRP showed higher healing potential compared to curcumin and *C*. CLAE-treated groups. Therefore, findings of the present study revealed that *C. longa* have efficient antioxidant activities, which might have accelerated the wound healing process by preventing lipid peroxidation mediated damage to the biological structures, and by scavenging free radicals. Our findings further suggest that pure curcumin might not be the only active pharmacological moiety present in *C. longa* for exhibiting wound healing property.

Keywords: *Curcuma longa*, Curcumin, cutaneous wound model, wound healing

Introduction

Healing process consist of a sequence of molecular and cellular events which occur after the onset of a tissue lesion in order to restore the damaged tissue [1]. During inflammatory phase of healing process, the inflammatory cells like neutrophils and macrophages infiltrate the wound area and secrete pro-inflammatory cytokines and growth factors [2]. These cytokines and growth factors facilitate the wound contraction and closure through production of collagen from fibroblasts, differentiation of fibroblasts to myofibroblasts, angiogenesis and re-epithelialization. Reactive oxygen species (ROS) are crucial regulators of several phases of wound healing. Indeed, low levels of ROS are required for the fight against external damage [3]. However, excessive amounts of their level often found in chronic or impaired acute wound, cause oxidative stress, which further damage cells and delays healing [4].

Therefore, reduction/termination of the persistent inflammation and elimination of free radicals by the introduction of an anti-inflammatory and antioxidant agent into wound therapy could be an important strategy to improve healing of wounds.

In spite of several advancements in the field of synthetic drug chemistry and antibiotics, plants continue to be one of the major raw materials for drugs treating various disease and ailments [5]. No satisfactory therapy is available for chronic wounds. There is an undeniable necessity of a promising treatment modality which is safe, cost effective and practicable and having potential wound healing effect [6]. *C. longa* is a popular Indian spice that has been used for centuries in herbal medicines. Till date *C. longa* and its active component curcumin is one of the highest studied compounds in ethnopharmacology [7]. It is highly regarded as universal panpharmacon in the ethnomedicine with a wide spectrum of pharmacological activities like anti-inflammatory, anti-bacterial, anti-fungal, anti-protozoal, anti-coagulant, anti-diabetic, analgesic, antioxidant, anti-infective, anti-mutagenic and anti- carcinogenic activities [7]. *C. longa* contains numbers of phytoconstituents like curcumin I (diferuloylmethane), curcumin II (bisdemethoxycurcumin), curcumin III (demethoxycurcumin), p-coumaric-acid, procurcumadiol, curcumene, curcumenol, copper/zinc, campesterol, cholesterol, Ar-turmerone arturmerone, curcumene, turmerone germacrone, Ar-curcumene[8]. However, to the best of our knowledge, there is no scientific data/information available on the comparative study of effect of different forms of *C. longa* (i.e Curcumin, alcoholic extract, and rhizome powder) on wound healing. We had hypothesized that topical application of different preparations of *C. longa*, such as, fine powder, alcoholic extract and pure curcumin on cutaneous wound might be producing variable stimulatory effect on healing process. So, we had planned a comparative study to evaluate the effect of different preparations of *C. longa* preparations on cutaneous wound healing in rats.

Materials and Methods

Experimental rats

Healthy adult male Wistar rats (6 weeks, 150-170 g) were procured from Laboratory Animal Resource Section, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.). The animals were housed in polypropylene cages with free access to standard feed and water. A balanced feed from Feed Technology Unit, IVRI was fed to the rats @ 15 g/rat, twice daily in morning and evening time throughout the study period. All rats were kept in the laboratory condition for a minimum of 7 days for acclimatization [9]. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee, Indian Veterinary Research Institute, Izatnagar, India as per permission No. IAEC/07.07.2020/S2.

Wound model

The rats were anesthetized by an intra-peritoneal injection of ketamine (50mg/kg) and xylazine (5mg/kg) combination. A ~ 2x2 cm^2 (400 mm^2) open excision wound was created on the back (dorsal thoracic region) of the rats to the depth including panniculus carnosus. Animals, after recovery from anaesthesia, were individually housed in properly disinfected cages [9].

Ointments preparation and application

Ointment base consisting of soft paraffin (90%), hard paraffin (5%) and lanolin (5%) was used to prepare the ointment [10]. Of different *C. longa* preparations in the concentration of 0.3% curcumin, 5% CLAE and 20% CLRP. Ointments were applied topically, twice a day in morning and evening time on the wound for 14 days.

Experimental design

After post wounding, 24 male rats were r and only divided into four groups of six animals each. Details of the experimental design are given in table 1.

Groups	No. of animals	*C. longa* preparations	Collection of tissue
I	6	Vehicle control (ointment base)	Day 14
II	6	CLRP (20 % w/w)	Day 14
III	6	CLAE (5 % w/w)	Day 14
IV	6	Curcumin (0.3 % w/w)	Day 14

Table 1: Experimental design to evaluate the wound healing potential of *C. longa* preparations.

Photographic evaluation and wound contraction measurements

Wounds were photographed and measured on days 0, 3, 7, 11 and 14 post-wounding to assess the quality of wound healing. Wound area (mm^2) was measured by image analysis software ImageJ v1.53a. The percent wound contraction was calculated by Wilson's formula [10] as follows

Collection of tissue

The granulation tissue was harvested immediately after euthanizing the rats by cervical dislocation under diethyl ether an-

aesthesia on day 14 post-wounding. The harvested tissue was divided into 3 parts for further processing. The first part was stored at appropriate temperature for estimation of hydroxyproline and glucosamine levels. The second part was snap frozen in liquid nitrogen and stored at -80ºC for oxidative parameters studies. The third part was stored in 10% normal buffer saline for conducting histopathological study [11].

Assessment of pro-healing parameters in granulation tissue

To indirectly assess the collagen content and extracellular matrix (ECM) deposition in the granulation tissue, pro-healing parameters like hydroxyproline and glucosamine levels were estimated in the granulation tissue by following Reddy and Enwemeka (1996) [12], and Rondle and Morgan (1955)[13] protocols, respectively. The tissue samples were acid hydrolysed (50mg tissue/2ml 6N HCL) in a tube, which was tightly sealed and autoclaved at 50-pound pressure for 3 h. The hydrolysate, obtained, was used for estimating hydroxyproline and glucosamine levels.

Protein isolation

Briefly the frozen tissues were thawed, weighed and minced finely by keeping over ice box using a pair of sharp scissors. Then samples were homogenized in ice-cold lysis buffer (0.785g Tris HCL, 0.8776g Nacl and 0.5ml Triton X 100, and finally volume made to 100ml with distilled water) at 10% w/v. To prevent protein degradation by proteases, the protease inhibitor cocktail was added to the lysis buffer at 1X concentration. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was collected, and again to prevent protein degradation, the protease inhibitor cocktail was added to it at 1X concentration. The aliquots of the supernatant were prepared and stored at -80ºC for antioxidant parameters. The total protein present in the supernatant was estimated by Lowry's kit method (GeNei, India) [11].

Estimation of anti-oxidative activity of *C. longa* preparations

Antioxidant activity of *C. longa* preparations was assessed in tissue lysate by determining different parameters such as (a) Reduced glutathione (GSH) [14], (b) Super oxide dismutase (SOD) activity [15], (c) Lipid peroxidation (malondialdehyde or MDA) level [16], (d) Catalase (CAT) activity [17].

Histological analysis of granulation tissues

The histopathological changes in the granulation tissues, harvested on day 14 post-wounding, were evaluated by H and E staining. The granulation tissues were fixed for 72 h in 10% neutral buffer formalin. After fixation, the tissue was washed overnight in running tap water, dehydrated in ascending grades of alcohol, cleared in benzene and were embedded in paraffin [11].

Hematoxylin and Eosin (H and E) staining

The 5 µm thick sections were obtained from paraffin embedded tissue and stained with H and E stain to evaluate the gross morphological changes in the granulation tissue as per standard protocol, and visualized under light microscope (OLYMPUS, BX 41, USA) at 10x and 40x magnifications. The comparative assessment of the quality of healing wounds was done by scoring the histological parameters through a semi-quantitative method. Sections were evaluated according to the scale: 0, 1, 2, 3 and 4 by two independent observers. The mean value was used for statistical comparison [11].

Scale	Epithelialisation	PMNL	Fibroblasts	New vessels	Collagen
0	Thickness of cut edges	Absent	Absent	Absent	Absent
1	Migration of cells (< 50%)	Mild ST	Mild-ST	Mild-SCT	Minimal-GT
2	Migration of cells (≥ 50%)	Mild DL/GT	Mild-GT	Mild-GT	Mild-GT
3	Bridging the excision	Moderate DL/GT	Moderate-GT	Moderate-GT	Moderate-GT
4	Keratinization	Marked DL/GT	Marked-GT	Marked-GT	Marked-GT

Table 2: Explanation of histological scoring scale used in the semi-quantitative evaluation of histolpathological changes in H and E tissue sections.

(Where ST: Surrounding Tissue, i.e., tissue out of GT; DL: Demarcation Line; SCT: Subcutaneous Tissue; GT: Granulation Tissue).

Statistical analysis

Results are expressed as mean ± S.E.M, and the statistical significance between the treatment and control values was analysed by applying two-way analysis of variance followed by Bonferroni post tests using GraphPad Prism V.5. Software program (San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant when compared between groups. Values bearing superscripts not in common differ significantly [11].

Results

Gross photographic evaluation of wound healing

Representative wound images, presented in figure 1, revealed that the wound size was progressively and considerably reduced in time dependent manner in all the treatment groups compared to control group. Moreover, among treatment groups, the reduction in wound size was considerably higher in CLRP-treated group, followed by CLAE and curcumin-treated groups.

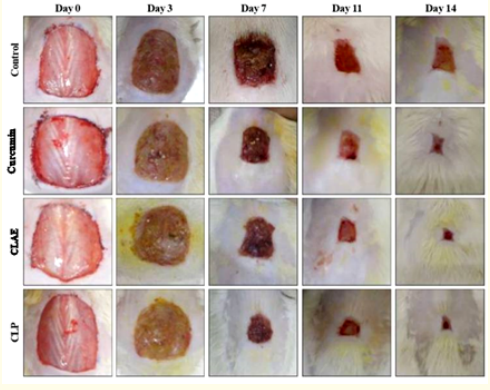

Figure 1: Representative gross images of wounds of control and *C. longa* preparations-treated groups on days 0, 3, 7, 11 and 14 post-wounding.

Wound closure (mm^2)

As evident from the table 3, on 3rd day post-wounding, there was no considerable difference in wound area of treatment groups compared to control group. On 7th day, the wound area was considerably reduced in treatment groups compared to control group. Further, on 11th and 14th day, all treatment groups, specifically CLRP and CLAE-treated groups, showed noticeable reduction in wound area compared to control group.

Wound contraction (%)

Compared to control group, topical application of *C. longa* preparations considerably reduced the wound size by significantly increasing the percent wound contraction on all days except day 3 post-wounding (Table 4 and Figure 2). Among treatment groups, wound contraction was highest in CLRP-treated group.

Prohealing parameters

The level of pro healing parameters like hydroxyproline and glucosamine was estimated in granulation tissue the results are presented in table 5 and figure 3 There was significant increase in level of both hydroxyproline and glucosamine in all treatment groups compared to control group. Among treatment groups, CLRP-treated group showed highest level of hydroxyproline. Curcumin and

Groups	Day 0	Day 3	Day 7	Day 11	Day 14
Control	440.63 ± 17.14	435.94 ± 8.56	242.19 ± 11.12	146.9 ± 5.56	78.12 ± 7.32
Curcumin	428.13 ± 12.36	423.44 ± 6.78	229.69 ± 7.15	73.44 ± 8.40	46.90 ± 2.26
CLAE	439.06 ± 15.65	434.38 ± 9.54	217.50 ± 8.48	62.50 ± 6.24	43.75 ± 6.48
CLRP	431.25 ± 10.23	429.69 ± 4.28	209.38 ± 5.78	48.44 ± 2.62	29.69 ± 4.64

Table 3: Effect of *C. longa* preparations on wounds closure (area mm^2).

Groups	Day 3	Day 7	Day 11	Day 14
Control	7.53 ± 0.84^a	52.11 ± 0.95^a	77.80 ± 0.92^a	83.16 ± 0.65^a
Curcumin	10.91 ± 0.92^a	61.45 ± 0.85^{bc}	86.20 ± 0.49^b	89.64 ± 0.37^{bc}
CLAE	12.66 ± 0.90^a	60.28 ± 0.72^c	87.76 ± 0.50^{cd}	90.58 ±0.58^c
CLRP	15.84 ± 0.60^a	63.03± 0.30^d	90.49 ± 0.34^d	94.20 ± 0.57^d

Table 4: Effect of topical application of *C. longa* preparations on wound contraction (%).

Groups	**Hydroxyproline** (µg/mg tissue)	**Glucosamine** (µg/mg tissue)
Control	8.75 ± 0.46^a	4.11 ± 0.02^a
Curcumin	13.25 ± 0.87^{bc}	6.03 ± 0.75^{bc}
CLAE	14.05 ± 1.16^{cd}	6.85 ± 0.63^c
CLRP	17.82 ± 0.28^d	8.60 ± 0.13^d

Table 5: Effect of *C. longa* preparations on level of hydroxyproline and glucosamine.

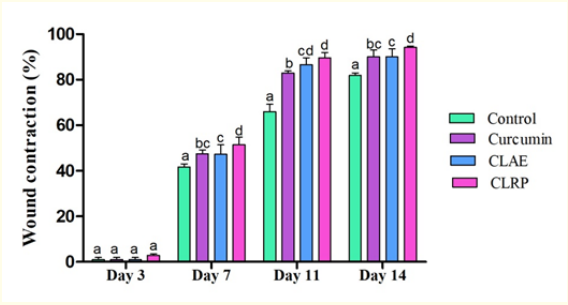

Figure 2: Effect of topical application of *C. longa* preparations on wound contraction (%) on days 0, 3, 7, 11 and 14 post-wounding.

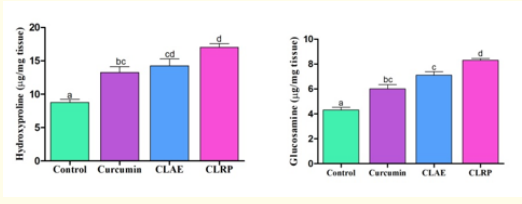

Figure 3: Effect of topical application of *C. longa* preparations on level of hydroxyproline and glucosamine in granulation tissues harvested on day 14 post-wounding.

CLAE-treated groups showed almost same level of glucosamine, while, CLRP-treated group showed highest level of glucosamine.

Oxidative stress related parameters

The level, of oxidant and antioxidant parameters, was estimated in granulation tissue. The obtained results (Table 6 and Figure 4) revealed that MDA level was significantly decreased in all treatment groups compared to control group, while, the level of GSH, SOD and catalase were significantly increased in all treatment groups compared to control group.

Histological analysis of granulation tissues

H and E staining

Representative images of H and E-stained granulation tissue sections are presented in figure 5 (10X and 40X), where inset boxes represent lower magnification. Tissue section of treatment groups showed the formation of thick regenerated epithelial layer with compact keratinization covering over healed tissue. Moreover, there was formation of mature granulation tissue with well-organized collagen fibres, new blood vessels and less number of PMNL cells. While, the control group showed incomplete formation of epithelial layer with no keratinization, less number of fibroblast and high number of PMNL cells. Histological scoring of parameters like

Groups	MDA nmol/mg protein	SOD Unit/mg protein	GSH nmol/mg protein	Catalase nmol/min/mg protein
Control	7.75 ± 0.38^{a}	11.54 ± 0.56^{a}	2.66 ± 0.06^{a}	121.51± 5.09^{a}
Curcumin	6.16 ± 0.10^{bc}	13.60 ± 0.49^{ab}	3.47 ± 0.09^{b}	137.11 ±5.77^{abc}
CLAE	5.42 ± 0.18^{c}	14.77 ± 0.53^{b}	3.96 ± 0.12^{c}	149.89 ± 5.42^{bc}
CLRP	3.81 ± 0.13^{d}	17.46 ± 0.42^{c}	5.23 ± 0.24^{d}	152.00 ± 5.64^{c}

Table 6: Effect of *C. longa* preparations on oxidative stress related parameters.

epithelialization, angiogenesis, fibroblasts, collagen deposition, done in H and E-stained granulation tissue, revealed significant difference between treatment and control groups (Figure 6).

Discussion

Wound contraction is the movement of wound edges towards each other in a centripetal fashion [18]. Contraction of the wound begins soon after wounding, and peaks at 2 weeks. During granulation tissue formation, fibroblasts are migrated at the injured site, and gradually transformed into the myofibroblasts [19]. Myofibroblasts are the predominant mediator of this contractile process because of their ability to extend and retract. In the present study, we observed significant increase in per cent wound contraction, so, there was considerable reduction in wound size and absolute

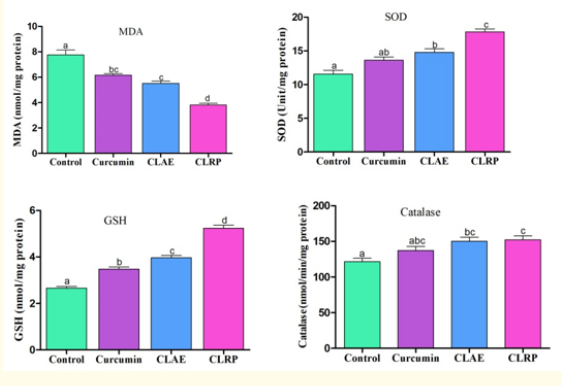

Figure 4: Effect of topical application of *C. longa* preparations on levels of oxidative stress related parameters in granulation tissues.

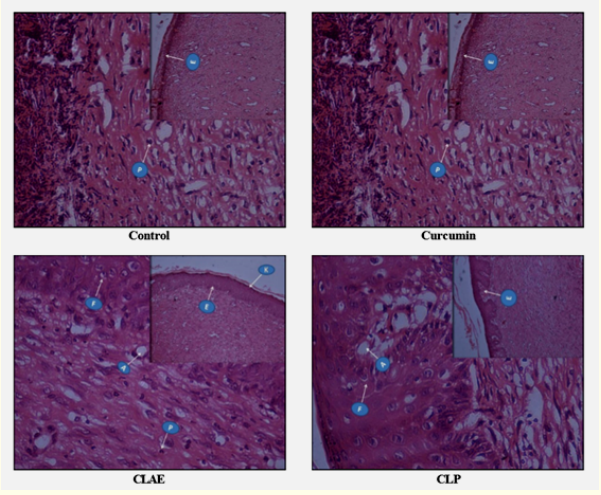

Figure 5: Representative images of H and E-stained granulation tissue sections of control and *C. longa* preparations-treated groups on day 14 post-wounding (10X and 40X magnification). Where, inset boxes in the right upper corner represent lower magnification. P: PMNL, F: Fibroblast, A: Angiogenesis, E: Epithelialization, K: Keratinization.

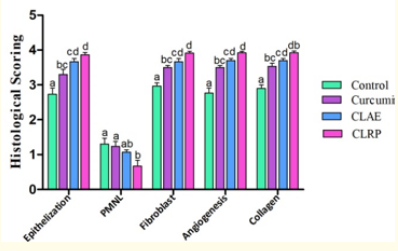

Figure 6: Scoring of H and E-stained tissue sections of control and *C. longa* preparations-treated groups.

wound area in *C. longa* preparations-treated groups from day 7 to 14 compared to control group. Among treatment groups, CLRP-treated group showed enhanced wound contraction, which was followed by CLAE and curcumin-treated groups. So, our findings suggests that there was marked acceleration of wound healing process after *C. longa* preparations application on wound, which may be due to the stimulatory effect of *C. longa* preparations on fibroblasts migration and their subsequent transformation to myofibroblasts, thus, promoted the granulation tissue formation. Our study results are supported by previous reported finding, topical application of paraffin-based CLP ointment reduced post-surgical algesia and fastened wound closure, which may be attributed to its anti-inflammatory effect and enhanced collagen deposition, respectively [10].

Hydroxyproline is a biochemical marker for assessing the collagen synthesis and deposition in the tissue, and its high level in the injured tissue is a positive indication of the progression of healing process [20]. Glucosamine is a prominent precursor in the biochemical synthesis of hyaluronic acid [21]. Hyaluronic acid (HA) interacts with fibrin to form HA-fibrin matrix, which plays a major role in tissue regeneration process by stimulating the migration and mitosis of mesenchymal and epithelial cells [22]. In our study, hydroxyproline and glucosamine levels were significantly high in all treatment groups compared to control group. CLRP-treated group showed highest levels of hydroxyproline and glucosamine. Our findings are in agreement with the previous study, where it has been found that, in proliferative phase, curcumin ameliorates the wound healing process by increasing the hydroxyproline and collagen synthesis [23]. The significantly higher content of hydroxyproline and glucosamine levels, in *C. longa*-treated groups compared to control group, revealed faster maturation of the wounds, which might be due to promotion of sharp twisting of collagen helix, necessary for wound contraction, and stimulation of the migration and mitosis of mesenchymal and epithelial cells at the injured site.

ROS have been implicated as important mediators of cell signaling and inflammation in wound repair. Low levels of ROS play crucial role in wound healing by working as mediators of intracellular signaling [24]. However, excessive amounts of their level, often found in chronic or impaired acute wound, cause oxidative stress, which further damage cells and delays healing. Antioxidant mechanism plays an important role in scavenging free radicals [25]. In the present study, levels of MDA, GSH, SOD and catalase were assessed in the granulation tissue of rats. MDA production is an index of lipid peroxidation. The production of free radicals increases the peroxidation of lipid molecules, which is harmful to the biological structures. SOD catalyses the dismutation of superoxide radical into oxygen and H_2O_2, thus decreases the ROS generation and oxidative stress. Catalase neutralize the excessive H_2O_2 accumulated in the wound tissues due to enhanced activity of SOD [26]. GSH protect the cellular components from oxidative damage caused by ROS. Our results showed significantly low level of MDA in treatment groups compared to control group, while, the level of GSH, SOD and catalase activities were significantly increased in treatment groups compared to control group. Moreover, our findings are in agreement with the previous findings where they showed that *C. longa* contains the anti-oxidant activity and it acts as an antioxidant by scavenging ROS, by restoring abnormal changes induced by external factors, and by suppressing transcription factors related to oxidation [27]. *C. longa* extracts were obtained from using various techniques exhibited the strongest antioxidant

activities [28]. Therefore, our findings suggests that, in the present study, *C. longa* preparations has exhibited efficient antioxidant activity, which might have prevented lipid peroxidation mediated damage to the biological structures by scavenging free radicals and thus, promoted wound healing by curtailing the undue prolongation of the inflammatory phase.

H and E staining showed further evidence of accelerated wound healing process in *C. longa* preparation-treated groups through enhanced epithelialization, angiogenesis, fibroblasts proliferation, collagen deposition and less number of PMNL. Superior granulation tissue is characterized by the presence of fibroblasts with ECM formation and well-formed blood vessels in the perpendicular direction. In addition to the formation of ECM in healing tissue, its progressive degradation and remodeling in a regulated manner is essential to form mature healed wound tissue. *C. longa* stimulate wound healing by earlier re-epithelialization, improved neovascularization, increase fibroblast proliferation and collagen deposition by regulating different cytokines and growth factors [29]. In the present study, tissue sections, from *C. longa* treated groups, showed more number of fibroblasts, and well organised collagen bundles in a time-dependent manner as evidenced in histopathological observations, which indicates that topical application of *C. longa* facilitated a well synchronized process of wound repair. The increased formation of ECM and collagen in the *C. longa* treated groups were also evident from significantly high levels of glucosamine and hydroxyproline in the granulation tissues respectively.

Conclusions

Based on study results we concluded that CLRP have higher healing potential in excision wound model compare to alcoholic extract and curcumin. *C. longa* accelerates healing process by different mechanisms such as wound contraction, collagen deposition, formation of ECM and anti-oxidant activities. Curcumin might not be the only active pharmacological moiety present in turmeric for exhibiting wound healing property.

Future Prospects

Further studies are required for identification and isolation of the different active pharmacological moieties present in the *C. longa*, their role in wound healing process and for confirming its use as medicine in animals.

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