POTENTIAL OF ETHANOLIC EXTRACT OF FICUS BENGHALENSIS ON OPEN WOUNDS AND INFLAMMATION

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Abstracts
The present study deals with evaluation of antioxidant, wound healing and anti-inflammatory activity of ethanolic extract of *Curcuma longa* Linn rhizomes. The ethanolic extract prepared by maceration technique was subjected to screen for antioxidant activity using DPPH radical scavenging method and wound healing activity using incision, excision, histopathological and dead space wound model and the study was supported with evaluation of granuloma tissue to estimate hydroxyproline content and histopathological evaluation. The anti-inflammatory study was carried out by using carageenan induced rat paw odema method. The tested extract of different dilutions in range 200µg/ml to 1000 µg/ml shows activity in range of 9.34% to 18.55%. Significant increase in wound closure rate, skin breaking strength, granuloma breaking strength was observed. The hydroxyproline content was also increased with decrease in scar area. The initial healing action might be due to increased collagen deposition and better alignment, with the obtained results it can be concluded that Curcuma longa extract has significant wound healing activity and initial healing may be due to presence of some terpenoids and antimicrobial agents. The extract shows prominent anti-inflammatory activity as compared to that of standard (Ibuprofen gel). The extract shows good anti-inflammatory activity on carageenan induced rat paw odema method.
Keywords: antioxidant, wound healing, *Curcuma longa*, anti-inflammatory

1. Introduction

Curcuma longa Linn (Zingiberaceae) is distributed in India, Pakistan, China and other tropical countries. The colouring matter present in the rhizomes of *Curcuma longa* is curcuminoides that include curcumin, demethoxy curcumin and bis demethoxy curcumin[1]. These compounds are diaryl heptanoid compounds of dark yellow colour. The rhizomes also consist of dicaffeoylmethane, caffeoylferuloylmethane, essential oil, zingiberine, turmerone sesquiterpines, monoterpinoids and monosaccharides. Curcuminoids have been reported to have very strong antiinflammatory, anti-carcinogenic, anti-oxidant, antiallergic, anti-bacterial, and anti-tumor activities[2-5]. Curcumin has recently attracted increased attention because of s potent anti-leishmanial properties[6], and ability to reduce alcohol-induced liver disease [7] and Alzheimer's disease[8]. Several methods have been developed to separate the curcuminoids: thin-layer chromatography (TLC)[9], supercritical fluid chromatography (SFC)[10], gas chromatography (GC)[11], high performance liquid chromatography (HPLC)[12,13], and HPLC–mass spectrometry (LC–MS)[11, 14].

Wounds are visible results of individual cell death or damage and can be classified by site, size, depth and causation – surgery, accident or circulatory failure[15]. Wound healing is a process which is fundamentally a connective tissue response. Initial stage of this process involves an acute inflammatory phases followed by synthesis of collagen and other extracellular macromolecules which are latter remoulded to form scars[16,17]. Inflammation is the process which may be due to release of histamine, kinins, serotonins and prostaglandin[18]. Anti-inflammatory agents are the agents which normally inhibit the release of these inflammatory mediators.

The present study deals with the use of traditionally claimed use of *Curcuma longa*. The invitro antioxidant activity was screened by DPPH method. The wound healing study was evaluated by incision and excision and dead space wound model followed by histopathological study. Anti-inflammatory study was screened by carageenan induced paw odema method.

2. Material and Methods

2.1 Plant material and Preparation of Herbal Extract: *Curcuma longa* rhizomes was purchased from the local market and authenticated in Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University Campus, Nagpur. Initially these botanicals were washed with fresh water to remove adhering dirt and foreign particles. Excess of water shake off and dried at 35 - 40°C in an oven for 24 hours. The dried roots were crushed and grinded and the coarse powder mass of rhizomes was weighed and allowed to undergo maceration technique.

The crushed mass were weighed and then placed with ethanolic solution in a cylinder. 200g of *Curcuma longa* rhizomes in 1.0 liter of ethanolic solution were macerated for 7 days. The mensturm was removed and concentrated by vaccum distillation. Again the crude material was allowed to undergo maceration for 4 days followed by 2 days for complete extraction of *Curcuma longa* rhizomes. The mensturm was collected and concentrated by vaccum distillation and then air dried in an evaporating dish till constant weight was obtained. The percent yield of *Curcuma longa* rhizomes extract is 17.26%.

2.2 Animals: In the present study male wistar rats (150-200g) were used for the study. They were individually housed and maintained on normal standard diet (Gold Muhor Brand, Lipton India limited) and water *ad libitum.* Temperature was maintained at 23±1°C with 12hr light and dark cycle.

2.3 Antioxidant activity: The DPPH (1,1-diphenyl-2-picryl hydrazyl) method was used for the determination of in vitro antioxidant activity for crude extract. DPPH is stable free radicals scavenge by antioxidant present in test solution.

To 1.0 mL of 0.01mM Solution of DPPH, 1.0mL of ethanolic solution and 0.95 mL of tris HCl buffer of 0.05M was added. To this solution 50µl of extract of specific strength was added. It was kept for 20 min for reaction at room temperature and then absorbance was recorded at 517 nm. Control solution was also carried out without extract. Percent scavenging of DPPH radical was calculated by comparing absorbance between the test and diluted control mixture. [19]

2.4 Wound Healing activity: Animals were wounded under light ether anesthesia, semiaseptically. The animals were assigned in to three groups, each group containing six animals. First group was untreated group which was taken as control (Group1). In Second group (Group-2) wounds received topical application of *Curcuma longa* extract, while animals in Group- 3 (Reference Standard) received treatment of Framycetine Sulphate Cream (FSC) in both excision and incision wound models. No other topical or systemic therapy was given to animals during the course of this study. The experimental protocols were approved by the Institute Animal Ethical Committee.

2.5 Excision wounds[20]: Hairs were removed from dorsal thoracic central region of anaesthetized rats. Full thickness from the demarketed area was excised to produce wound measuring around 300 mm^2 as shown in Fig-1. Wound was cleaned with cotton swab soaked in alcohol. The test extract and FSC cream was applied on the excised wound once daily for 24 days starting from the first day of wounding. Wound contraction was measured as percent reduction in wound area[21].

2.6 Resutured Incision wounds: Animals were anaesthetized and paravertebral incisions (2.5 - 3.0 cm long) were made through the entire length of skin. After the incision was made, the parted skin was kept together and stitched with nylon thread at 0.5 cm apart with curved needle (No. 11).The Photographic representation of incision wound model with the stitches on the incised skin are shown in Fig 2.The sutures were removed on day 8 and healing (tensile) strength was measured on day 10[22].

2.7 Dead space wound model: Under light ether anesthesia, subcutaneous dead space wounds were inflicted in the region of axilla and groin, by making a pouch through a small nick in the skin. Granuloma formation was induced by implanting either sterile cotton pellets or grass piths. Two sterile cotton pellets weighing 10 mg (sterilized by autoclaving) were implanted in axilla by technique of D'Arcy et al as describe by Turner [23], but the granuloma were removed on 10^{th} day. Thus one animal had two cottons. The sutures were mobbed with an alcoholic swab and animals were placed into their individual cages after recovery from anesthesia.

Physical, mechanical and histopathological changes in granuloma tissue were studied in this model. Excision of granuloma from the surrounding tissues were performed on the 10^{th} post wounding day under light ether anesthesia cotton pellet granuloma excised from dead space wound were dried overnight at 60°C so as to obtained a constant dry weight, their weights were noted and expressed as mg/100gm body weight [24]. The excised tissue was cut into two approximately equal halves. One half of the

granuloma tissue was used for determination of hydroxyproline content [25]. The other part is kept in 10% formalin solution for histopathological studies to evaluate the effect of extract on collagen formation.

2.8 Histopathological study: The histopathology study was done to evaluate the healing promoters like keretenization, epithelization, collagenation, fibrosis and neovascularisation, which were evidenced to promote wound healing in the experimental animals. The section from 10 day old regenerated tissue of incision wound was taken and washed with normal saline solution to study the healing markers. After fixing the section in 10% formalin solution the tissues were dehydrated with 90% ethanol, cut into thin sliced section (7µm thick), stained with haemotoxyline-eosin dye and observed under light microscope for keretinization, epithelization, fibrosis, collagen formation and neovascularization[26]. The interpretation of the results were numbered from 1 to 5 of which 5 stands for maximum similarity and 1 stands for least similarity form normal tissue around the wound area in the *Curcuma longa* treated and untreated wounds[27].

2.9 Anti-inflammatory Study: Anti-inflammatory activity of *Curcuma longa* rhizomes extract was studied by carageenan induced paw odema method. The animals were divided into three groups containing six animals in each group. Group – 1 (control) untreated group, Group – 2 topical application with *Curcuma longa* rhizomes extract, Group 3- topical application of Ibuprofen gel. Group 2 and 3 received topical application of *Curcuma longa* rhizomes extract and Ibuprofen gel for comparison of anti-inflammatory activity. One hour after the application of *Curcuma longa* rhizomes extract 0.1 ml of carageenan (1%) was injected into sub planar region of hind paw of rat. Measurement of paw volume (ml) were made by mercury displacing techniques using plethysmometer immediately before and 1, 2, 3 and 4 hr after carageenan injection. Percentage inhibition of inflammation after 1, 2, 3 and 4 hr was calculated by newbould's method [28].

2.10 Statistical Analysis: Statistical difference between the groups were evaluated using one way analysis of variance (ANOVA) followed by Turkey's Kramer Multiple comparison test ($P < 0.001$).

3. Result:

The present study deals with evaluation of antioxidant, wound healing and anti-inflammatory activity of ethanolic extract of *Curcuma longa* rhizomes. The method used to determine the peroxide value or scavenging activity is based on decomposition or scavenging of DPPH which is a very stable free radical. The comparative percent peroxide value of *Curcuma longa* rhizomes extract is shown in Fig. 3. Percent peroxide value for *Curcuma longa* extract is in the range of 9.34% to 18.55% for the extract with 200µg/ml to 1000 µg/ml strength extract. The wound healing study was screened by four models incision, excision, dead space wound model and histopathological study. The anti-inflammatory study was performed by carageenan induced rat paw odema method. The acute toxicity study of ethanolic extract of *Curcuma longa* rhizomes do not show any signs of toxicity up to 3g/kg body weight. Since there was no mortality at higher dose $1/10^{th}$ of maximum dose of extract tested for acute toxicity was screened for evaluation of wound healing activity i.e., 300mg/kg.

In the excision wound study the wounds are treated with FSC cream and *Curcuma longa* rhizomes extract shows complete healing in day 24-25. The results of these groups were compared with the healing activity of untreated group which took more than 30 days for wound closure and fall of eschar. The results are shown in Table 1.

In incision wound study the tensile strength was measured on day 10 of regenerated tissues. The tensile strength of wounds treated with FSC cream and *Curcuma longa* rhizomes extract is 333.78±3.55 and 379.00±6.32 respectively was compared with mean tensile strength ±SEM of untreated group i.e., 281.30±5.82. The comparative tensile strength is shown in Fig. 4.

In histopathological study some healing markers were evaluated like keretinization, epithelization, fibrosis, Collagenation and neovascularization. After the histopathological evaluation of slides the *Curcuma longa* treated wound shows prominent promotion of keretinization, epithelization, fibrosis and collagen formation, the results are compared with untreated group and wounds treated with FSC cream was consider as reference standard. The comparative promotion of these markers are shown with the help of photomicrographs in Fig. 5 (a), (b) and (c).The promotion of healing markers is four times more than that of untreated wound. The results are shown in Table 2.

Similarly in dead space wound model there was significant increase in granuloma breaking strength, dry granuloma weight and

hydroxyproline content of granulation tissues as compared to controls. The results are shown in Table 4.

The topical anti-inflammatory activity of *Curcuma longa* extract was carried out. The percentage protection (inhibition) of odema for *Curcuma longa* extract and Ibuprofen gel was found to be 47.76 (1hr), 71.49 (2hr), 27.96 (3hr), 56.79 (4hr) and 36.54 (1hr), 64.85 (2hr), 63.83 (3hr), 67.62 (4hr) respectively. The tested extract showed significant anti-inflammatory activity when the results are compared with untreated group and decreased in inflammation by Ibuprofen gel was taken as reference standard. The results are highly significant ($p< 0.001$) and are shown in Table 3.

4. Discussion:

A herbal drug for the treatment of chronic diseases or as raw material from which more or less complex chemical compounds with particular biological activity are isolated, the deeply held beliefs in the efficiency of natural products/herbals should be proven by scientific investigation bringing the tradition and experience in the ancient knowledge within the realm of sciences[29].

In excision wound healing study from the observed values it were assumed that *Curcuma longa* extract shows better and faster healing as compared to untreated group. During the initiation of the study day "0" the wound closure with *Curcuma longa* extract was very slow as compared to untreated and FSC treated wounds. As the study progress the wound healing efficiency increases shows complete healing on day 18-21 which is same for group 3 (wounds treated with FSC cream), But the untreated group took more time to complete wound closure. The study was carried out till fall of eschar leaving no scar behind. This shows healing potential of *Curcuma longa* extract with better and faster wound closure.

The result obtained for tensile strength shows higher tensile strength for *Curcuma longa* extract treated wounds as compared to wounds of untreated group. The increase in tensile strength may be due to promotion of collagen formation which significantly contributing to effective and better wound healing. The *Curcuma longa* extract also promotes healing markers as compared to that of control group. In addition to this increase in granuloma breaking strength, dry granuloma weight and hydroxyproline content strongly emphasize the positive wound healing potential of ethanolic extract of *Curcuma longa* rhizomes. Accordingly hydroxyl proline (marker of collagen) was significantly increased in treated group as compared to control and further histopathological studies of granulation tissue, which recorded increase in collagen content in treated group.

The determination of anti-inflammatory activity is based on plethysmographic measurement of odema produce by sub planer injection of carageenan in hind paw of rat. The increase odema in animal treated standard (Ibuprofen gel) and *Curcuma longa* extract were composed with increase in odema of untreated control animals at constant interval of 1, 2, 3 and 4hrs. The percentage inhibition of odema at known interval in treated animals was used for the purpose of calculating the percent inhibition of odema of control. The present study revealed that the *Curcuma longa* extract showed significant inhibition of odema. The maximum activity showed during 1st and 2nd hrs, the results are highly significant ($p<0.001$) as compared to standard. The anti-inflammatory activity may be due to inhibition of release of histamine, serotonin and kinins in first hour after injection of carageenan and also retard the release of prostaglandin and like substances[30], shows anti-inflammatory activity of *Curcuma longa* extract.

It has been reported that terpenoids posses an ability to increase the collagen content, which is one of the factor for promotion of wound healing [31]. As the title plant is rich in curcuminoids, it may be responsible for wound healing activity. The plant extracts also shows antioxidant activity i.e., free radical scavenging activity which might reduces lipid peroxidation, may not only prevent or slows down the onset of necrosis but also improve vascularity[32]. The experimental plant also shows antimicrobial activity as reported earlier these are the factors count for wound healing activity of *Curcuma longa* rhizomes.

Thus it may be concluded that the rhizomes of *Curcuma longa* shows significant antioxidant, wound healing and anti-inflammatory activity. Further studies are in progress to isolate the bioactive component of plant extract.

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Table- 1 Effect of topical application of *curcuma longa* extract on excision wound

Post wounding days	Wounding area (mm^2)			F values
	Group 1	Group 2	Group 3	
0	311.86±3.03 (0)	298.56±2.616 (0)	369.50±4.38 (0)	F(2,15) = 120.92
3	272.51±5.06 (12.62)	258.33±2.550 (13.47)	308.61±1.68 (16.47)	F(2,15) = 57.651
6	212.29±4.86 (27.76)	203.90±2.009 (31.70)	245.89±2.04 (33.45)	F(2,15) = 41.447
9	184.11±4.28 (41.01)	147.05±1.956 (50.74)	217.23±4.62 (41.20)	F(2,15) = 84.814
12	160.72±4.49 (46.54)	83.37±1.540 (72.07)	157.87±3.94 (57.27)	F(2,15) = 151.55
15	135.81±3.76 (56.45)	54.50±1.137 (81.74)	129.09±3.79 (65.06)	F(2,15) = 204.48
18	126.46±3.04 (59.45)	24.07±1.168 (91.95)	92.63±2.86 (74.93)	F(2,15) = 434.09
21	114.44±5.71 (63.30)	3.485±0.551 (98.83)	44.56±3.24 (87.94)	F(2,15) = 217.32
24	97.26±3.58 (68.81)	0.00 (100)	13.85±1.32 (96.25)	F(2,15) = 570.40
27	66.69±2.57 (78.61)	0.00 (100)	0.00 (100)	F(2,15) = 671.29

Values are mean ± SEM of animals (n=6) in each group. Number in parenthesis indicates percentage of wound contraction. All the values are significant at $p<0.001$ as compared to Group 1

Table- 2 Histopathological examinaion of wounds treated with *curcuma longa* extract at the end of 10 days

Parameters	Group 1	Group 2	Group 3	F values
Keretinization	0.48±0.03	3.9±0.07	3.66±1.22	F(2,15) = 511.05
Epithelization	1.23±0.55	3.65±0.18	4.16±0.04	F(2,15) = 186.75
Fibrosis	2.01±0.07	3.13±0.16	4.20±0.05	F(2,15) = 101.04
Collagenation	1.85±0.42	3.45±0.24	4.43±0.05	F(2,15) = 76.377
Neovascularization	0.50±0.06	4.0±1.11	4.42±0.12	F(2,15) = 405.41

Values are reading of mean ± SEM of six animals, Value 5 refers to maximum similarity and 0 refers to least similarity of wound from normal tissues. All the values are significant at p<0.001. Group 1 – Untreated group, Group 2 – Treated with *Curcuma longa* extract, Group 3 – Treated with framycetine sulphate cream

Table – 3 Topical Anti-inflammatory activity of *Curcuma longa* extract

Group	Dose	Percent inhibition of paw volume at different time intervals			
		1hr	2hr	3hr	4hr
Standard (Ibuprofen gel)	300	36.54	64.85	63.83	67.62
Test (*Curcuma longa* extract)	300	20.00	21.62	31.57	47.82

Table – 5 Effect of *Curcuma longa* extract on dead space wound models

Dead Space Wound Models			
Parameter Studied	Granuloma Breaking Strength (g)	Dry Granuloma weight (mg % of B.W.)	Hydroxyproline content
Control	278.89±2.603	43.08±2.381	6.89±0.4604
Ethanolic extract	299.25±1.048	65.28±1.257	8.57±1.092
F values	1.925	1.096	2.082

FIG. – 1: Excision wound model

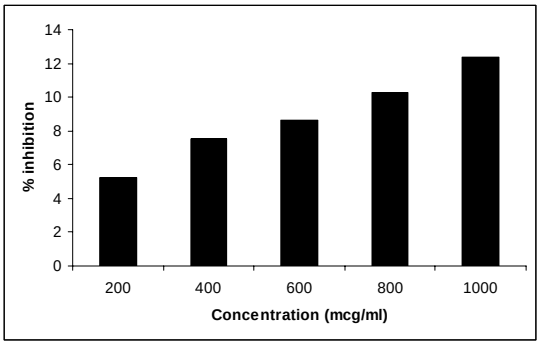

Fig. 3: Percent Peroxide value for *curcuma longa* extract

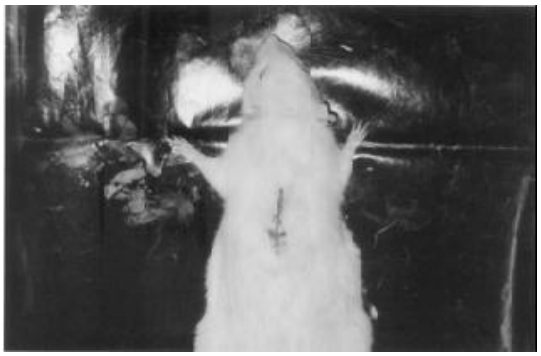

Fig. 2: Incision wound model

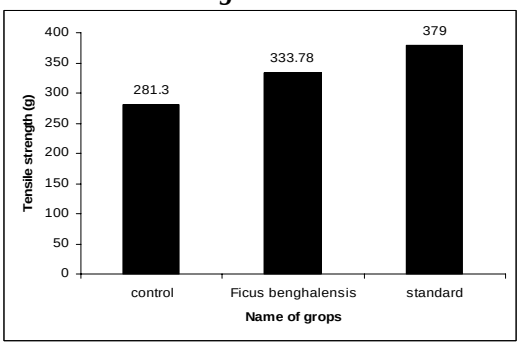

Fig. 4: Effect of *Curcuma longa* on tensile strength

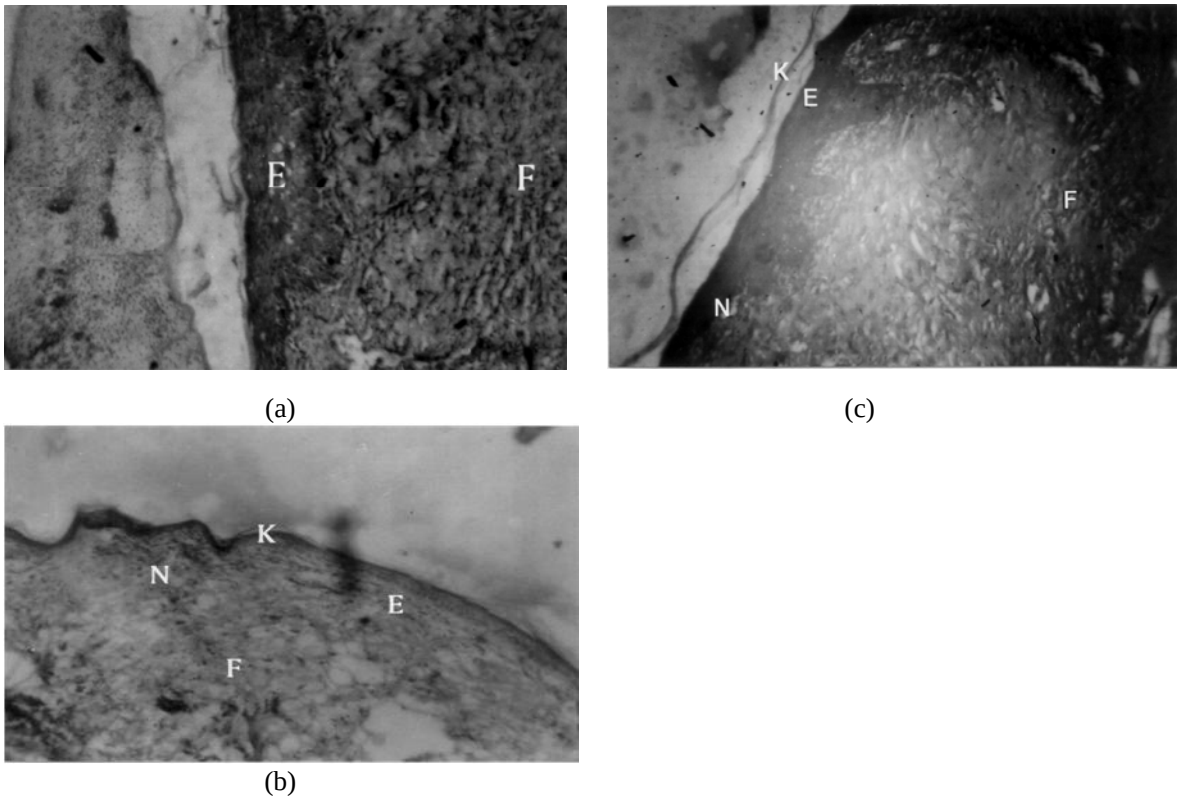

Fig. – 5: Histopathology of 10 D old regenerated tissue

Photomicrographs of histology of regenerated tissues collected from a) Untreated control, b) *Curcuma longa* treated wounds, and c) FSC (1% w/w) treated after 10 d. Maginification si 100 X. K - Keretinization, E - Epithelization, F- Fibrous tissue and N- Neovascularization