Exploring the role of curcumin containing ethanolic extract obtained from Curcuma longa (rhizomes) against retardation of wound healing process by aspirin

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ABSTRACT

Aim: The aim of the study was to assess the curcumin containing ethanolic extract (EtOH) obtained from Curcuma longa (Cl) against retardation of wound healing by aspirin.

Materials and Methods: Wound healing process was retarded by administering the dose of 150 mg/kg body weight of aspirin orally for 9 days to observe the effect of EtOH obtained from Cl using excision and incision wound model in rats. The various parameters such as % wound contraction, epithelialization period, hydroxyproline, tensile strength were observed at variant time intervals and histopathological study was also performed.

Results: Curcumin containing 5% and 10% ethanolic extract ointment have shown significant ($P < 0.01$) wound healing activity against an aspirin (administered 150 mg/kg body weight orally for 9 days) retarded wound healing process. Topical application of ointment showed significant ($P < 0.01$) difference as compared to the control group. Histopathological studies also showed healing of the epidermis, increased collagen, fibroblasts and blood vessels.

Conclusion: Ethanolic extract of Cl ointment (EtOHCl) containing 10% curcumin displayed remarkable healing process against wound retardation by aspirin.

KEY WORDS: Aspirin, Curcuma longa, excision wound, incision wound, wound contraction

Introduction

Wound healing is the dynamic self-recovery body mechanism from an injury which has been characterized in four different overlapping phase homeostasis, inflammatory, proliferative, and remodeling. These phases and their biophysiological functions must occur at a specific time, and maintain for a specific duration at an optimal strength.[1] Wounds associated with different diseases make it more complex to heal such as impaired wound healing in diabetes and ischemic wounds. Diabetes and impaired wound are now a major problem in wound healing this will lead to chronic wounds.[2] Wound healing is currently a clinical challenge due to inconsistencies encountered in the healing processes.[3]

Wound management involves pharmacological intrusion ranging from analgesics to antibiotics, which is often expensive. Some of these agents affect the healing process positively and some negatively; for example nonsteroidal anti-inflammatory drugs (NSAIDs) are known to retard healing.[4] NSAIDs are administered as analgesics or anti-inflammatory agents in fracture healing and postoperative orthopedic interventions.[5] Nonspecific and cyclooxygenase-2 (COX-2) selective NSAIDs function by inhibiting the COX isoenzymes and effectively reduce pain and inflammation attributed to acute or chronic musculoskeletal pathologies. NSAIDs are frequently used by patients in the period immediately following surgery. Clearly, the inhibitory effects of these drugs on wound healing represent a potential impediment to the patient’s recovery.[6]

Curcuma longa (Cl) is a rhizomatous perennial herb that belongs to the family Zingiberaceae, is a small perennial herb native to India bearing many rhizomes on its root system which are the source of its culinary spice known as turmeric...
and its medicinal extract called curcumin. In traditional medicine, it has been used for centuries due to its antimicrobial, antimalarial, and antioxidant properties. It contains three major curcuminoids, namely, curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin is the most abundant of the three.}

Curcumin, known chemically as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a naturally occurring low molecular weight polyphenolic phytoconstituents. Curcumin is the product obtained by solvent extraction of turmeric that is, the ground rhizomes of Cl. (Curcuma domestica Valetin) and purification of the extract by crystallization. Curcumin has been reported for various pharmacological activities like wound healing.

Materials and Methods

Plant Material

The rhizomes of Cl identified and collected from the local market of Bhopal. The plant was authenticated by botanist, Dr. Zia Ul Hassan, at Safia College of Science, Bhopal Voucher specimen no: 343/Botany/Safia/2012. The plant was cleaned, dried in the shade, pulverized into moderately coarse powder and stored in airtight container for further use.

Animals

Animal care and handling was done according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). This guideline has been issued by the Ministry of Environment and Forests. Animal study was performed at VNS Group of Institutions, Faculty of Pharmacy, Bhopal (Madhya Pradesh) with due permission from Institutional Ethical Committee (CPCSEA Reg. no: 778/PO/a/03/ CPCSEA; 03/09.). Healthy albino rats of either sex (100–150 g) with no prior drug treatment were selected to carry out all the present in vivo studies. The animals were used after an acclimatization period of 10 days to the laboratory environment. They were housed in standard metal cages and provided with food and water ad libitum.

Preparation of Extract

The dried rhizomes of Cl are subjected to size reduction to a coarse powder using dry grinder and passed through a sieve. 250 g powder material is taken and packed in soxlet apparatus and extracted successively with petroleum ether, ethanol. The solvent will be removed by distillation. Last traces of solvent will remove under vacuum and extract was stored in a desiccator and used subsequently.

Preliminary Phytochemical Screenings

Phytochemical screening was performed on EtOHCl for the assessment of the presence of various phytoconstituents.

Thin layer Chromatography

For that thin layer chromatography (TLC) analysis was carried out. From the literature survey, it was known that pure curcumin have Rf value is 0.6. Preparation of Ointment of Extract

Ointment of EtOHCl was prepared by fusion method. 5% and 10% ointment is prepared by using white soft paraffin wax as an ointment base.

Skin Irritation Studies

The ointment was applied on shaven skin of the rat. Skin irritation potential of 5% and 10% ointment of EtOHCl was assessed by carrying out patch skin irritation test on albino rats (150–200 g). Rats were acclimatized for 7 days before the study. The fur from the dorsal surface of rats was removed with electronic hair remover without damaging the skin, 24 h prior to the experiment. Animals were divided into three groups containing three rats in each group. Group I was kept as control applied topically ointment base only, Group II was treated group applied topically with 5% EtOHCl ointment and Group III was treated with 10% EtOHCl ointment. The formulations were applied topically to approximately 1 cm2 in area of the skin. The animals were then returned to their cages and were examined at 24, 48, and 72 h after the application of the formulation. The sites were inspected for dermal reactions such as erythema and edema. The mean erythemal and edemal scores was recorded on the basis of degree of severity: No erythema/edema = 0, slight erythema/edema = 1, moderate erythema/edema = 2, severe erythema/edema = 3.

Drug Administration

Aspirin neutralized with calculated amount of sodium carbonate solution before use, was fed to the rats for 9 days through a oral gavage, starting 1-day before wound induction at the dose of 150 mg/kg of body weight were given to different groups of rats.

Experimental Design

For the assessment of wound healing activity of curcumin containing EtOH obtained from Cl (rhizomes) against retardation of wound healing process by aspirin was performed on excision and incision wound model in rats. The animals are grouped into four groups each group contain six animals. Group I was served as normal control (only ointment base is applied topically), Group II was kept as negative control (aspirin fed orally for 9 days at dose of 150 mg/kg of body weight and ointment base applied topically on wound), Group III and IV are shown as test group (aspirin fed orally for 9 days at dose of 150 mg/kg of body weight and 5 and 10% ointment of ethanolic extract of curcumin, respectively). Wound induction was done on 2nd day of aspirin feeding and served as the initial day (zero) of postwound day in all groups excluding Group I.

Excision Wound Model

Excision wound was created by according to the method described by Thakur, et al., 2011 with some modification and animals were categorized as according to selected evaluation parameters. The administration of aspirin started from day 1 before the wound creation and after the formation of wounds, aspirin is orally administered regularly in all groups for 9 days except normal control group. After 9 days, discontinue the aspirin administration and applied only test sample ointments. For excision wound formation, the particular skin area was shaved 1-day prior to the experiment. The rats were anesthetized by administering ketamine and xylazine (50 mg/kg and 5 mg/kg i.p. body weight). A full thickness of the excision wound of the circular area was made on the shaved back of the rats 15 min later the administration of anesthesia. The shaved portion and an excision wound were inflicted by cutting away a 2 cm2 full thickness of the skin from a
predetermined shaved area. Wounds were left undressed to the open environment. In this model, % Wound contraction, period of epithelization, and hydroxyproline content were measured.\textsuperscript{[16]}

**Incision Wound Model**

Incision wound was created by according to the method described by Thakur \textit{et al}., 2011\textsuperscript{[16]} with some modification. All animals were anesthetized (ketamine and xylazine 50 mg/kg, 5 mg/kg i.p. body weight) before wound creation and two full thickness paravertebral long incisions were made through the skin at the distance of about 1 cm from midline on each side of the deplilated back of rat. The both edges kept together and stitched with black silk surgical thread (no. 000), and a curved needle (no. 11) was used for stitching. The continuous threads on both wound edges were tightened for good closure of the wound. The aspirin are administered regular all groups in 9 days. When wounds were cured thoroughly, the sutures were removed on the 8th postwounding day and the percentage wound contraction was determined using the following formula:

\[
\text{% wound contraction} = \frac{\text{Healed area}}{\text{total wound area}} \times 100
\]

Epithelization time refers to the number of days taken by the wounds to appear completely closed with no moist granulation tissue, and the wound was covered with new epithelium.\textsuperscript{[21]}

**Tensile Strength**

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under tension and may designate in part of repaired tissue. For that healed tissue is excised on 10\textsuperscript{th} day of wound healing using anesthesia and subjected to tensiometer. The instrument used for measurement is called Tensiometer. Tensile strength was measured with the help of the instrument made in the laboratory which resembles a tensiometer.\textsuperscript{[17]}

**Hydroxyproline Estimation**

Hydroxyproline (C\textsubscript{5}H\textsubscript{9}O\textsubscript{2}N) is an uncommon amino acid present in the collagen fibers of granulation tissues. The measurement of hydroxyproline can be used as an index for collagen turnover. For the determination of hydroxyproline content, the animals from each group were anesthetized using diethyl ether. The wound tissues were excised on day 12 and dried in a hot air oven at 60°C–70°C to constant weight and were hydrolysed was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. the reaction was terminated by the addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60°C. The absorbance was measured at \(\lambda_{max} = 557\ nm\) using a spectrophotometer. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline in the same time.\textsuperscript{[10,19]}

**Histopathological Studies**

Histological analyses after fixation with 4% paraformaldehyde for 24 h at room temperature, the specimens were embedded in paraffin and sectioned in a plane perpendicular to the incision. Sections 5 \(\mu\)m thick were mounted on polylysine-coated slides, dewaxed, rehydrated to distilled water, and stained with hematoxylin and eosin. Evaluation of all sections was performed by two experienced pathologists who were blinded to the previous treatment. Sections were semi-qualitatively assessed under a light microscope (Labomed, CXR3, Labo America Inc., California) and observed with respect to fibroblast proliferation, collagen formation, neovascularization, granulation tissue, and epithelialization on day 12.\textsuperscript{[20]}

**Statistical Analysis**

The mean and standard deviation and the level of significance for the difference between means were determined by Tukey’s Cramer multiple comparison test\textsuperscript{[21]} and were analyzed by GraphPad Prism 4, (Graph Pad Software, Inc., USA).

**Results**

The plant material cleaned, dried in the shade, and pulverized into moderately coarse powder was subjected to extraction using soxhlet apparatus. The yields were found to be 20.017 g (8.0% w/w of crude drug) of petroleum ether extract with semisolid mass of yellowish color; 18.1 g (11.83% w/w of crude drug) of EtOH with orange color semisolid mass for Cl.

Preliminary phytochemical screening shows the presence of glycosides, tannins, and flavonoids as shown in Table 1. The \(R_f\) value of the pure curcumin was reported as 0.6 as given in the literature. The calculated \(R_f\) value from TLC was also 0.69, when the pure curcumin sample was applied. After derivatization of the extract, there were 4 spots observed. The \(R_f\) value of Spot 1 was calculated as 0.38, Spot 2 was 0.49, Spot 3 was 0.69 and Spot 4 was 0.92. So it was concluded that curcumin was present in the EtOHCl as \(R_f\) value of pure curcumin and Spot 3 was calculated same, that is, 0.69 it shown in Figure 1.

In skin irritation studies, there is no sign of erythema and edema was found on upto72 h after application of EtOHCl ointment.

The epithelialization time was measured from the initial day. The time taken for complete epithelialization of the excision wound was 18 days in Group I and 28 days in Group II, 26 days in Group III, and 24 days in Group IV (Table 2).

| Phytoconstituents          | Ethanal extract of Curcuma longa (Rhizome) |
|----------------------------|--------------------------------------------|
| Alkaloids                  | -                                          |
| Glycosides                 | +                                          |
| Tannins                    | +                                          |
| Flavonoids                 | +                                          |
| Amino-acids                | -                                          |
| Proteins                   | +                                          |

**Table 1:**

**Phytochemical analysis of ethanolic extracts of Curcuma longa**

\[**=Presence, - =Absent\]
A remarkable healing pattern was observed after the 9th day when the experimental groups stopped receiving aspirin. A better healing pattern with complete wound closure was observed in Group I, Group III, and Group IV in comparison to Group II as shown in Table 3 and Figure 2. The wound contraction in Group I was 100% on 21th day significant (P < 0.001) different from Group II, while it was found only 83.1% in Group II and 90.3% in Group III, 91.6 in Group IV. Reduction in time of wound closure was recorded as significant (P < 0.01) in both treatment group with a comparison of Group II. The complete healing was observed on 30, 28, 26 days of postwound healing in Group II, III, and IV respectively.

There was no remarkable increase in hydroxyproline content during the administration of aspirin as shown in Table 4. In Group I, hydroxyproline content observed was 19.5 μg/mg of tissue. Group II, hydroxyproline content observed was 15 μg/mg of tissue showed significantly (P < 0.001) decrease level of hydroxyproline content in comparison of Group I. It was highest in Group IV that is 18.8 μg/mg of tissue showed significantly (P < 0.01) increased level of hydroxyproline content, and decreases aspirin effect, compared with group II. Groups III and IV show higher hydroxyproline content on day 12 in healed tissue when compared to Group II. Group III also shows the significant (P < 0.01) increase in hydroxyproline in comparison of Group II. This result shows increase in collagen synthesis in treated groups.

The study of the healing of the wounds in rats has shown that the aspirin has brought retardation during 1st day to 9 days in Table 3. In the incision wound model, a significant (P < 0.01) increase in wound breaking strength in the group III was observed 213.33 g/cm, and in Group IV was 238.3 g/cm. A significant (P < 0.01) decrease in the wound breaking strength in the group II was observed 110.2 g/cm in comparison to Group I was 110.33 g/cm [Table 5].

In control group rats (ointment base), histopathology showed inflammatory changes, increased collagen, fibroblasts and blood vessels are observed in Figure 3. Epidermis is completely healed. In Group II, rats were shown inflammatory changes, less collagen, fibroblasts and area with cellular necrosis. In Group III, animals treated with 5% ethanolic extract ointment area with cellular necrosis, incomplete epidermis, and small collagen is

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**Table 2:**

Effect of curcumin containing ethanolic extract obtained from *Curcuma longa* in wound retardation by aspirin on Epithelialization period in excision wound model in rats

| Groups                                      | Epithelialization period (mean time in days) |
|--------------------------------------------|-----------------------------------------------|
| Group I normal control (Ointment base)      | 18±0.25                                       |
| Group II negative control (Aspirin+Ointment base) | 28.4±0.56**                                   |
| Group III (5% *Curcuma longa* extract ointment+Aspirin) | 26.6±0.36*                                   |
| Group IV (10% *Curcuma longa* extract ointment+Aspirin) | 24.7±0.585*                                  |

n=6 albino rats per groups; values are represents mean±SD. **P<0.001, *P<0.01 (Comparison of I with II, and III&IV with II)

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**Figure 1:** Thin layer chromatography (TLC) of ethanolic extract (*Curcuma longa*), stationary phase: Aluminum base TLC silica gel 60 F254, mobile phase: Chloroform:ethanol/glacial acetic acid (95:5:1), derivatizing agent: Iodine vapors

**Figure 2:** Effect of curcumin containing ethanolic extract obtained from *Curcuma longa* in wound retardation by aspirin on % wound contraction in excision wound model in rats

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Table 3:
Effect of curcumin containing ethanolic extract obtained from Curcuma longa in wound retardation by aspirin on % wound contraction in excision wound model in rat

| Post wounding days | Group I normal control (Ointment base) | Group II negative control | Group III 5% Curcuma longa extract ointment+aspirin | Group IV 10% Curcuma longa extract ointment+aspirin |
|--------------------|----------------------------------------|---------------------------|---------------------------------------------------|---------------------------------------------------|
| 0 day              | 0±0                                    | 0±0                       | 0±0                                               | 0±0                                               |
| 3 day              | 30±1.72                                | 10.3±8.0**                | 14±2.64                                           | 16.3±4.6                                          |
| 6 day              | 50±4.22                                | 18.3±8.0**                | 19.6±5.07                                         | 21.3±6.3                                          |
| 9 day              | 70.5±1.8                               | 24.7±0.7.01**             | 25±4.35                                           | 28.3±9.81                                         |
| 12 day             | 93±1.71                                | 30±1.7.3**                | 33.6±14                                           | 34.6±9.2                                          |
| 15 day             | 98±0.9                                 | 54.4±1.9**                | 65.9±7.1                                          | 64.8±3.0                                          |
| 18 day             | 99±0.5                                 | 70.5±1.8**                | 76.5±7.0                                          | 83.9±3.7                                          |
| 21 day             | 100                                    | 83.1±2.05**               | 90.3±1.13*                                        | 91.6±1.52*                                        |
| 24 day             | -                                      | 92.6±1.8**                | 94.2±1.13*                                        | 98.7±0.7**                                        |
| 26 day             | -                                      | 97.4±0.7                 | 99.4±0.3*                                         | 100*                                              |
| 28 day             | -                                      | 99±0.3                   | 100*                                              | -                                                 |
| 30 day             | -                                      | 100                      | -                                                 | -                                                 |

n=6 albino rats per groups; values are represents mean±SD. **P<0.001, *P<0.01 (Comparison of I with II, and III&IV with II)

Table 4:
Hydroxyproline content (μg/mg tissue) in different groups at day 12

| Groups                                   | Hydroxyproline content (μg/mg tissue) |
|------------------------------------------|---------------------------------------|
|                                          | 12th day                              |
| Group I normal control (Ointment base)   | 19.5±0.32                             |
| Group II negative control (Aspirin+Ointment base) | 15±0.53**                           |
| Group III (5% Curcuma longa extract ointment+Aspirin) | 16.52±0.4*                           |
| Group IV (10% Curcuma longa extract ointment+Aspirin) | 18.8±0.30*                           |

n=6 albino rats per groups; values are represents mean±SD. **P<0.001, *P<0.01 (Comparison of I with II, and III&IV with II)

Table 5:
Tensile strength in (g/cm) of healed wound, in different groups at 10th day

| Groups                                   | Tensile strength in (g/cm) |
|------------------------------------------|---------------------------|
| Group I normal control (Ointment base)   | 322±19.0                  |
| Group II negative control (Aspirin+Ointment base) | 110.33±10.0**            |
| Group III (5% Curcuma longa extract ointment+Aspirin) | 213.33±15.2*             |
| Group IV (10% Curcuma longa extract ointment+Aspirin) | 238.3±16.07*             |

n=6 albino rats per groups; values are represents mean±SD. **P<0.001, *P<0.01 (Comparison of I with II, and III&IV with II)

Discussion

Wound healing or wound repair, is an intricate process in which the skin (or another organ) repairs itself after injury. In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) exists in steady-state equilibrium, forming a
phytochemical constituent like flavonoids, triterpenoids, and the wound healing activity may be due to flavonoids (curcumin). The antioxidant properties of curcumin in accelerating wound peroxidase activities were significantly increased exhibiting while the levels of superoxide dismutase, catalase, glutathione by increasing cellular proliferation and collagen synthesis at the wound site by increasing total protein and Type III collagen content of wound tissues. ETOHCl-treated wounds were found to heal much faster as indicated by improved rates of epithelialization, wound contraction, and increased tensile strength which were also confirmed by histopathological examinations. Effect of aspirin on wound healing retardation was minimized by regular topical application of ointment of ethanolic extract of C. I may probably be due to the curcumin present in the plant or could the additive effects of the other phytoconstituents.

All the results were found significant \( P < 0.001 \) when compared with negative control group to others. Thus, it can be concluded that ethanolic extract (10% ointment preparation specially) has significant wound healing potential. Also some flavonoids (e.g. curcumin) possess wound healing potential by increasing cellular proliferation and collagen synthesis at the wound site, as evidenced by increase in DNA, total protein and Type III collagen content of wound tissues. Curcumin treatment was shown to decrease the levels of lipid peroxides while the levels of superoxide dismutase, catalase, glutathione peroxidase, activities were significantly increased exhibiting the antioxidant properties of curcumin in accelerating wound healing. Since, flavonoid was found positive in ethanolic extract; the wound healing activity may be due to flavonoids (curcumin).

Recent studies with other plant extracts have shown that a phytochemical constituent like flavonoids, triterpenoids, and tannins are known to promote healing process. Preliminary screening of ETOHCl has been shown to possess flavonoids and tannins. Furthermore, phenolic contents were found to be present in ethanolic extract. The chromatographic studies, qualitative, and spectrophotometric studies show that curcumin may be present in the ethanolic extract.

Application of herbal extract offers hope for the development of drugs to enhance wound to heal. ETOHCl increases cellular proliferation and collagen synthesis at the wound site by increasing total protein and Type III collagen content of wound tissues. ETOHCl-treated wounds were found to heal much faster as indicated by improved rates of epithelialization, wound contraction, and increased tensile strength which were also confirmed by histopathological examinations. Effect of aspirin on wound healing retardation was minimized by regular topical application of ointment of ethanolic extract of C. I may probably be due to the curcumin present in the plant or could the additive effects of the other phytoconstituents.

Conclusion

Aspirin acts on key skin cells called keratinocytes, delaying skin repair at wound sites. A better understanding of this retardation process and observation of the healing by the application of herbal extract offers hope for the development of drugs to enhance wound to heal. ETOHCl increases cellular proliferation and collagen synthesis at the wound site by increasing total protein and Type III collagen content of wound tissues. ETOHCl-treated wounds were found to heal much faster as indicated by improved rates of epithelialization, wound contraction, and increased tensile strength which were also confirmed by histopathological examinations. Effect of aspirin on wound healing retardation was minimized by regular topical application of ointment of ethanolic extract of C. I may probably be due to the curcumin present in the plant or could the additive effects of the other phytoconstituents.

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