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Evaluation of aqueous extract of leaves of *Ocimum kilimandscharicum* on wound healing activity in albino wistar rats

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ABSTRACT : The present study was aimed to evaluate the wound healing potential of aqueous extract of leaves of *Ocimum kilimandscharicum*. It is a well-known plant in Indian traditional medicine. On the basis of traditional use and literature references, this plant was selected for evaluation of wound healing potential. An aqueous extract of leaves was examined for wound healing activity at two different doses (200 and 400 mg/kg) in three types of wound models on rats: the excision, the incision and dead space wound model. Significant increase in skin breaking strength, granuloma breaking strength, wound contraction, dry granuloma weight and decreased in epithelization period was observed in animals of both the treatment groups compared to control. Granuloma tissue was subjected to histological examination to determine the pattern of lay-down for collagen using Van Gieson and Masson Trichome strains. Biochemical parameters viz; L-Hydroxyproline, Hexose amine, Ascorbic acid and Malondialdehyde also confirmed its potential wound healing activity at either dose. Thus, the enhanced wound healing may be due to free radical scavenging action and the antibacterial property of the phytoconstituents present in it which either due to their individual or additive effect fastens the process of wound healing.

Key words: *Ocimum kilimandscharicum*, aqueous extract, wound healing, ascorbic acid, L-Hydroxy Proline.

INTRODUCTION

Wound, a clinical entity contemporary to mankind is a common clinical problem till today, hence it is often challenging to clinicians.

Wound is a distruction of anatomical or functional continuity of living tissue, produced by physical, chemical, electrical or microbial insult to the tissue[1].Wound healing is a complex phenomenon involving a number of processes, including induction of an acute inflammatory process, regeneration of parenchymal inflammatory process, migration and proliferation of both parenchymal and connective tissue cells, synthesis of extracellular matrix (ECM) proteins, remodelling of connective tissue and parenchymal components, and acquisition of wound strength[2].

Injury of the skin induces repair mechanism that restores its functions in protecting the individual against environmental factors that might be harmful. Three different phases constitute the physiologic process of wound-healing; (i) substrate phase, (ii) proliferative phase and (iii) remodeling phase[3]. All these steps are orchestrated in controlled manner by a variety of cytokines including growth factor[4]. Some of these growth factors like platelet derived growth factor (PDGF), transforming growth factor B (TFG-B), fibroblast growth factor (FGF) and epidermal growth factor (EGF) etc. have been identified in self healing wounds.

Ocimum kilimandscharicum (Lamiaceae) is an aromatic undershrub with pubescent quadrangular branchlets; found throughout India and also cultivated. The leaves are acrid, thermogenic, aromatic, antibacterial, insecticidal, antiviral and deodorant. It is employed as indigenous medicine for a variety of ailments like cough, bronchitis, viral infections, foul ulcers, anorexia and wounds[5]. Keeping this in view, wound healing potential of aqueous extracts of leaves of *Ocimum kilimandscharicum* has been investigated on different parameters of wound healing in albino rats.

MATERIALS AND METHODS

Plant material and Preparation of Extracts

The fresh leaves of *Ocimum kilimandscharicum* were collected in the month of July 2008 and authenticated from Dr. Harsha Hegde, Chief Botanist, Indian Council of Medical Research (RMRC), Belgaum branch.

Preparation of Extract

Around 1kg of leaves were separated and dried under shade for about a week and extracted by hot percolation method using soxhlet apparatus[6]. Air-dried, powdered leaves were added with 3000ml boiling water. After mixing thoroughly, it was macerated in a suitable percolator for 2h. The percolation process was continued at moderate rate by gradually adding boiling water until the extraction process was completed (indicated by fade coloured menstrum). The percolate was evaporated on a hot water bath, not more than 800ml; cooled and alcohol was added as a preservative to the concentrated percolate. Preliminary phytochemical screening of the extract gave positive test for proteins, carbohydrates, volatile oils, fatty acids, flavonoids, tannins, saponins, sterols and triterpenoids.

Pharmacological activity

Experimental animals

Albino wistar rats of either sex (150-200g weight) were procured from Venkateshwara Enterprises, Bangalore. The animals were divided into three groups comprising of six animals in each group. They were kept in polypropylene cages at 23±1 C in 12:12h dark: light cycle, with free access to standard pellet feed (Chakan Oil Mill, India) and water *ad libitum*. This project was cleared by Institutional Animal Ethical committee.

Acute Oral Toxicity study

Swiss albino mice of either sex (18-22g weight) were used for acute oral toxicity study. The study was carried out as per the guidelines set by OECD[7] and up to 2000mg/kg b.w. of extract there were no signs and symptoms seen when the animals monitored for 48h. Based on the results obtained from this study, the dose for wound healing activity was fixed to be 200mg/kg b.w. and 400mg/kg for dose dependent study.

WOUND HEALING ACTIVITY

Animals were divided into three groups (n=6). They were starved for 12h prior to initiate the experiment. Group I served as Control, which received normal saline. Group II and III served as test, received aqueous extract at dose of 200mg/kg and 400mg/kg b.w. respectively.

Excision wound model

An impression was made on the dorsal thoracic region 1cm away from vertebral column and 5cm away from ear using a round seal of 2.5cm diameter on the anaesthetized rat. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2 diameters. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Contractions, which contribute for wound closure in the first two weeks, were studied by tracing the wound on a transparency paper initially. Then an impression was taken on a millimeter scale graph paper, scar area after complete epithelization and time for complete epithelization in days was evaluated to calculate the degree of wound healing[8].

Incision wound model

In the incision model[9], the rats were anesthetized by anesthetic ether and two longitudinal paravertebral incisions of 6cm length were made through the skin and cutaneous muscle at a distance of about 1.5cm from the midline on each side of the depilated back. After the incision, the parted skin was sutured 1cm apart using a surgical thread (No. 000) and curved needle (No. 11). The wounds were left undressed. The extracts were given by oral route once a day, till complete healing. The sutures were removed on eighth post-wound day. The skin-breaking strength of the 10-day-old wounds was measured by the method of Lee[10].

Dead Space Wound Model

In this model the physical and mechanical changes in the granuloma tissue were studied. Under light ether anesthesia the hairs in the axilla and groin were clipped out and a subcutaneous dead space wound were inflicted in the same region, by making a pouch through a small nick in the skin. Granuloma formation was induced by implanting both sterile cotton pellets and grass piths. Two sterile cotton pellets weighing 10g (sterilized by autoclaving) were used to grow granuloma by the technique as described by, but the granulomas were removed on 10^{th} day. As described above two cylindrical grass piths measuring (25x3mm) were also introduced in the subcutaneous pouch in each animal. The sutured wounds were mopped with an alcoholic swab and animals were placed into their individual cages. The granuloma was excised from the surrounding tissue on 10^{th} post-wounding day under light ether anesthesia. Cotton pellet granuloma excised from dead space wounds were dried overnight 60°C so as to obtain constant dry weight. Their weights were expressed as mg/100g body weight as suggested by Dispaquale and Meli[11].

Granuloma surrounding grass piths were excised and slit opened by a longitudinal incision in one plane so as to obtain rectangular strips. The breaking strength of a strip of granuloma measuring about 15mm in length and 8mm in width (obtained by trimming the rectangular strip

of granuloma tissue) was measured employing the method described under incision wounds.

Collection of granulation tissue

Granulation tissues from both control and treated rats were collected, washed well in cold saline (0.9% NaCl) to remove blood tissues and stored for various parameters. Granulation tissues were lyophilized for collagen and hexose amine analysis.

Collection of Blood sample

Blood sample were collected by cervical decapitation and sterile syringe rinsed with EDTA was used to collect blood. Plasma was separated for malondialdehyde and ascorbic acid estimation.

Biochemical Parameters

L- Hydroxy Proline[12]

Samples of varying concentrations were taken for analysis. Hydroxyl proline was oxidized by adding 1ml of Chloramine T to each tube. The contents were mixed thoroughly by shaking and allowed to stand for 20min at room temperature. Adding 1ml of 70% perchloric acid to each tube then destroyed the Chloramine T. The contents were mixed and allowed to stand for 5min. finally 1ml of PDAB (Para Dimethyl Amino Benzaldehyde) solution was added and the mixture was shaken well. The color developed was read spectrophotometrically at 557 nm. The collagen content was then calculated by multiplying the hydroxyl proline content by the factor 7.46 and was expressed as mg/100 mg of dry weight of the sample.

Hexose Amine[13]

Samples of varying concentrations were taken for analysis. The solutions were treated with 1ml of freshly prepared 2% acetylacetone in 0.5M Na_2C0_3 in capped tubes and kept in boiling water bath for 15min. After cooling in tap water, 5ml of 95% ethanol and 1ml Ehrlich's reagent were added and mixed thoroughly. The purple red color developed was read after 30min at 530nm.

Malondialdehyde[14]

To 0.1ml of sample 0.9ml of 10% TCA and 2ml of 0.67% thiobarbituric acid reagent were added and kept in boiling water bath for 20min. The tubes were cooled after centrifugation and the absorbance of the supernatant was read at 532nm.

Ascorbic acid[15]

To 0.5ml of plasma, 0.5ml of ice cold 10% TCA was added and mixed thoroughly and centrifuged for 20min. At 3500g, supernatant (0.5ml) was mixed with 0.1ml of DTC reagent mixed well and incubated at 37^0C for 3h. Then 0.75ml of ice cold 65% H_2S0_4 was allowed to stand at room temperature for 30min. The yellow color developed was read at 520 nm. Ascorbic acid was used as standard.

Histopathology

A section of granuloma tissue was subjected to histopathological examination so as to determine the pattern of lay-down for collagen using two special strains i.e. Van Gieson and Masson Trichome[16].

Statistical Analysis

Results are expressed as Mean ± S.D. The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The results were considered statistically significant when $P<0.0001$.

RESULTS

In the present investigation, preliminary phytochemical analysis of aqueous extract revealed the presence of flavonoids, tannins, saponins, sterols, carbohydrates, proteins and triterpenoids. The effects of the aqueous extracts of *Ocimum kilimandscharicumi* leaves on various wound healing parameters using different wound models have been shown in Tables 1, 2, 3 and 4.

In excision wound model, the percentage closure of wound area was significantly increased by the curative effect of both the doses (200mg/kg b.w. and 400m/kg b.w.) by decreasing the time of epithelization period (21.50±0.4282, $P<0.0001$ and 21.00±0.3651, $P<0.0001$ respectively) as evidenced by shorter period for fall of escher as compared to control. The drug extract also facilitated the rate of wound contraction significantly at both the dose levels from 4^{th} day to 20^{th} day. The extract at both the doses (200mg/kg b.w. and 400m/kg b.w. respectively) showed significant decrease in mean scar area (31.00±0.5774, $P<0.0001$ and 27.83±0.6009, $P<0.0001$ respectively) as compared to control (40.83±0.7923) as shown in Table 1.

In incision wound model, extract at both the doses (200mg/kg b.w. and 400m/kg b.w.) showed highly significant (428.3±7.010, $P<0.0001$ and 470.0±8.376, $P<0.0001$ respectively) in breaking strength when compared to control (299.3±6.987) as shown in Table 2.

In studies using dead space wound model, the extract at both the doses (200mg/kg b.w. and 400m/kg b.w.) showed highly significant increase in breaking strength (262.4±7.776, $P<0.0001$ and 303.8±4.968, $P<0.0001$ respectively) and dry weight of granulation tissue (44.28±1.195, $P<0.0001$ and 50.92±1.046, $P<0.0001$ respectively) as compared to control (Table 3).

In biochemical parameters studies, extract at both the doses have shown highly significant results when tested for L-Hydroxy proline, Hexose amine, Malondialdehyde and Ascorbic acid contents when compared to control group (Table 4).

Histopathological studies of granulation tissue of control group showed accumulation of more macrophages and very few collagen fibres (Fig.3 and 4). Aqueous extract at 400mg/kg b.w. dose evidenced very few macrophages and enhanced collagen deposition (Fig. 1 and 2).

DISCUSSION

Wound healing involves various phases. Initially involves acute inflammatory phase followed by the synthesis of collagen and other extra cellular macromolecules, which are later removed to form a scar[17]. Drugs, which influence one phase, may not

necessarily influence another. Hence different models have been used in our study to assess the effect of various phases, which run concurrently, but independent of each other.

The results of present study showed that the aqueous extract of leaves of *Ocimum kilimandscharicum* possesses a definite pro-healing action. This is demonstrated by a significant increase in the rate of wound contraction and by enhanced epithelization.

Significant increase was also observed in skin breaking strength and hydroxyproline content which was a reflection of increased collagen levels by increased cross linking of collagen fibres. In addition, increase in dry granulation tissue weight indicated the presence of higher protein content[18]. The breakdown of collagen liberates free hydroxyl proline and its peptides and elevated level of hydroxyl proline is the index of increased collagen turnover and that was further supported by histological evidence and gain in granuloma breaking strength.

Ascorbic acid is reported to have free radical scavenging activities and inhibition of lipid peroxidation. In the present study, it was found that the ascorbic acid levels was higher in the test group when compared to the control group and hence a decline in the lipid peroxidation.

Hexose amine content increases in the early stages of wound healing and indicated that the fibroblasts actively synthesized, ground substances (mucopolysaccharides) on which the collagen can be laid on[19]. This pattern of hexose amine increase was found among the treated rats in this present study.

The cytokine cascade activated after an injury with stimulation of phagocytic cells that result in the formation of oxygen free radicals and lipid peroxidation. The control group showed an elevation in the lipid peroxidation levels which indicates the decreased scavenging capacity of the wounded tissues. Lipid peroxidation is oxidative deterioration of PUFA. It leads to cell injury leading to generation of peroxides and lipid peroxides.

Phytochemical screening revealed the presence of tannins, flavonoids, proteins and other important constituents. Flavonoids have been documented[20] to possess potent antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing. Thus, the enhanced wound healing may be due to free radical scavenging action and the antibacterial property of the phytoconstituents present in it which either due to their individual or additive effect fastens the process of wound healing.

Table 1: Effect of aqueous extract of *Ocimum kilimandscharicumi* on excision wound model

Wound model		**Control**	**AqE 200#**	**AqE 400^**
Wound closure (days)		25.17±1.014	21.50±0.4282**	21.00±0.3651**
Mean scar area(mm^2)		40.83±0.7923	31.00±0.5774***	27.83±0.6009***
%wound contraction by day				
	4th	473.4±2.529 (5.32)	427.6±3.636*** (14.48)	394.3±5.039*** (21.14)
	8th	358.5±4.700 (28.30)	295.5±2.971*** (40.90)	252.0±3.431*** (49.60)
	12th	239.7±3.031 (52.06)	165.7±2.468*** (66.86)	128.9±2.912*** (74.22)
	16th	118.8±3.605 (76.24)	65.97±3.010*** (86.80)	24.93±1.973*** (95.01)
	20th	33.23±1.858 (93.35)	19.75±1.316*** (96.05)	11.05±0.6682*** (97.79)

AqE 200#: Aqueous extract 200mg/kg b.w., AqE400^: Aqueous extract 400mg/kg b.w.
(Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test, *P* values: ***P<0.001 considered as significant when compared to control

Table 2: Effect of aqueous extract of *Ocimum kilimandscharicumi* on healing of incision wound

Group(N)	Breaking strength (g)
Control	299.3±6.987
AqE 200#	428.3±7.010***
AqE 400^	470.0±8.376***

AqE 200#: Aqueous extract 200mg/kg b.w., AqE400^: Aqueous extract 400mg/kg b.w.
(Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test, *P* values: ***P<0.001 considered as significant when compared to control

Table 3: Effect of aqueous extract of *Ocimum kilimandscharicumi* on healing of dead-space wound

Groups	Breaking Strength (g)	Granulation tissue Dry Weight (mg/100g)
Control	217.6±2.728	35.43±1.056
AqE 200#	262.4±7.776***	44.28±1.195***
AqE 400^	303.8±4.968***	50.92±1.046***

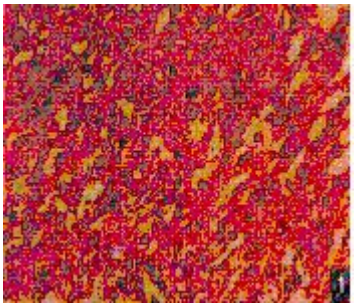
AqE 200#: Aqueous extract 200mg/kg b.w., AqE400^: Aqueous extract 400mg/kg b.w.
(Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test, *P* values: ***P<0.001 considered as significant when compared to control

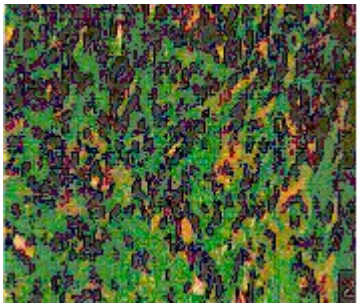
Table 4: Biochemical Parameters

Groups	L-Hydroxy Proline	Hexose Amine	Malondialdehyde (Lipid Peroxide)	Ascorbic acid
Control	0.1442±0.003070	0.07017±0.003719	7.102±0.06814	6.950±0.04797
AqE200#	0.1880±0.004336***	0.1212±0.004012***	4.776±0.1042***	7.371±0.1079**
AqE400^	0.2100±0.004604***	0.1540±0.003950***	3.526±0.1095***	9.049±0.05130***

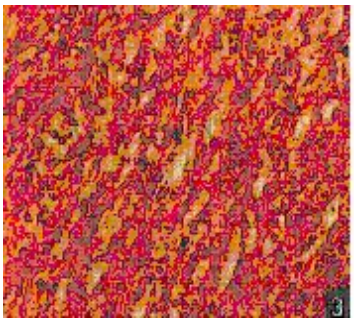
AqE 200#: Aqueous extract 200mg/kg b.w., AqE400^: Aqueous extract 400mg/kg b.w.
(Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test, *P* values: ***P<0.001 considered as significant when compared to control

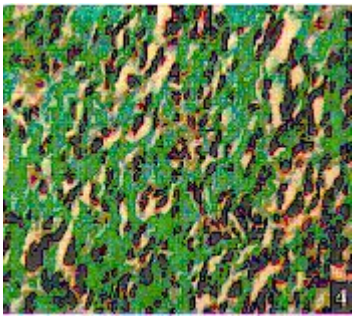
Fig: 1-4 Comparative Histopathological study of control group and treated group





Figs 1 and 2: Histological section of granulation tissue of the AqE 400 treated group showing few macrophages and increased collagen deposition (100X)





Figs 3 and 4: Histological section of granulation tissue of the control group showing more macrophages and less collagen deposition (100X)

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