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WOUND HEALING ACTIVITY OF THE CRUDE EXTRACTS OF THE PLANT *LEUCAS ASPERA*

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Abstract:

The wound healing effect of different extracts of Leucas aspera was studied. Three wound models viz. incision, excision and dead space wounds were used in this study. Parameters studied were breaking strength in case of incision wounds, epithelialization and wound contraction in case of excision wound and granulation tissue dry weight, breaking strength and hydroxyproline content in case of dead space wound. Extracts of *Leucas aspera* exhibited wound healing activity in all three models used in rats, but the significant and maximum wound healing activity was produced by petroleum ether extract of plant *Leucas aspera*.

Key words: *Leucas aspera*, wound healing activity, Incision wound, Excision wound, Dead space wound

1. INTRODUCTION

Leucas aspera belonging to the family Labiate is used as anti-inflammatory, stimulant, in jaundice, cough, asthma, conjunctivitis, diabetes, malaria, headache, otalgia, skin diseases, snake bite, toothache, and wound healing etc.[1] Leucas aspera is studied for anti-inflammatory activity[2,3,4],analgesic activity[5], cobra venom induced mortality in mice[6], anti-parasitic activity[7], antibacterial activity against Micrococcus pyrogenes, V.aureus and Escherichia Coli[8], toxic to the filarial vector mosquito[9], antinociceptive, antioxidant and cytotoxic activity[10]. Preliminary chemical examination of entire plants of L. aspera revealed presence of triterpenoids[11], contains oleanolic acid, ursolic acid and 3-sitosterol[12], and aerial parts are reported to contain nicotine, sterols, two new alkaloids (compound A m.p. 61.2°, α-sitosterol and β-sitosterol m.p. 183.4^{o}),

reducing sugars (galactose), glucoside (230.1^{o})[13]. The present study was undertaken to verify the claim and evaluate the wound healing activity of the plant Leucas aspera.

2. EXPERIMENTAL

2.1 Plant Material: The plants of Leucas aspera were collected from local areas around the Mangalore, Karnataka, India, and after authentication by botanist separate voucher specimen (NIMS/2010/NLA) is being maintained in laboratory of Phytochemistry and Pharmacognosy, NIMS Institute of Pharmacy, Shobha Nagar, Jaipur, India, for the plant Leucas aspera. Whole plants then, including root, stem, leaves and flower were shade dried and chopped into small pieces separately.

2.2 Preparation of extracts

The shade dried plants were powdered (300g) and extracted by successive extraction method and 3 different extracts (Petroleum ether, ethanol and aqueous) were obtained. Extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50^{0} C) using flash evaporator.

2.3 Preliminary phytochemical investigation

Preliminary phytochemical investigation of petroleum ether extract, ethanolic extract, and aqueous extract of the plant materials was carried out for qualitative determination of the groups of organic compounds present in them, by using different tests for Alkaloids, Carbohydrates, Proteins, Steroids etc.[14]

2.4 Acute oral toxicity study

A preliminary pharmacological study was conducted to assess the acute pharmacological effects and LD50 of the drug extract. The acute oral toxicity study was done by 'Up-and- Down' method in healthy adult female albino rats according to CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) recommended 'OECD' guideline 425.[15]

Healthy adult female albino rats (200-250 g body weight) were randomly selected and kept in their cages for 5 days prior to dosing to allow for acclimatization to the laboratory conditions. The animals were fasted over-night prior to dosing. Following the periods of fasting, rats were weighed and divided into different groups, each consisting of 6 animals. The different drug extracts (prepared as aqueous suspension in 0.6% w/v sodium CMC) were administered in a single dose by gavage using a stomach tube, at a fixed dose levels of 175, 550

and 2000 mg/kg (Note: according to OECD guideline 425, a starting dose level of 175 mg/kg can be used, if no estimate of the substance's lethality is available) to different groups of animals.

Following the dosing, the animals were observed individually once during the first 30 minutes, periodically during the first 24 hours (with special attention given during the first 4 hours) and daily thereafter, for a total of 14 days. The parameters observed during study period include ANS profiles (e.g. heart rate, salivation, respiration, pupil size diuresis, righting reflex and diarrhoea), CNS profiles (e.g. spontaneous motor activity, sedation, sedation, convulsion (tremors), body weight and deaths. Observations were tabulated and the LD50 value was determined by Karber's method.[16]

2.5 Test animals

Male wistar albino rats (160 – 200 g) were used in the experiment. Animals maintained under standard environmental conditions, were fed with a standard diet (Hindustan Lever, India) and water ad libitum. The animals were fasted for 16h before experimentation but allowed free access to water. Institutional animal Ethics Committee's permission was obtained before starting the experiments on animal.

The acute oral toxicity study was done by 'Up-and- Down' method in healthy adult female albino rats according to CPCSEA recommended 'OECD' guideline 425. There were no changes from dose level of 175 mg/kg. p.o, to 2000 mg/kg, p.o. Drug extracts did not cause any death upto 2000 mg/kg. The LD_{50} calculated is 2000 mg/kg for all the extracts, so one tenth of the maximum tested dose (i.e. 200 mg/kg, p.o.) was selected for each extract for the evaluation of the wound healing activity.

2.5.1 Effect of Leucas aspera extracts on wound healing activity

The Albino wistar rats of either sex (150-200g weight) were starved for 12 h prior to wounding. The rats were divided into four groups (n = 6). Animals were depilated at the dorsal thoracic region before wounding. Group I served as control similarly group II, III and IV received, petroleum ether, alcoholic and aqueous extract by oral route at a dose of 200 mg/kg body weight by oral route daily for 10 consecutive days in incision and dead space wound model and for 20 days in the excision wound model.[17].

2.5.1.1 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 millimetre scale graph paper, scar area after complete epithelization and time for complete epithelization in days was evaluated to calculate the degree of wound healing. The parameters studied were wound closure, epithelization time and scar features. The observation of the percentage wound closure were recorded on 4th , 8th, 12th, 16th and 20th post wounding day and also for epithelization and size and shape of scar area.[18]

2.5.1.2 Incision wound model

In this model the rats were anesthetized by anaesthetic 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. Sutures were removed on eighth post-wound day. Skin-breaking strength of 10-day-old wounds was measured by the method of Lee. [19]

2.5.1.3 Dead Space Wound model

For the dead space wound four groups of six animals each were used. Dead space wound was made by implantation of polypropylene tube (0.5cmX2.5cm), beneath the dorsal Para vertebral skin. On the 10th day the granuloma tissue form on the dead space wound was dissected and tensile strength was determined. The excess tissue was cut into two approximately equal halves. One of the granuloma tissues was dried in an oven at 60° C and the dry weight was noted. The granulation tissue so harvested was subjected to hydroxyproline estimation. [20]

2.6 Statistical analysis

All the results were analyzed by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The level of significance was set at $P<0.05$.

3. RESULTS

3.1 Preliminary phytochemical study

The % yield of petroleum ether, ethanolic and aqueous extracts was found to be 4.2, 6.1 and 8.3. Preliminary phytochemical screening of the crude extracts of the plant L. aspera showed the presence of steroids, alkaloids, glycosides, saponins, flavonoids, tannins and carbohydrates. Results are tabulated in table 1.

Table-1: Phytochemical screening of different extracts of plant L. *aspera.*

Extracts	Steroids	Alkaloids	Glycosides	Saponins	Flavonoids	Tannins	Carbohydrates
Petroleum ether	+	+	-	+	+	-	-
Ethanol	+	+	+	+	+	+	+
Aqueous	-	-	+	-	+	+	+

(+) = present; (-) = absent

3.2 Toxicological study

The acute oral toxicity study was done by 'Up-and- Down' method in healthy adult female albino rats according to CPCSEA recommended 'OECD' guideline 425. There were no changes from dose level of 175 mg/kg. p.o, to 2000 mg/kg, p.o. drug extracts did not cause any death upto 2000 mg/kg. The LD_{50} calculated is 2000 mg/kg for all extracts as per the calculation given in the table 2. So, 1/10 of the LD_{50} was selected as an oral dose.

Table-2: LD_{50} determination of different extracts of plant L. *aspera.*

Group	Dose (mg/kg)	Dose difference (a)	Dead	Mean mortality(b)	(a × b)	Σ (a ×b)	LD_{50} = $Dose_{(max)}$- [Σ (a ×b)/No of animals]
1	175	-	00	-	-	0	2000 mg/kg
2	550	375	00	-	-		
3	2000	1450	00	-	-		

3.3 Acute oral toxicity study

The acute oral toxicity study was conducted to assess acute pharmacological effects and showed no changes in autonomic profiles (writhing, heart rate, defecation, light reflex, etc), neurological response (abdominal tone, twitching, grip strength, limb tone, etc.) and behavioral response (alertness, irritability, fearfulness, touch response, etc.). The results are tabulated in the table 3.

Table 3: Acute oral toxicity study of different extracts of plant L. *aspera*

	Alertness	Sterotype	Irritability	Fearfulness	Touch response	Pain response	Spontaneous activity	Grooming	Restlessness	Righting reflex	Limb tone	Grip strength	Twitching	Abdominal tone	Pinna reflex	Corneal reflex	Tremors	Convulsions	Writhing	Defecation	Urination	Heart rate	Respiratory	Pupil size	Skin colour
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Petroleum ether extract	**N**	**N**	-	-	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**
Ethanolic extract	**N**	**N**	-	-	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**
Aqueous Extract	**N**	**N**	-	-	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**	-	-	**N**	**N**	**N**	**N**	**N**	**N**	**N**

(-) = not present; (N) = normal; (+) = present

3.4 Wound healing Activity

In an excision wound model, petroleum ether extract of Leucas aspera showed significant wound healing activity on 20^{th} day compared to control. It also showed complete epithelization. When compared to control petroleum ether extract showed a significant reduction in the scar area as depicted in Table 4. In incision study, the petroleum ether extract showed significant breaking strength when compared to control as given in Table 5. Petroleum ether extract also showed significant increase in breaking strength, dry weight of granulation tissue and hydroxyproline content when compared to control as illustrated in Table 6. Histological studies of the tissue obtained from the petroleum ether and ethanolic extract treated group showed significant increase in collagen deposition, few macrophages, tissue oedema and more fibroblasts. The wound healing was more significant in petroleum ether treated group of animals.

Table-4: Effect of oral administration of L. aspera extract (Excision wound).

GROUP	Dose	Excision wound						
		% Wound Contraction					Mean size of scar area mm^2	Period of epithelization (days)
		4th day	8th day	12th day	16th day	20th day		
I	Control	12.46 ± 0.623	24.62 ± 0.241	39.15 ± 0.514	68.83 ± 0.301	78.64 ± 0.398	16.2 ± 0.964	23.48 ± 0.265
II	200 mg/kg of LAPE	18.24 ± 0.502*	46.25 ± 0.564*	68.23 ± 0.826*	86.53 ± 0.619*	98.52 ± 0.204*	9.89 ± 0.716*	17.89 ± 0.314*
III	200 mg/kg of LAEE	16.22 ± 0.192	31.26 ± 0.865	57.30 ± 0.524	77.45 ± 0.816	87.43± 0.186	12.30 ± 0.462	19.80 ± 0.213
IV	200 mg/kg of LAAE	14.40 ± 0.173	26.85 ± 0.640	51.91 ± 0.794	72.68 ± 0.858	83.81 ± 0.598	13.10 ± 0.511	21.19 ± 0.213

***** indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analysed by One-way ANOVA followed Dunnett's test.

Table-5: Effect of oral administration of extracts of L. aspera (Incision wound).

Group	Dose	Wound breaking strength
I	Control	138.62± 7.80
II	Rats treated with 200 mg/kg of LAPE	289.92±4.94*
III	Rats treated with 200 mg/kg of LAEE	248.18±7.10*
IV	Rats treated with 200 mg/kg of LAAE	171.53±5.75

* indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analysed by One-way ANOVA followed Dunnett's test.

Table-6: Effect of oral administration of extracts of L. aspera (Dead space).

Group	Dose	Breaking strength (g)	Granulation tissue dry weight (mg/100g)	Hydroxy-proline (µg/100mg)
I	Control	198.15±6.406	46.86 ± 1.79	1278 ± 12.44
II	Rats treated with 200 mg/kg of LAPE	374.23± 7.543*	75.78 ± 1.17*	2342 ± 19.95*
III	Rats treated with 200 mg/kg of LAEE	294.43± 4.641	64.32 ± 1.14	1887 ± 15.32
IV	Rats treated with 200 mg/kg of LAAE	274.34± 5.235	51.24 ± 1.92	1335 ± 13.46

* indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analysed by One-way ANOVA followed Dunnett's test.

LAPE:- Leucas aspera petroleum ether extract

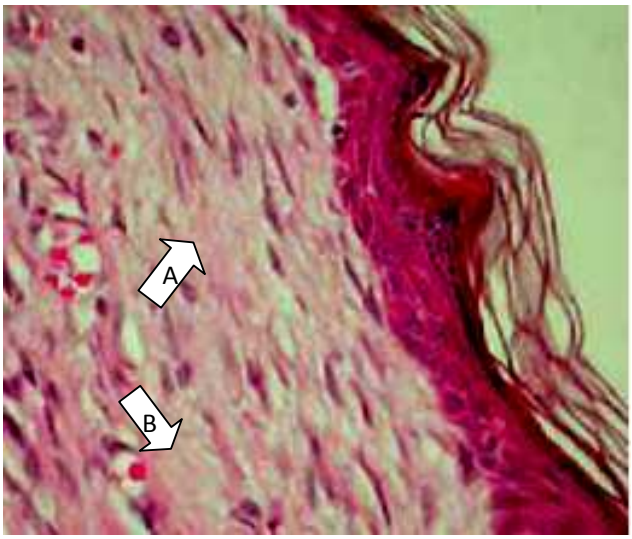
LAEE:- Leucas aspera alcoholic extract

LAAE:- Leucas aspera aqueous extract

4. DISCUSSION

The collagen composed of amino acid (hydroxyproline) is the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of the hydroxyproline could be used as an index for collagen turnover. The data depicted in table 6 showed that the hydroxyproline content of the granulation tissue of the animals treated with petroleum ether extract of Leucas aspera was significantly increased when compared to the control indicating increased collagen turnover. In addition, increase in dry tissue weight also indicated the presence of higher protein content. The extract of Leucas aspera contains flavanoids and triterpenoids. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, preventing the cell damage and by promoting the DNA synthesis. Flavonoids[21], triterpenoids[22] are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property (Leucas aspera has antimicrobial activity[8]), these factors seems to be responsible for wound contraction and increased rate of epithelialisation.

Figure 1:- Histology of the Granulation tissue of Leucas aspera petroleum ether treated animal showing more collagen and less macrophages and a well formed epidermis with hair follicle formation in the dermis.



A Macrophages, B collagen fibres

5. CONCLUSION

The data obtained from this study indicates the non toxic nature of plant, the acute toxicity study revealed non toxic nature of the drug. The extracts of the plant Leucas aspera are capable of exhibiting significant wound-healing activity. However further investigations are required to isolate active constituents responsible for these activities and to elucidate exact mechanisms of action.

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