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Antimicrobial, antioxidant and wound healing properties of *Leucas lanata* Wall. ex Benth

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Abstract

Leucas lanata Wall. ex Benth. (Lamiaceae) is an important plant commonly known as Biskapra or Gumma and used to treat many ailments by traditional healers and local peoples. This study was designed to evaluate wound healing potential of *Leucas lanata* through the excision wound model and functional changes in biochemical indicators of antioxidant parameters. This study also investigated the antimicrobial as well as antioxidant activity of *L. lanata*. The 50% EtOH extract of *L. lanata* was found to contain 0.74% of phenolic and 0.21% of flavonoid content. The IC5O value was 122.56 µg/ml and reducing power increased with the increasing concentrations. The antimicrobial activity of the extract was more effective against bacterial strains compared to fungal strains. Remarkable wound healing activity was observed with the 10% (w/w) ointment of *L. lanata* 50% EtOH extract. In the study of uninfected wounds, epithelization period was reduced from 24.66±0.97 for the control group treated with blank ointment to 12.16±0.36 for the group treated with 10% LLEE ointment. Similarly, in case of infected wounds with Staphylococcus epidermidis, the percentage of wound contraction was significantly enhanced. Both doses of extract significantly increased superoxide dismutase, catalase, reduced glutathione when compared with the control group of infected and uninfected wound. HPLC analysis showed the presence of gallic, protocatechuic, chlorogenic, caffeic and ferulic acids. These compounds have important biological activities and responsible for antimicrobial and wound healing activity. The study provided sufficient evidences that, *L. lanata* might be indeed potential sources to treat many diseases.

Keywords: *Leucas lanata*, Soxhlet extraction, Antimicrobial, Wound healing, Antioxidant.

Introduction

Medicinal Plant has long been a very important source of drug and many have been screened if they contain compounds with therapeutic activity.[1] Many of these isolations were based on the uses of the medicinal plant by traditional healers.[2] The medicinal value of these plants lies in their secondary metabolites, which create a specific physiological action on the human body. The most important bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds.[3] Plant-based antioxidants are now preferred to the synthetic ones because of safety concerns.[4] These factors have stimulated the widespread screening of plants for possible medicinal, antimicrobial and antioxidant properties.[5]

Wounds are simply physical injuries that results loss of cellular and functional continuity of living tissue. It can be caused by physical, chemical, microbial, immunological insults typically associated with the loss function.[6] Wound healing is an interaction of a complex cascade of cellular and biochemical actions, healing to the restoration of structural and functional integrity with regaining the strength of injured tissues. Therefore, the aim of treating a wound is either to shorten the time required for healing or to accelerate the wound healing process. Although the process of wound healing is natural, an infection can delay healing.[7] Medicinal plants have been used for the treatment of various dermatological ailments, especially cuts, wounds and burns.[8, 9] Some of them owe their direct effect on the wound healing process and some to their antioxidant, anti-inflammatory and antimicrobial properties. Antimicrobial therapy of wound care mainly controls further microbial contamination, colonization and consequent proliferation which promoting the healing of wounds.[10]

Leucas lanata Wall. ex Benth. (*Lamiaceae*) is vernacularly known as Biskapra or Gumma grows generally on dry slopes among the grasses in the Himalayas and the hills of South India at an altitude range of 700 m – 3,000 m. Ethnomedicinally, the juice of the whole plant has been traditionally used by local peoples to treat stomach-ache[11], headache[12], whooping cough[13] and as an antidote for reptile poison[14]. Leaves and flowers with cold water or milk are also used in cold, cough and dysentery.[15] Fresh leaves are placed on the affected area for absorbing pus[16] and applied externally for wound healing in the

form of paste[17]. This plant also reported to have antibacterial[18], free radical scavenging and antiepileptic[19] and antiparkinson activities[20].

However, the medicinal values of this plant pertaining to antimicrobial, antioxidant and wound healing activities have not yet been reported. This study was designed to explore the healing effects of topically applied 50% EtOH extract of *L. lanata* in Sprague Dawley rats and functional changes in biochemical indicators in antioxidant parameters.

Materials and Methods

Chemicals and microorganisms

Sodium acetate, potassium chloride, 2-diphenyl-1- picrylhydrazyl-hydrate (DPPH), Folin–Ciocalteu reagent, catechol, beta carotene, Quercetin, Gallic acid, Tween-20, sodium carbonate, sodium hydroxide, acetone, butanol, chloroform, ethanol and aluminium chloride were obtained from Merck India, Mumbai. For HPLC method Gallic, Protocatechuic, caffeic, ferulic acids and electronic-grade methanol and acetonitrile were procured from Sigma-Aldrich company. Whatman No.1 filter paper and disc were used for filtration of the samples and antimicrobial assay respectively. Microorganisms such as *Salmonella enterica* ser. *typhi* (MTCC-733), *Salmonella enterica* subsp. *enterica* ser. *typhimurium* (MTCC-3224), *Staphylococcus epidermidis* (MTCC-3382), *Aspergillus fumigatus* (MTCC-10561), *Candida krusei* (MTCC-9215) were obtained from the Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh. Various Media for analysis were purchased from Hi-media laboratories Pvt. Ltd., Mumbai. All the chemicals used for analysis were of analytical grade.

Collection and authentication of plant

The whole plant of the *L. lanata* was collected from the local areas of Pachmarhi, Hoshangabad, Madhya Pradesh, India in the month of September, 2014. For future reference, voucher specimens (Collection No.:260612) were deposited in the LWG herbarium, CSIR-NBRI, Lucknow.

Extraction and phytochemical screening

The material was dried and pulverized to a coarse powder. The powdered material was passed through a 40 mesh sieve and extracted with of 50% (v/v) EtOH at 38^0C on a water bath using Soxhlet extractor for 24 h. The extract was filtered and concentrated under reduced pressure in a rotavapour (Buchi R-200 USA) below 40^0C. The resulting crude extract was then stored at 4^0C until further analysis of wound healing and antimicrobial activity. Phytochemical studies were carried out on the 50% EtOH extract of *L. Lanata*[21, 22] and it revealed the presence of tannins, phenolics, saponins, flavanoids, terpenoids and carbohydrates. The percentage yield of the extract was found to be 10.5% w/w.

A stock solution of 1mg/ml plant extract (using extract solvent) was prepared. 0.5ml stock solution was estimated for total flavonoid and phenolic content according to the Woisky and Salatino[23] and Folin–Ciocalteu method[24] respectively.

2, 2'-diphenyl-1-picrylhydrazyl (DPPH˚) radical scavenging assay

The capacity to scavenge the stable free radical DPPH was evaluated according to the method of Blois[25] with some modifications. Briefly, various concentrations (200-50µg/mL) of extract were prepared using 50% EtOH and mixed with equal volume of 50% EtOH solution containing DPPH radicals (0.135mM). The mixture was shaken vigorously and left to react in the dark for 30 minutes at room temperature (until stable absorption values were obtained). The reduction of the DPPH radical was determined the absorption at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\% \text{ RSA} = \frac{[\text{Absorbance of DPPH} - \text{Absorbance of test Sample}]}{\text{Absorbance of DPPH}} \times 100$$

The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid was used as standard.

Reducing power assay

The reducing power of the sample was determined by the method of Oyaizu[26] with some modifications. An aliquot of the sample (1.0 mL) at various concentrations (50-200 µg/mL) was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm using an appropriate blank; higher absorbance indicates higher reducing power. Measurements were done in triplicates.

Antimicrobial Assay

Agar disc diffusion method was performed to evaluate the antimicrobial activity of plant extract.[27] Briefly, all concentrations of 50% EtOH extract were prepared using dimethyl sulphoxide (DMSO). For the inoculums (10^8 cfu/mL), test bacteria and fungi were grown in sterile Muller–Hinton broth and Sabouraud dextrose broth tubes respectively overnight. The inoculums of bacteria and fungi were aseptically plated using sterile cotton swabs, into petri dishes with Muller–Hinton agar and Sabouraud dextrose agar respectively. The filter paper disc was impregnated with the different concentration to obtain 200, 150, 100, 50 µg/disc samples and placed on the prepared agar surface. The petri dishes were pre-incubated at room temperature, allowing the complete diffusion of the samples and incubated at 37°C for 24 hours (for bacteria) and 48 hours (for fungi). Tetracycline and nystatin were used as standard antibacterial and antifungal antibiotics respectively. The experiments were performed in triplicate. After incubation the inhibitory potential of the extract was quantified by measuring the diameter of the zone of inhibition in mm. Antimicrobial activity was assessed using the parameters according to Quinto & Santos[28]: inhibition zone <10 mm, inactive; 10-13 mm, partially active; 14-19 mm, active; >19 mm, very active.

Excision wound model

Experimental animals

Sprague Dawley rats (120-180 g) of either sex were taken from the animal house of the National Laboratory Animal Centre, Lucknow, India. All experiments were performed according to the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals[29] approved by the Institutional Committee for Ethical use of Animals and Review Board (CPCSEA/IAEC/7-18). Two types of formulations with different concentrations 5 and 10% (w/w) of 50% EtOH of *L. lanata* extract was prepared in simple ointment base (USP) by the tituration method in a ceramic mortar and pestle.[30] Standard (betadine ointment) was compared with the formulations to check the wound healing potential.

Wound without Infection

The animals were divided into four groups of six animals each and anaesthetized with slight vapour inhalation of anaesthetic ether in anaesthesia chamber. The dorsal surface of animals was shaved and full skin thickness was excised from the sterile dorsal marked area to get a wound measuring about 8 mm diameter. The animals were placed singly in individual cages. The wound was left undressed to the open environment. Wounds were left open and the ointment was applied topically twice a day (once in the morning and evening) onto

each rat till the wound was completely healed.[31] Then the treatment was started in the following manner to the different groups:

Group I: Test group with 5% w/w ointment of LLEE

Group II: Test group with 10% w/w ointment of LLEE

Group III: Test group with standard drug ointment (Betadin)

Group IV: No treatment and served as controlled.

Wound with Infection

The methodology used for wound formation and treatment was the same as in without the infection model. The wound of each animal was inoculated with an overnight (18 h old) *S. epidermidis* culture and it were left for 24 h to set the infection in and then the treatment was started, till the wound was completely healed.

Parameters evaluated for excision wound healing

Measurement of wound contraction: The wound area was measured with a translucent paper and traced on every 3rd day. Wound contraction was expressed as percentage of the reduction in wound size.[32]

Percentage of wound contraction = [(Initial wound area – Specific day wound area) / Initial wound area] x 100

Epithelialization period: Number of days required for falling of the eschar without any residual raw wound gave the period of epithelization. It was measured in days from wounding day (day zero) till the full epithelialization.

Antioxidant parameters

After day 12 post wounding, Superoxide dismutase (SOD), Catalase (CAT) and reduced Glutathione (GSH) were estimated in wound tissue homogenate. Briefly, wound tissue were excised by using the same punch (8 mm diameter), which excised wounded area without contaminating it with normal skin. The tissue was collected in Phosphate buffered saline (PBS, pH 7) and sample preparation was done according to Shukla *et al.*[33]

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitro blue tetrazolium formazan. The activity of SOD was calculated in terms of units defined as the amount of SOD that inhibits nitro blue tetrazolium reduction by 50%.[33]

CAT measurement was done based on the ability of catalase to oxidize hydrogen peroxide. One unit (U) of catalase is the enzyme, which decomposes one mM of H_2O_2/min at 25°C.[34]

GSH activity in the homogenate was estimated using a fluorometric method in which GSH with ortho-phthalialdehyde (OPT) yields a highly fluorescent product at alkaline pH.[35]

HPLC studies

1mg/ml stock solution of 50% EtOH extract of *L. lanata* was prepared in 50% (v/v) methanol. Qualitative and quantitave analysis of sample extract and standard polyphenoles (Gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid) was performed by HPLC-UV.[36]

Statistical analysis

The statistical significance of the results were analyzed by one way analysis of variance (ANOVA) followed by Student–Newman–Keul's procedure. Experimental results concerning this study were mean ± (standard error mean) SEM of six parallel observations and $p<0.05$, $p<0.01$ and $p<0.001$ was considered as significant.

Results

After estimations of the 50% EtOH extract of *L. lanata* was found to contain 0.735±0.017% of total phenolic (Figure 1) and 0.21±0.020% of total flavonoid content (Figure 2). The IC_{50} is used for the interpretation of the results from the DPPH method and is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color). The IC_{5O} value for 50% hydro alcohol extract was 122.56 µg/ml. The IC_{50} value for Ascorbic acid was shown to be 42.23 µg /ml. The reducing power of 50% EtOH extract at various concentrations, increased with the increasing concentrations.

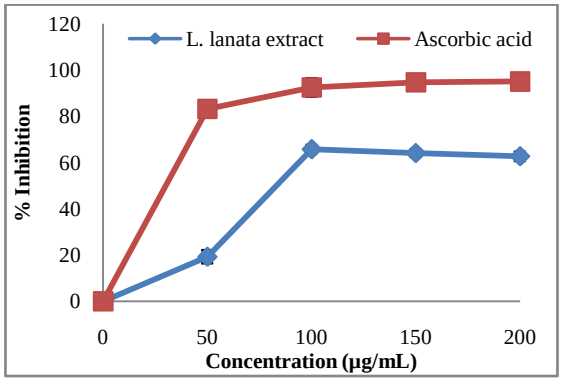


Figure 1: DPPH radical scavenging activity of 50% EtOH extract of *L. lanata*

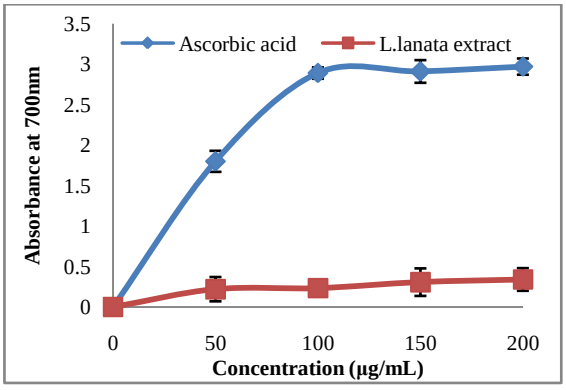


Figure 2: Reducing power of 50% EtOH extract of *L. lanata*

Antimicrobial assay

The result data pertaining to the antimicrobial potential of 50% EtOH extract of *L. lanata* were presented in table 01. A maximum zone of inhibition (20.91±0.69 mm) exhibited by 50% EtOH extract of *L. lanata* against Gram positive bacteria *Staphylococcus epidermidis* at 200µg/disc concentration, followed by Gram negative bacteria *Salmonella typhi* (20.56±0.37) and *Salmonella typhimurium* (19.76±0.37). In case of fungal strains, the highest concentration of the extract showed maximum activity against *Candida krusei* (17.82±0.72) in comparison of *Aspergillus fumigatus* (16.52±0.97) at 200µg/disc concentration. While lowest concentration (50µg/disc) showed inactive or partially active antimicrobial activity against all tested bacteria and fungi.

Table 1: Antimicrobial activity of *L. lanata* against different pathogens compared to standard antibiotics

Concentration	Inhibition Zone (mm)				
	Staphylococcus epidermidis	***Salmonella typhi***	***Salmonella typhimurium***	***Candida krusei***	***Aspergillus fumigatus***
LLEE 50 µg/disc	11.61±0.43[a]	9.15±0.81	10.31±0.27[a]	7.85±0.56	0.0± 0.0
LLEE 100 µg/disc	13.46±0.64[a]	13.35±0.48[b]	12.26±0.35[b]	10.43±0.81[a]	9.17±0.82
LLEE 150 µg/disc	17.37±0.82[c]	16.81±0.51[b]	15.34±0.51[b]	14.52±0.64[c]	14.15±0.45[a]
LLEE 200 µg/disc	20.91±0.69[b]	20.56±0.37[c]	19.76±0.37[c]	17.82±0.72[c]	16.52±0.97[b]
Tetracycline 20 µg/disc	30.16±0.11	22.56±0.09	21.06±0.13	0.0± 0.0	0.0± 0.0
Nystatin 20 µg/disc	0.0± 0.0	0.0± 0.0	0.0± 0.0	24.2±0.08	25.2±0.14

Values are expressed as mean ±SEM; n=3, One Way ANOVA followed by Student-Newmen-Keuls t-test; t-value denoted significance at a: P<0.05; b: P<0.01; c: P<0.001 respectively

Wound contraction studies

In the study of the excision wound model without infection, the rate of wound healing was higher. 50% EtOH extract of *L. lanata* showed significant dose dependent (5% and 10% w/w ointment, topically) wound healing potency. The studies revealed that, when compared to control (4.57±0.13) on day 15, 5% LLEE ointment (0.99±0.32) showed significant improvement (p<0.001), while 10% LLEE ointment and standard showed wounds full contraction (Table 2). It was observed that there is complete healing of the wounded area in different group of animals were clearly visualized in 12 to 15 days of the experimental period.

In the case of infected wounds, reduction of wound area of different treatment groups on the 18th day for the excision wound model was recorded. The studies revealed that, when compared to control (4.33±0.31) on day 15, 5% LLEE ointment (1.02±0.13), 10% LLEE ointment (0.23 ± 0.36) and standard (0.09 ± 0.36) showed significant improvement (p<0.05 to p<0.001) in wound contraction (Table 3). It was observed that there is complete healing of wound in the group of 10% (w/w) LLEE ointment and standard (Betadin) on the 15 to 18days. The study reveals that all the four groups of both infected and uninfected wound model showed decreased wound area from day to day (Figure 3).

Table 2: The effect of *L. lanata* ointment on the uninfesected excised wound in rats

Treatment group	Wound contraction (mm^2) (percentage wound closure in parenthesis)							Epithelization period (Days)
	0 Day	3rd Day	6th Day	9th Day	12th Day	15th Day	18th Day	
LLEE ointment (5% w/w)	8.74±0.37[a] (0.00)	7.14±0.30[b] (18.31)	5.68±0.38 (35.01)	4.03±0.27[c] (53.89)	2.59±0.24[b] (72.65)	0.99±0.32[c] (88.67)	0.00 (100)	16.08± 0.77
LLEE ointment (10% w/w)	8.53±0.40[b] (0.00)	6.29±0.23[b] (26.26)	4.54±0.28[c] (46.78)	2.37±0.33[c] (72.23)	0.39±0.32[c] (95.43)	0.00 (100)	0.00 (100)	12.16±0.36
Betadin ointment (10% w/w)	8.67±0.37[c] (0.00)	5.98±0.30[b] (31.03)	4.37±0.34[c] (49.60)	1.94±0.40[c] (77.62)	0.17±0.27[c] (98.04)	0.00 (100)	0.00 (100)	12.98±0.64
Blank ointment	8.41±0.30 (0.00)	7.37±0.21 (12.37)	6.96±0.34 (17.24)	5.94±0.23 (29.37)	5.31±0.11 (36.86)	4.57±0.13 (45.67)	3.13±0.19 (62.78)	24.66±0.97

Values are expressed as mean ±SEM; n=6, One Way ANOVA followed by Student-Newmen-Keuls t-test; t-value denoted significance at a: P<0.05; b: P<0.01; c: P<0.001 respectively.

Table 3: The effect of *L. lanata* ointment on the infected excised wound in rats

Treatment group	Wound contraction (mm2) (percentage wound contraction in parenthesis)							Epithelization period (Days)
	0 Day	3rd Day	6th Day	9th Day	12th Day	15th Day	18th Day	
LLEE ointment (5% w/w)	8.26±0.23[a] (0.00)	6.91±0.36[c] (16.34)	5.89±0.13[b] (28.69)	4.11±0.30[b] (50.24)	2.74±0.17[a] (66.83)	1.02±0.13[c] (87.65)	0.35±0.23[c] (95.73)	19.06±0.56
LLEE ointment (10% w/w)	8.14±0.24[a] (0.00)	6.00±0.37[b] (26.29)	4.79±0.31[c] (41.16)	2.89±0.28[c] (64.50)	1.35±0.20[c] (83.42)	0.23±0.36[c] (97.17)	0.00 (100)	15.83±0.30
Betadin ointment (10% w/w)	8.39±0.18[b] (0.00)	5.82±0.35[c] (30.63)	4.81±0.27[c] (42.66)	2.37±0.31[c] (71.75)	1.17±0.32[c] (86.05)	0.09±0.36[c] (98.93)	0.00 (100)	15.09±0.38
Blank ointment	8.32±0.39 (0.00)	7.41±0.36 (10.94)	6.99±0.31 (15.99)	6.03±0.24 (27.52)	5.67±0.33 (31.85)	4.33±0.31 (47.95)	3.15±0.22 (62.14)	25.26±0.77

Values are expressed as mean ±SEM; n=6, One Way ANOVA followed by Student-Newmen-Keuls t-test; t-value denoted significance at a: p<0.05; b: p<0.01; c: p<0.001respectively.

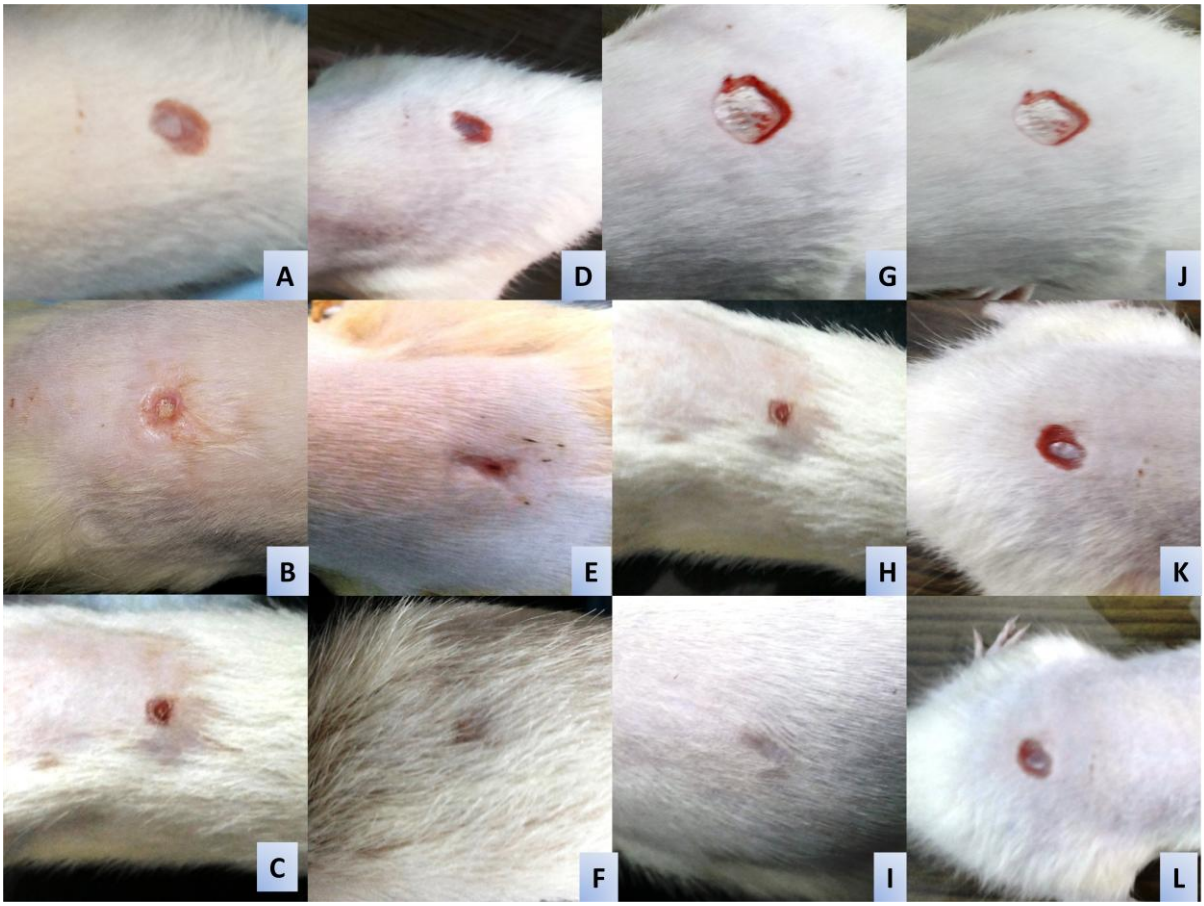


Figure 3: Sprague dawley rats dorsal wound area photographed at 0, 6th and 15th day by Excision wound model (Uninfected): (A-C) Group I- treated with 5% w/w ointment of LLEE ; (D-F) Group II-treated with 10% w/w ointment of LLEE; (G-I) Group III- treated with standard drug (10% w/w Betadin) (J-L) Group-IV – Not treated

Epithelialization period

The epithelialization was observed from the first day. The data in the observation tables reveals that extent of percentage closure by epithelialization in case of uninfected wound is higher than excised infected wound. It was observed that in case of uninfected excision wound, epithelization period was reduced in a dose related manner from 24.66±0.97 for the blank ointment control to 12.16±0.36 for the treated group with 10% (w/w) LLEE ointment (Table 2). Similarly, in case of infected excised wound model epithelization period was equally reduced in a dose related manner from 25.26±0.77 for the blank ointment control to 15.09±0.38 for the standard treated group (Table 3).

Antioxidant Parameters

SOD activity in wound tissue was significantly increased in the case of rats treated with LLEE 10% w/w ointment ($p<0.01$) and LLEE 5% w/w ointment ($p<0.01$) in the excision uninfected wound model. Similarly, the activity was also significantly increased in treated rats with LLEE 10% w/w ointment ($p<0.001$) and LLEE 5% w/w ointment ($p<0.001$) in uninfected excision wound models, when compared with control wounds (Figure 4).

Catalase activity found to be elevated significantly in experimental rats treated with LLEE 10% w/w ointment ($p<0.01$) and LLEE 5% w/w ointment ($p<0.05$) in the excision uninfected wound model. While in the case of infected excision wounds significant increase in different treated groups ($p<0.01$) was observed over blank ointment treated group (Figure 5).

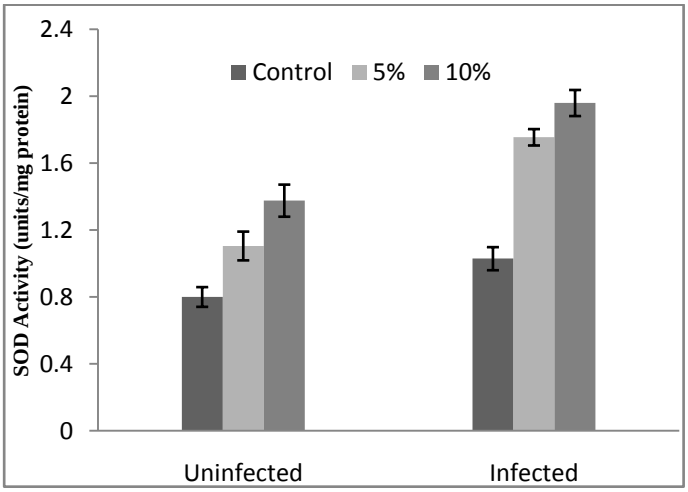


Figure 4: Effect of topical 5% and 10% ointment of *L. lanata* on SOD activity. Values are mean ±SEM (n=6).

Reduced glutathione, which is an important free radical scavenger showed a trend towards significant increase in treated rats with LLEE 10% w/w ointment ($p<0.05$) and LLEE 5% w/w ointment ($p<0.01$) of the uninfected wound model. Similarly, application of LLEE 10% w/w ointment and LLEE 5% w/w ointment resulted significant elevation in the level of GSH, $p<0.05$ and $p<0.001$ respectively, when compared to blank ointment treated rats (Figure 6).

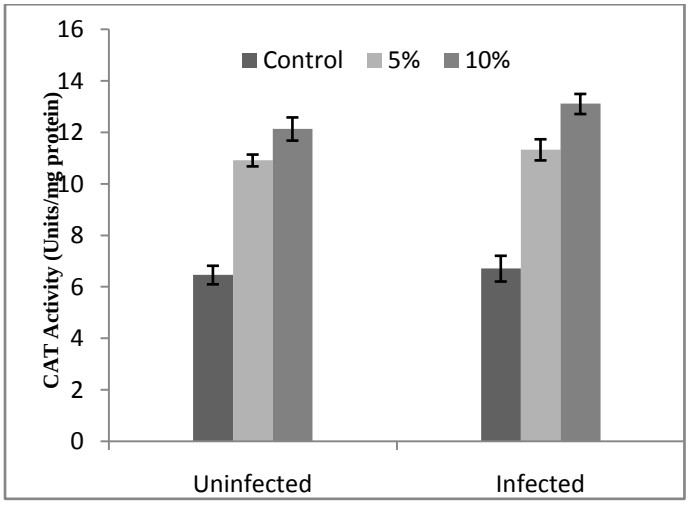


Figure 5: Effect of topical 5% and 10% ointment of *L. lanata* on CAT activity. Values are mean ±SEM (n=6).

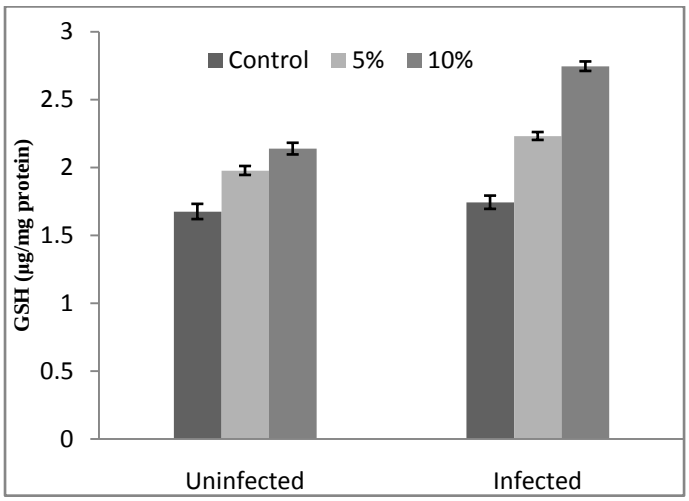
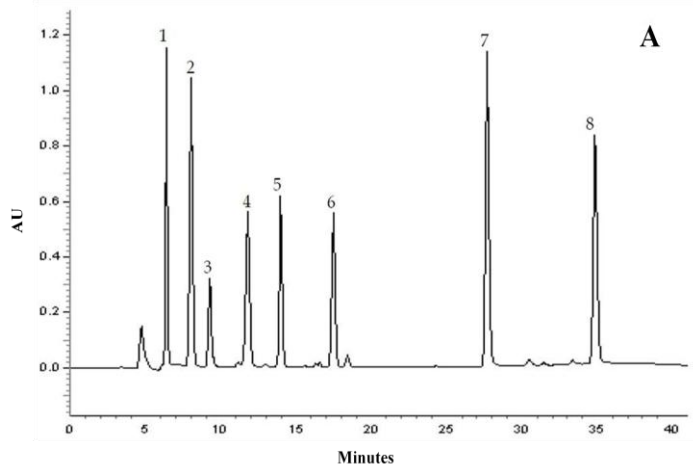


Figure 6: Effect of topical 5% and 10% ointment of *L. lanata* on GSH level. Values are mean ±SEM (n=6)

HPLC studies

From the HPLC study chromatogram (Figure 7) of distinct peaks for all five polyphenols of following compounds have been identified in the total cell-free 50% EtOH extract of *L. lanata*. The amount of compounds present in extract of *L. lanata* were: gallic acid (0.683%), protocatechuic acid (0.291%), Chlorogenic acid (0.856%), caffeic acid (0.198%), ferulic acid (0.317%) and Quercetin (0.045%).



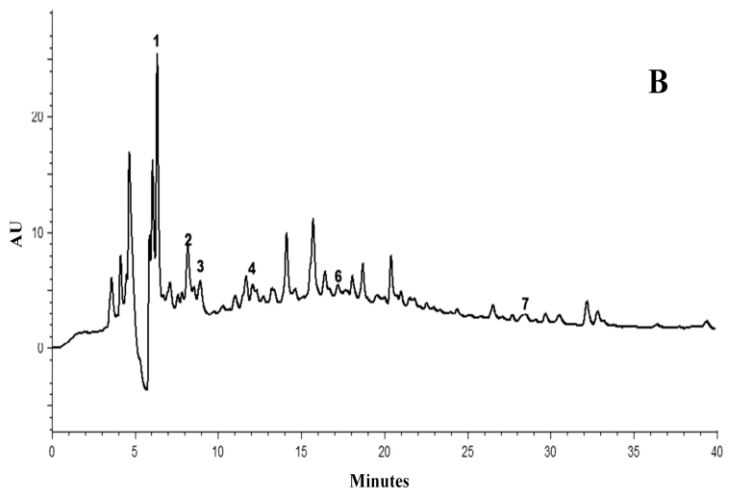


Figure 7: HPLC chromatograms obtained from Standarad (A) and *L. lanata* (B) extract at 254 nm: 1, gallic acid; 2. protocatechuic acid; 3. chlorogenic acid; 4. caffeic acid; 5. rutin,; 6. ferulic acid; 7. quercetin; 8. kaempferol.

Discussion

The present study was carried out to evaluate antioxidant, antimicrobial activity and wound healing effects of 50% EtOH extract of *L. lanata*. Phytochemical compounds such as alkaloids, flavonoids, tannins, phenolics, saponins, terpenoids and other aromatic compounds are secondary metabolites that are produced in plants as a response to stress or as a part of their defence mechanism against prediction by many microorganisms, insects and other herbivores.[37] Plants phenols and flavonoids are important groups of natural antioxidants and having greater antimicrobial activity. The principle of the DPPH method based on the production of free radical[38] and the effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. The results indicating that, total phenolic and flavonoid content in the EtOH extract of *L. lanata* were responsible for its antioxidant activities. Numerous studies exhibited a strong relationship between total phenolic, flavonoid content and antioxidant activity in fruits, vegetables, and medicinal plants.[39-41]

Screening of antimicrobial properties of medicinal plants is being increasingly reported from all over the world. The above results of antimicrobial activity showed that, all tested concentrations were significant against studied pathogenic microorganisms. Overall, the antimicrobial activity of the *L. lanata* was noticeably more effective against the growth of bacterial strains compared to the fungal strains. The results obtained in this assay revealed that extracts were found to be not inactive against any organism and highest concentration of the extract was more effective against all microorganisms but showing highest zone of inhibition against Gram positive bacteria. The results showed that the mean zone of inhibition produced by positive controls (tetracycline and nystatin), was larger than those produced by all concentrations of 50% EtOH extract of *L. lanata*. This may be attributed to the fact that plant extracts being in crude form contain smaller concentrations of bioactive compounds.[42]

Wound healing is a complex and intricate process initiated in response to an injury that restores the function and integrity of damaged tissues. The four phases of wound healing[43] requires the collaborative efforts of different tissues. There are three stages of wound healing process such as inflammation, proliferation and remodeling. These are all classic symptoms of inflammation due to the release of prostaglandins, leukotrienes, and ROS. This involves blood clotting and platelet aggregation, inflammatory response to injury, fibrin formation, altered ground substances, re-epithelialization and angiogenesis. Depletion in wound area is made by the action of myofibroblasts, which set up a clench on the wound edges and contract themselves.

The inhabitation of pathogenic bacteria such as Staphylococcus, Streptococcus and Pseudomonas in wounds normally may lead to infection of wounds which may result in the formation of chronic wounds.[44] In the present study, topical application of 50% EtOH extract of *L. lanata* showed significant ($p<0.05$ to $p<0.001$) increase in wound contraction and reduced the epithelization periode on the both infected and uninfected wounds. This increased epithelization may be due to the effect of *L. lanata* extract on collegen synthesis. Wounds are known to be easy portals for infections and provides a suitable medium for the growth of microorganism and it can be delayed wound healing process. [45]

Several phytochemicals like flavonoids, saponins, tannins, terpenoids, especially polyphenoles are known to promote wound healing activity mainly due to their antimicrobial and astringent property, which seem to be responsible for wound closure and enhanced epithelization as shown in the present study.[46] High-performance liquid chromatography analysis of the 50% EtOH extract of *L. lanata* showed the presence of gallic acid, caffeic acid, chlorogenic acid, protocatechuic acid and ferulic acid as major components. These compounds have important biological activities and responsible for antimicrobial activity as well as wound healing activity. Earlier reports also suggested that, polyphenoles such as caffeic acid[47], protocatechuic acid[48] and ferulic acid[49] having a significant positive role in the healing of wounds. Another important observation of HPLC chromatogram was that a group of unidentified peaks were present, which represent some non-identified phytochemical presence in the sample. These unidentified phytochemicals could also be responsible for the different biological activities, especially antimicrobial activity.

There are many evidences suggesting that increased production of reactive oxygen species (ROS) results in oxidative stress play an important role in delayed wound healing. Therefore, elimination of ROS could be an important strategy in healing of chronic wounds. [50] In the present study, we found that in LLEE ointment treated wounds showing increased level of all antioxidants, which may be responsible and favorable for faster wound healing and this plant extract may be useful in the management of abnormal healing and hypertrophic scars. Although further studies are highly recommended to elucidate the active compounds and investigate the specific mechanism of action in cellular and macromolecular levels.

Conclusion

The results obtained from the study showed remarkable antimicrobial and wound healing activity of *L. lanata*. The observations also indicated that 50% EtOH extract of *L. lanata* exhibited free radical scavenging activity against DPPH radicals and significantly reducing power. HPLC analysis revealed the presence of important biologically active compounds which may be responsible for antimicrobial and wound healing activity. The study provided sufficient evidences that, *L. lanata* might be indeed potential new herbal antimicrobial and wound healing agents.

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References

1. Rosy BA, Joseph H, Rosalie. Phytochemical, pharmacognostical, antimicrobial activity of *Indigofera spalathoides* Vahl. (Fabaceae). Int J Biol Technol. 2010; 1: 12-15.

2. Cragg GM, Newman DJ. Medicinals for the Millennia. Annals NY Acad Sci. 2001; 953: 3-25.

3. Edeoga1 HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotech. 2005; 4: 685-688.

4. Akinmoladun AC, Ibukun EO, Afor E, Akinrinlola BL, Onibon TR, Akinboboye AO, Obuotor EM, Farombi EO. Chemical constituents and antioxidant activity of *Alstonia boonei*. Afr J Biotechnol. 2007;6: 1197-1201.

5. Jayaprakasha GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed (*Vitrus vinifera*) extracts on peroxidation models in vitro. Food Chem. 2001; 73: 285-290.

6. Padmaa M Paarakh, Chansouria JPN, Khosa R.L. Wound healing activity of *Annona muricata* extract. J Pharm Res. 2009; 2: 404-406.

7. Subramoniam A, Evans DA, Rajasekharan S, Nair GS. Effect of *Hemigraphis colorata* (Blume) H. G. Hallier leaf on wound healing and inflammation in mice. Indian J Pharmacol. 2001; 33:283-285.

8. Biswas TK, Mukherjee B. Plant medicines of Indian origin for wound healing activity: a review. Int J Low Extrem Wounds. 2003; 2:25-39.

9. Muthu C, Ayyanar M, Raja N, Ignacimuthu S. Medicinal plants used by traditional healers in Kancheepuram district of Tami Nadu. Indian J Ethnobiology and Ethnomedicine. 2006; 2:43-53.

10. Veerapur VP, Palkar MB, Srinivasa H, Kumar MS, Patra S, Rao PGM, Srinivasan KK. Effect of ethanol extract of *Wrightia tinctoria* bark on wound healing in rats. J. Natural Remedies. 2004; 4(2): 155-159.

11. Girach RD, Aminuddin PA, Siddioui PA, Khan S.A.Traditional plant remedies among the Kondh of district dhenkanal (Orissa). Int J Pharm. 1994; 32:274-283.

12. Chagnon M. General pharmacologic inventory of medicinal plants of Rwanda. J Ethnopharmacol. 1984; 12: 239-251.

13. Nazir AP, Negi AK, Todaria NP. Traditional uses of medicinal plants of Pauri Garhwal, Uttarakhand. Nature & Science. 2010; 8(6): 57-61.

14. Yanfg LL, Yen KY, Kiso Y, Kikino H. Antihepatotoxic actions of formosan plant drugs. J Ethnopharmacol. 1987; 19:103-110.

15. Verma S, Chauhan NS. Indigenous medicinal plants knowledge of Kunihar forest division, District Solan. Indian J Tradit Know. 2007; 6:494-497.

16. Singh H. Importance of local names of some useful plants in ethnobotanical study. Indian J Tradit Know. 2008; 7: 365-370.

17. Lin CC. Crude drugs used for the treatment of diabetes mellitus in Taiwan. Am J Chin Med. 1992; 20:269-279.

18. Unani BG, Borah A, Wann SB, Singh HR, Devi B, Bhattacharjee M. Phytochemical and antibacterial study of traditional medicinal plants of North East India on Escherichia coli. Asian J Exp Sci. 2009; 23 (1): 103-108.

19. Ramalingam R, Ravinder AN, Bindu MB, Nagulu M, Bala AS. Free radical scavenging and antiepileptic activity of *Leucas lanata*. J Pharm Res. 2013a; 6:368-372.

20. Ramalingam R, Ravinder AN, Bindu MB, Nagulu M, Bala AS. Antiparkinson's and free radical scavenging study of ethyl acetate fraction of ethanolic extract of *Leucas lanata*. Drug Invention Today 2013b; 5: 251-255.

21. Trease GE, Evans WC. Pharmacognosy. 13th ed. Bailliere Tindall Ltd, London; 1989, pp. 176-180.

22. Harborne JB. Phytochemical method. 3rd ed. Chapman and Hall, London; 1993, pp.135-203.

23. Woisky R, Salatino A. Analysis of propolis: some parameters and procedures for chemical quality control. J. Apic. Res. 1998; 37: 99-105.

24. Bray HG, Thorp WV. Analysis of phenolic compounds of interest in metabolism. Methods in Biochemistry Analysis. 1954; 1:27-52.

25. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181: 1199-1200.

26. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition. 1986; 44: 307-315.

27. Ahlam AA, Hazaa AM, Afaf W, Sadri S, Amzad H, Sohail A. In vitro antioxidant, cytotoxic and antimicrobial screening of the leaves of *Acridocarpous orientalis*, native to Sultanate of Oman. British J Pharm Res. 2013; 3(4): 734-742.

28. Quinto E & Santos M. Microbiology, In: edited by Guevarra BQ (Espana Manila, Philippines: University of Santo Tomas Publishing House), 2005.

29. Zimmerman M. Ethical guidelines for investigation of experimental pain in conscious animals. Pain. 1983; 16:109-110.

30. Cooper JL, Gunns C. Dispensing for pharmaceutical students, 12th ed. In: Carter SL (Ed.), CBS Publisher and Distributors, Delhi, 1987, pp. 199-200.

31. Nayak SB, Pinto Pereira LM, Maharaj D. Wound healing activity of *Carica papaya* L. in experimentally induced diabetic rats. Indian J Exp Bio. 2007; 45: 739–743.

32. Tramontina VA, Machado MA, Nogueira F, Gda R, Kim SH, Vizzioli MR, Toledo S. Effect of bismuth subgallate (local hemostatic agent) on wound healing in rats. Histological and histometric findings. Braz. Dent. J. 2002; 13: 11-16.

33. Shukla A, Rasik AM, Patnaik GK. Depletion of reduced glutathione, ascorbic acid, vitamin E and antioxidant defence enzymes in a healing cutaneous wound. Free Radical Res. 1997; 26, 93-101.

34. Aebi HU. Catalase in Methods in Enzymatic Analysis, vol. 3, Academic Press, New York, NY, USA, edited by H.U. Bergmeyer; 1983.

35. Cohn VH, Lyle J. A fluorometric assay for glutathione. Anal Biochem. 1966; 14: 434-440.

36. Niranjan A., Barthwal J., Lehri A., Singh D.P., Govindrajan,R., Rawat A.K.S., Amla D.V. Development and validation of an HPLC-UV-MS-MS method for identification and quatification of polyphenols in *Artimisia pallens* L. Acta chromartographica. 2009; 21(1): 105-116.

37. Bonjar GHS, Nik AK, Aghighi S. Antibacterial and antifungal survey in plants used in indigenous herbal-medicine of south east regions of Iran. J Biol Sci. 2004; 4:405-412.

38. Hossain MA, Rahman SM. Total phenolics, flavonoids and antioxidant activity of tropical fruist pineapple. Food Res Int. 2011; 44(3): 672-676.

39. Ghasemzadeh A, Jaafar HZE, Rahmat A. Antioxidant Activities, Total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). Molecules. 2010; 15:4324-4333.

40. Velioglu YS, Mazza G, Gao, L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetable and grain products. J Agric. Food Chem. 1998; 46: 4113-4117.

41. Dorman HJD, Kosar M, Kahlos K, Holm Y, Hiltunen R. Antioxidant properties and composition of aqueous extracts from Mentha species, hybrids, varieties and cultivars. J. Agric. Food Chem. 2003; 51: 4563-4569.

42. Chew AL, Jeyanthi JAJ, Sasidharan S. Antioxidant and antibacterial activity of different parts of *Leucas aspera*. Asian Pac J Trop Biomed. 2012; 2(3): 176-180.

43. Puratchikody A, Nithya C, Nagalakshmi G. Wound healing activity of *Cyperus rotundus* Linn. Indian J Pharm Sci. 2006; 68: 97-101.

44. Frantz RA. Identifying infection in chronic wounds. Nursing. 2005; 35(7):73.

45. Bowler PG, Duerden BI, Armstrond DG Wound microbiology and associated approaches to wound management. Clin. Microbiol Rev. 2001; 14:244-269.

46. Ilodigwe EE, Ndunagu LU, Ajaghaku DL, Utoh-Nedosa UA. Evaluation of the Wound Healing Activity of a Polyherbal Remedy. Ann Bio Res. 2012; 3(11): 5393- 5398.

47. Song HS, Park TW, Sohn UD, Shin YK, Choi BC, Kim CJ, Sim SS. The Effect of Caffeic Acid on Wound Healing in Skin-incised Mice. Korean J Physiol Pharmacol. 2008; 12: 343-347.

48. Borate AR ,Suralkar AA, Birje SS, Malusare PV, Bangale PA. Antihyperlipidemic effect of protocatechuic acid in fructose induced hyperlipidemia in rats. International Journal of Pharma and Bio Sciences. 2011; 2 (4):456.

49. Ghaisas MM, Kshirsagar SB, Sahane RS. Evaluation of wound healing activity of ferulic acid in diabetic rats. Int Wound J. 2014; 11(5):523-532.

50. Dissemond J, Goos M, Wagner SN. The role of oxidative stress in the pathogenesis and therapy of chronic wounds. Hautarzt. 2002; 53: 718–723.