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Original Article

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ABSTRACT

Objective: Oxidative stress was found to be the underlying mechanism in many disorders especially in neurodegenerative disorders like epilepsy. The main aim of the investigation was to evaluate *invitro* free radical scavenging potential and *invivo* antiepileptic activity of selected fraction of *Leucas lanata*.

Methods: Ethanolic extract of *L. lanata* was taken for its phytochemical screening, *invitro* free radical scavenging, cytotoxic studies, fractionation with ethyl acetate and its HPTLC studies. Further the fraction was evaluated for *invivo* acute toxicity studies and antiepileptic activity in mice.

Results: After the estimation of 64.412 ± 8.44 mgGAE/g of phenolic and 63.723 ± 8.01 mgRE/g of flavonoids contents, *invitro* free radical scavenging studies of ethanolic extract of the plant revealed its almost equal potential as quercetin. HPTLC studies of ethyl acetate fraction of extract revealed the presence of 3 unknown flavonoids with R_f values of 0.03, 0.48 and 0.93. In animal studies there was a decrease in the duration of hind limb extension like 7.8 ± 0.1 s, 7.4 ± 0.2 s and 7.1 ± 0.2 s linearly with doses. In forced swim test animals were found to be active without any decreased locomotory activity indicated by more immobilized time in diazepam treatment (233.33 ± 5.03 s) and less immobilized time with the treatment of fraction (161.66 ± 5.686 s). From the studies of malondialdehyde estimation, there was a comparative decrease in the content which was not linear with dose.

Conclusion: From the results of *invitro* and *invivo* studies it can be concluded that, flavonoid and phenolic rich ethyl acetate fraction of *L. lanata* can be used to treat epilepsy without any induction of depression.

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1. Introduction

Epilepsy is the most common neurological disorder characterized by recurrent spontaneous seizures affecting 1–2% of the population worldwide.¹ The most underlying mechanism in the development and progression of epilepsy and several other neurological disorders is oxidative stress.² Oxidative stress is caused by excessive production of reactive oxygen species such as hydroxyl, superoxide anion radical, nitric oxide and hydrogen peroxide.³ There are so many drugs available to treat epilepsy but none of them are free from side effects such as depression, ischemia, impaired cognition, motor disability and etc.⁴ Among all, depression is the common side effect produced by most of the antiepileptic drugs and that remains untreated.⁵ It has been observed that seizure activity during epilepsy increases the amount of free radicals and decreases the antioxidant defense mechanism in the brain which further induce the oxidative stress.³ The extract obtained from plants of the genus *Leucas* display a wide range of pharmacological activities such as antioxidant, hepatoprotective, antiinflammatory, antidiabetic, antimicrobial, antidiarrhoeal and antinociceptive activity.^{6–9} No research or scientific work has been done on *Leucas lanata*, therefore the present study is aimed at exploring the potential of free radical scavenging activity along with its capability to treat epilepsy.

2. Materials and methods

2.1. Materials

1, 1-Diphenyl-2-picryl hydrazyl, 2-thiobarbituric acid, 1, 1, 3, 3-tetramethoxy propane and pentylentetrazole were obtained from Sigma–Aldrich, St Louis, MO, United States. Phenazine methosulphate, nitroblue tetrazolium and sulfanilamide were purchased from NR chemicals Pvt Ltd, Mumbai, India. Sodium nitroprusside was obtained from HiMedia Laboratories Pvt Ltd, Mumbai, India. 2-Deoxy-D-ribose and reduced nicotinamide adenine dinucleotide were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India and all other reagents and solvents used were of analytical grade and obtained from various other commercial sources.

2.2. Methods

2.2.1. Extraction and phytochemical screening

The whole plant of *L. lanata* was collected from Tirulmala hills, Andhra Pradesh, India. *L. lanata* was authenticated with vouchure number 1798. 500 g of air dried and powdered *L. lanata* was first defatted with petroleum ether at room temperature for 72 h. The defatted material was extracted with 95% ethanol at room temperature for 72 h. The resultant ethanolic extract was concentrated under reduced pressure at room temperature using rotary vacuum evaporator. Ethanolic extract of *L. lanata* was subjected for preliminary phytochemical screening to determine the presence of carbohydrate, alkaloid, amino acid, flavonoid, phenolic substance, steroid, protein, saponin and tannin.¹⁰

2.2.2. Estimation of total phenolic content and flavonoids content

0.5 ml of ethanolic extract was estimated for total phenolic and flavonoids contents by using UV spectrophotometric method.¹¹

2.2.3. 1, 1-Diphenyl-2-picryl hydrazyl radical scavenging activity

1 ml of different concentrations of ethanolic extract in ethanol were added to 3 ml of 0.1 mM methanolic solution of 1, 1-diphenyl-2-picryl hydrazyl. The mixture was shaken followed by incubating at room temperature for 30 min in dark. The absorbance against blank was measured at 570 nm by using UV spectrophotometer.¹²

2.2.4. Superoxide anion radical scavenging activity

1 ml of nitroblue tetrazolium solution (156 μ M in 100 mM phosphate buffer, pH 7.4), 1 ml of 2-deoxy-D-ribose and reduced nicotinamide adenine dinucleotide solution (468 μ M in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different concentrations of the ethanolic extract in ethanol were mixed. The reaction was started by adding 100 μ l of phenazine methosulphate solution (60 μ M in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance at 560 nm was measured against blank samples, containing all the reagents except phenazine methosulphate.¹³

2.2.5. Hydroxyl radical scavenging activity

0.2 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mM) and 0.2 ml of ethylene diamine tetra acetic acid (10 mM) mixed solution was prepared in a test tube, and 0.2 ml of 2-deoxyribose solution (10 mM), 0.2 ml of ethanolic extract in ethanol and phosphate buffer (pH 7.4, 0.1 M) were added to give a total volume of 1.8 ml. Finally, 200 μ l of H_2O_2 solution (10 mM) was added to this reaction mixture and the whole was incubated at 37 °C for 4 h. After this incubation, 1 ml each of a tri-chloro acetic acid solution (2.8%w/v) and thiobarbituric acid solution (1.0%w/v) were added to the reaction mixture and the resultant solution was boiled for 10 min in water bath, cooled in ice, and its absorbance was measured at 520 nm. The hydroxyl radical scavenging activity was calculated as the inhibition rate of 2-deoxyribose.¹⁴

2.2.6. Nitric oxide scavenging activity

0.1 ml of aqueous sodium nitroprusside (10 mM) in 0.2 ml of phosphate buffer (0.2 M, pH 7.8) was mixed with 0.5 ml of different concentration of ethanolic extract in ethanol and incubated at room temperature for 150 min. After incubation period, 0.2 ml of Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the reaction mixture was read at 546 nm against blank.¹⁵

2.2.7. Ethyl acetate fraction

After n-hexane fraction, in order to enrich flavonoid content, ethanolic extract was dissolved in ethyl acetate. Ethyl acetate soluble fraction was separated and evaporated to get dry residue. This ethyl acetate fraction was taken for further studies.

2.2.8. HPTLC studies

Ethyl acetate fraction and standard flavonoids (quercetin, rutin and kaempferol) were processed on the automated HPTLC system (CAMAG LINOMATS 5, Switzerland) with toluene: 1, 4-dioxan: glacial acetic acid (90:25:4) as mobile phase.¹⁶ The plate was photodocumented in day light and UV 366 nm mode using photo documentation (CAMAG Reprostar 3) chamber. After derivatization, the plate was fixed in scanner stage (CAMAG TLC scanner 3) and scanning was done at UV 366 nm. The software used was WINCATS 1.3.4 version.

2.2.9. Acute oral toxicity studies

Toxicity studies of the fraction in 0.2% CMC solution were performed according to OECD guidelines no: 423 using female non pregnant albino mice weighing 25–30 g at Technocrats Institute of Technology – Pharmacy, Bhopal with a Reference number of TIT/IAEC/831/P'col/2012/07.

2.2.10. Antiepileptic study

Male swiss albino mice weighing 25–30 g were employed for the antiepileptic study at Technocrats Institute of Technology – Pharmacy, Bhopal (Reference number. TIT/IAEC/831/P'col/2012/08). The ethyl acetate fraction was reconstituted by 0.2% CMC and was given orally. Diazepam was used as standard. The animals were divided in to 5 groups and were observed for duration of hind limb extension.^{17,18} Group 1 administered with 0.2% CMC and after 30 min followed by pentylene-tetrazole I.P., Group 2 with diazepam 2 mg/kg I.P. and after 30 min followed by pentylene-tetrazole I.P., Group 3 with 100 mg/kg fraction and after 30 min followed by pentylene-tetrazole I.P., Group 4 with 200 mg/kg fraction and after 30 min followed by pentylene-tetrazole I.P. and Group 5 with 300 mg/kg fraction and after 30 min followed by pentylene-tetrazole I.P.

2.2.10.1. Forced swimming test. After cessation of seizures the animals were subjected for forced swimming test to assess the depressive behavior. In this test, the animals were kept individually in glass cylinder (25 × 12 × 25 cm³) containing water at room temperature up to a level of 15 cm for 5 min and total immobility period in seconds was noted. The animals were judged to be immobile when they stopped struggling and remained floating motionless in water, making only those movements necessary to keep their head above water.^{17,18}

2.2.10.2. Malondialdehyde determination. The animals were sacrificed by decapitation at the end of experiment. The brains were quickly removed and were washed with cold saline solution. The brains were cut in to small pieces with sharp knife and the resultant tissues were homogenized in 4 volumes of ice cold tris-hydrochloride buffer (50 mM, pH 7.4). The homogenized tissue was mixed with 2 volumes of cold 10%w/v trichloro acetic acid to precipitate proteins. The precipitate was centrifuged, pelleted and an aliquot of the supernatant was mixed with 0.67%w/v of thiobarbituric acid for 15 min in a boiling water bath. After cooling the absorbance was measured at 532 nm. The results were expressed as nM/g of protein in brain tissues based on standard graph, which was plotted by using serial dilutions of standard 1, 1, 3, 3-tetra-methoxy propane.¹⁹

3. Results

The plant *L. lanata* was collected, authenticated and extracted with 95% ethanol. The % yield of the extract was found to be 5.7%w/w. The preliminary phytochemical studies revealed that the ethanolic extracts of *L. lanata* had given positive result for flavonoids, saponins, carbohydrates, tannins and phenolic compounds. They were found to give negative result for the phytochemicals like proteins, amino acids, alkaloids and steroids. After estimations the ethanolic extract of *L. lanata* was found to contain 64.412 ± 8.446 mgGAE/g of total phenolic and 63.723 ± 8.015 mgRE/g of total flavonoid content. The detailed values of free radical scavenging activity were given in Tables 1–4. Ethyl acetate fraction of the ethanolic extract of *L. lanata* was prepared and the percentage yield was found to be 0.248% w/w. From the HPTLC studies it was observed that, there were 3 flavonoids in the LLEA fraction and was not containing the standard flavonoids, quercetin, rutin and kaempferol. Among the identified flavonoids, flavonoid 1 was found at 0.03 R_f value with 1045.0 plot area and 6.55% relative percentage. Flavonoid 2 was found at 0.48 R_f value with 1292.1 plot area and 8.10% relative percentage. Flavonoid 3 was found at 0.93 R_f value with 822.1 plot area and 5.15% relative percentage. The R_f value of standard flavonoids, quercetin, rutin and kaempferol was found to be 0.20, 0.01 and 0.36 respectively. For antiepileptic activity the results of durations of hind limb extension, immobility times in forced swim test and malondialdehyde content in extracted brains of animals were given in Table 5.

4. Discussion

Most of the recent investigations have proved the free radical scavenging activity of the phytoconstituents especially flavonoids. Flavonoids are recently given considerable scientific and therapeutic interest and they offer protection from free radicals damage.²⁰ Phytoconstituents like glycosides from *Leucas* genus were found to have free radical scavenging activity.²¹ In our present investigation after phytochemical screening, the extract was found to contain considerable amounts of flavonoids (64.412 ± 8.44 mgGAE/g) and phenolic compounds (63.723 ± 8.01 mgRE/g). Studies on free radical scavenging activity revealed that, the IC₅₀ values of the extract were found to be almost equal to the IC₅₀ values of quercetin

Table 1 – % Inhibition of 1, 1-diphenyl-2-picryl hydrazyl radical by quercetin and extract.

Concentrations (µg/ml)	% Inhibition by quercetin	% Inhibition by extract
0	0	0
20	92.68 ± 1.29	47.98 ± 3.83
40	93.59 ± 0.5	55.59 ± 3.16
60	93.22 ± 0.27	63.69 ± 4.76
80	93.95 ± 0.22	69.645.47
100	92.58 ± 0.56	69.74 ± 1.44
IC 50 (µg/ml)	9.27 ± 0.75	25.46 ± 7.53

Table 2 – % Inhibition of superoxide anion radical by quercetin and extract.

Concentrations (µg/ml)	% Inhibition by quercetin	% Inhibition by extract
2	16.26 ± 9.061	29.715 ± 2.575
4	32.172 ± 2.213	40.57 ± 1.353
6	34.727 ± 1.319	46.42 ± 2.227
8	41.58 ± 2.521	51.578 ± 1.733
10	50.523 ± 4.688	57.506 ± 6.005
25	58.653 ± 4.186	68.36 ± 1.973
50	60.046 ± 5.44	70.978 ± 1.733
IC50 (µg/ml)	27.805 ± 3.37	25.588 ± 3.694

except for 1, 1-diphenyl-2-picryl hydrazyl radical scavenging. The preliminary studies indicated the presence of flavonoids and with the positive values from free radical scavenging activity, the presence of flavonoids was almost confirmed. The same was further confirmed from the HPTLC studies. There were 3 unknown flavonoids revealed from HPTLC run of ethyl acetate fraction of *L. lanata*.

Univalent reduction of oxygen produces free radicals and these are found to produce damage to blood vessels and parenchyma of the brain. Especially in seizures, these free radicals were involved in causation of lipid peroxidation, brain edema, dysfunction including coma and death.²² Even in current scenario, epilepsy continues to be a neurological disorder awaiting the use of safer drugs. For the antiepileptic studies in mice, pentylenetetrazole was used to induce seizures in mice. Pentylenetetrazole induced seizure activity mimics the increased oxidative stress in brain by altering membrane phospholipid metabolism and ultimately resulting in the release of free radicals.¹⁹ To assess the seizure activity, duration of hind limb extension was measured. In control group there might be damage in brain due to the free radicals produced by pentylenetetrazole and hence the duration of hind limb extension was more. In other groups there was a significant ($df = 4$, $F = 28.34$ and $p = 0.3961$) decrease in the duration of hind limb extension indicating the protective effect of the standard drug diazepam and fraction at all administered doses. Being potential free radical scavenger, the selected fraction might have protected the mice from oxidative damage and hence there was a decrease in the duration of hind limb extension. In forced swim test, the immobilized time was increased significantly ($df = 4$, $F = 189.18$ and $p = 0.6899$) in comparison with control group. The animals treated with all the doses of fraction were found

Table 3 – % Inhibition of hydroxyl radical by quercetin and extract.

Concentrations (µg/ml)	% Inhibition by quercetin	% Inhibition by extract
8	8.3612 ± 3.857	13.572 ± 5.049
16	13.823 ± 1.174	19.543 ± 6.416
24	18.506 ± 8.729	22.584 ± 6.114
32	25.641 ± 2.276	27.795 ± 2.955
40	30.99 ± 0.696	32.790 ± 1.639
IC 50 (µg/ml)	146.86 ± 13.860	114.386 ± 23.566

Table 4 – %Inhibition of nitric oxide by quercetin and extract.

Concentrations (µg/ml)	% Inhibition by quercetin	% Inhibition by extract
6	51.297 ± 1.713	0.483 ± 0.29
12	64.355 ± 4.872	1.529 ± 0.816
18	63.776 ± 1.839	6.007 ± 1.523
24	63.969 ± 1.157	11.223 ± 0.647
30	66.148 ± 2.577	20.206 ± 3.727
75	67.067 ± 2.658	62.403 ± 2.118
IC50 (µg/ml)	16.09 ± 1.798	63.749 ± 2.742

to be with increased alertness unlike diazepam treated group. There was an increased immobilized time in diazepam group indicating the depressive symptoms of the drug. 29% of the epileptic patients suffer from depression during the course of treatment.²³ The antiepileptic drugs were found to decrease the locomotor activity.²⁴ This might be the reason for the increase in immobilized time with diazepam. Repeated induction of seizures is also one of the reason for depression.²⁵ In control group there was less immobilized period may be due to single induction of seizures. The decrease in immobilized time with the administered doses of fraction indicates the positive antiepileptic activity without the induction of depression. This may be because of the flavonoids which are believed in literature to improve the synaptic signaling.²⁶ Another reason may be the mechanism of flavonoids to increase the levels of serotonin and noradrenalin by inhibiting monoamino oxidase²⁷ that catalyzes the oxidative deamination of serotonin and noradrenaline.²⁸ The decrease in the levels of serotonin and noradrenaline can lead to depression.²⁹ Further studies were continued with the estimation of malondialdehyde as it is an index of lipid peroxidation.² In these estimations the treatment per se caused non-significant changes ($df = 4$, $F = 1.07$ and $p = 0.4317$). Flavonoids can act as GABA agonist³⁰ as they are similar in structure with benzodiazepines and NMDA antagonist.³¹ This may be the strong evidence that, they are able to protect the animals from pentylenetetrazole, a GABA antagonist and NMDA agonist induced seizures. Oxidative stress is one of the underlying mechanisms of epilepsy. Ethyl acetate fraction of ethanol extract of *L. lanata* which is rich in flavonoids and phenolic contents can be an effective treatment for epilepsy without the induction of depression. The responsible flavonoids must

Table 5 – Various parameters estimated for antiepileptic activity.

Group	Duration of limb extension (s)	Immobility time (s)	Malondialdehyde (nM/g protein)
1	8.5 ^a ± 0.173	140.33 ^a ± 4.509	0.561 ± 0.0073
2	6.5 ^a ± 0.3	233.33 ^a ± 5.033	0.559 ± 0.007
3	7.8 ^a ± 0.1	161.666 ^a ± 5.686	0.552 ± 0.0009
4	7.4 ^a ± 0.2	185.33 ^a ± 3.511	0.603 ± 0.079
5	7.1 ^a ± 0.2	196 ^a ± 4.582	0.564 ± 0.010

a Indicates statistical significance $p < 0.001$ by ANNOVA.

be isolated and elucidated for their structure in further studies.

Conflicts of interest

All authors have none to declare.

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