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In-vitro Antioxidant and In-vivo Hepatoprotective activity of *Leucas ciliata* leaves

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Abstract: Objective of the present work was to evaluate ethanolic extract of *Leucas ciliata* leaves for possible antioxidant and hepatoprotective potential. Antioxidant activity of the extract was evaluated by using Diphenyl picryl hydrazyl (DPPH) radical scavenging, Nitric oxide (NO) radical scavenging, Iron chelation and Reducing power methods. Hepatoprotective activity of the extract was evaluated by carbon tetrachloride (CCl_4) induced liver damage model in rats. The extract demonstrated a significant dose dependent antioxidant activity comparable with ascorbic acid. In hepatoprotective activity study, CCl_4 significantly increased the levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP) and total bilirubin. Pretreatment of the rats with ethanolic extract of *L. ciliata* (100, 200 and 400mg/kg *po*) inhibited the increase in serum levels of SGPT, SGOT, ALP and total bilirubin and the inhibition was comparable with silymarin (100mg/kg po). The present study revealed that *L. ciliata* leaves have significant radical scavenging and hepatoprotective activity.

Keywords: Antioxidant; hepatoprotective; *Leucas ciliate*; DPPH; nitric oxide; CCl_4.

1. Introduction

Oxidative stress resulting from the toxic effects of free radicals on the tissue plays an important role in the pathogenesis of various pathological conditions such as ageing process, anemia, arthritis, asthma, inflammation, ischemia, mongolism, neurodegeneration, Parkinson's disease, and perhaps dementia. Antioxidants are radical scavengers, which protect the human body against free radicals [1, 2]. Free radical also induces liver damage. Likewise, metabolism of certain drugs like paracetamol, produce free radicals, which cause liver damage. Antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms and thereby help in preventing the free radical induced diseases [3].

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Leucas ciliata (Lamiaceae) commonly known as Burumbi is a shrub having ovate/ lanceolate leaves with serrate margins. It is frequently distributed along the ghats and in planes along forest edges in Ahmednagar, Dhule, Kolhapur, Mumbai, Satara, Sindhudurg and Thane regions of Maharashtra in India. Traditionally *L. ciliata* is used for wound healing and as antidote for snakebite [4,5]. In Chinese medicine it is used for its antibacterial and antifungal activity [6,7]. Preliminary phytochemical analysis of *L. ciliata* leaves indicated presence of relatively high levels of flavonoids. Several flavonoids have been reported to possess antioxidant and hepatoprotective properties [8]. Hence the present investigation was undertaken to determine the antioxidant and hepatoprotective potential of *L. ciliata* leaves.

2. Materials and Methods

2.1. Plant Material

Fresh leaves of *Leucas ciliata* were collected in the month of June form Nandurbar district of Maharashtra and were authenticated by Botanical Survey of India, Pune. Herbarium of plant specimen was deposited at Govt. College of Pharmacy, Karad, Maharashtra (Voucher no. QMNM2). The leaves were shade dried and crushed to coarse powder.

2.2. Preparation of extract and doses:

The dried powdered leaves (200g) were extracted with three liters of ethanol (95%v/v) by continuous extraction method for 36 hours. Solvent was distilled off and the extract was concentrated and dried under reduced pressure, which yielded a brownish green mass (5.2 g). It was stored at 2 to 4°C. The extract and silymarin were suspended in distilled water with Tween 80 (1%v/v) and 20mg/mL suspension was used for administration to the Albino rats in different doses.

2.3. Animals

Healthy Albino rats (120-150g) of either sex were procured from National Toxicology Center, Pune MS India and were kept in standard plastic animal cages in groups of 5 animals with 12 hours light and dark cycle. The rats were fed on standard rat chow and provided water *ad libitum*. Prior to initialization of experimentation the animals were acclimatized to laboratory conditions for a week. The experiments were carried out according to guidelines of 'Committee for Prevention and Control of Scientific Experimentation on Animals' (CPCSEA) New Delhi, and the procedures were approved by Institutional Animal Ethics Committee (IAEC), Govt. College of Pharmacy, Karad, Maharashtra, India.

2.4. Antioxidant activity

The antioxidant activity was determined by using Diphenyl picryl hydrazyl (DPPH) radical scavenging, Nitric oxide (NO) radical scavenging, Iron chelation and reducing power methods.

2.4.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was measured by spectrophotometric method. 1mL of ethanolic solution of extract of various concentrations (25–800 µg/mL) were mixed with 1mL of ethanolic solution of DPPH (200µM). Similarly 1mL ethanolic solutions of ascorbic acid (200 µg/mL)

was mixed with 1mL of DPPH solution. A mixture of 1mL of ethanol and 1mL of ethanolic solution of DPPH (200 µM) served as control. After mixing, all the solutions were incubated in dark for 20 minutes and absorbance was measured at 517 nm [9]. The experiments were performed in triplicate and percent scavenging activity was calculated as follows;

$$\text{Scavenging \%} = \frac{\text{Absorbance of control} - \text{absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.4.2. Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity was measured by using Griess' reagent. 5mL each of extract solutions of different concentrations (25–800 µg/mL) in standard phosphate buffer solution (pH 7.4) were incubated with 5mL of sodium nitroprusside solution (5mM) in standard phosphate buffer (pH 7.4) at 25°C for 5 hours. In an identical manner 5mL of ascorbic acid solution (200 µg/mL) in standard phosphate buffer solution (pH 7.4) was also incubated with 5mL of sodium nitroprusside solution (5mM) in standard phosphate buffer (pH 7.4). Control experiments without the test compound but with equivalent amount of buffer were also conducted. After incubation, 0.5mL of the incubation mixture was mixed with 0.5 mL of Griess' reagent (Sulphanilamide 1%, *O*-phosphoric acid 2% and naphthyl ethylene diamine dihydrochloride 0.1%) and the absorbance was measured at 546nm [10]. From the absorbance the percent scavenging activity was calculated using the same formula as described above. The experiments were performed in triplicate.

2.4.3. Iron chelating activity

Iron chelation activity is a measure of antioxidant activity. Different concentrations of extract of (25–800 µg/mL) and ascorbic acid solution (200 µg/mL) each as 2mL in 5% v/v methanol were incubated with methanolic *O*-phenanthroline solution (1mL, 0.05%w/v) and ferric chloride solution (2mL, 200 µM) at ambient temperature for 10 minutes. After incubation, the absorbance of solutions was measured at 510nm [11]. The experiments were performed in triplicate.

2.4.4. Reducing power

Reducing power of the extract was evaluated by Oyaizu method. 2.5mL of solutions of different concentrations of the extract (25–800 µg/mL) in standard phosphate buffer solution (pH 6.6) were incubated with 2.5mL of potassium ferricyanide solution (1% w/v) at 50°C for 20 min. In an identical manner solution of ascorbic acid (200 µg/mL) was also incubated. After incubation, 2.5mL of 10% trichloro acetic acid solution was added to each tube and the mixture was centrifuged at 650 rpm for 10 minutes. 5mL of the upper layer solution was mixed with 5mL of deionized water and 1mL of ferric chloride solution (1%w/v) and the absorbance was measured at 700 nm [12].

2.5. Hepatoprotective activity

The hepatoprotective activity of *L. ciliata* leaves extract was determined by using carbon tetrachloride induced hepatotoxic rat model. Rats were divided in six groups each comprising of five rats. Before treatment, the rats were fasted overnight with free access to water. Group I served as vehicle control and received Tween 80 (1%v/v) in distilled water (5mL/kg, po) for seven days. The

group II served as toxic control and was administered vehicle (5mL/kg po) daily and carbon tetrachloride in olive oil 1:1 v/v (0.7mL/kg, ip) on alternate days for seven days. Group III served as positive control and was administered silymarin (100mg/kg, po, daily) and groups IV, V and VI were administered ethanolic extract of *L. ciliata* (100, 200 and 400mg/kg, po, daily) along with carbon tetrachloride in olive oil 1:1 v/v (0.7mL/kg, ip) on alternate days for seven days. At the end of treatment, blood was withdrawn under light ether anesthesia by retro orbital cannulation. Then each rat was dissected to isolate liver and the liver weight was measured. The blood samples after coagulation were centrifuged at 2500 rpm for 10-15 minutes and the sera isolated were used for estimation of the biochemical markers of liver damage viz. SGOT, SGPT, ALP and Bilirubin levels.

2.6. Statistical analysis

Data obtained were analyzed by ANOVA followed by t-test. Values at $p<0.001$ were considered significant.

3. Results and Discussion

Alcoholic extract of *L. ciliata* leaves in graded concentrations was tested for antioxidant activity in four different in vitro models. It was observed that the test compounds scavenged free radicals in a concentration dependent manner in the models studied. Maximum percentage inhibition of DPPH radicals by the extract was about 69% at 800µg/mL concentration (Table 1). Standard drug ascorbic acid showed about 87% inhibition of the DPPH radicals at 200µg/mL.

In the nitric oxide radical scavenging model, the maximum percentage inhibition of nitric oxide radicals by *L. ciliata* was about 80% at 800µg/mL (Table 1). Ascorbic acid at 200µg caused about 95% inhibition.

The reducing power of *L. ciliata* extract was dose dependent and is presented in Table 1. The maximum absorbance of *L. ciliata* extract at 800µg/mL is comparable with ascorbic acid 200µg/mL.

The iron chelation of *L. ciliata* extract was also dose dependent and is presented in Table 1. *L. ciliata* extract at 800µg/mL produced maximum absorbance of 0.357 while ascorbic acid at 200µg/mL produced maximum absorbance of 0.153.

In the study of hepatoprotective activity, after treatment with CCl_4, a significant increase in levels of SGOT, SGPT, ALP, Bilirubin and also an increase in liver weight as compared to the normal control were observed. While in the groups of rats pretreated with silymarin the levels of SGOT, SGPT, ALP and Bilirubin were significantly lower. The groups of rats pretreated with *L. ciliata* extract, demonstrated dose dependent inhibition of elevation of the biochemical parameters. *L. ciliata* at a dose of 400mg/kg showed inhibition of elevation of the biochemical parameters comparable with silymarin (100mg/kg). Also the liver weight was significantly reduced in silymarin and *L. ciliata* treated groups (Table 2).

Table 1. Antioxidant activity of ethanolic extract of *L. ciliata*

Treatment group	% Inhibition (Mean ±SEM)		Absorbance (Mean ±SEM)	
	DPPH	NO	Iron Chelation	Reducing Power
L. ciliata (µg/mL)				
800	69.09±1.39	79.90±1.54	0.357±0.003	0.823±0.015
400	45.85±1.32	43.05±1.17	0.190±0.006	0.427±0.009
200	27.88±1.21	20.27±0.17	0.113±0.003	0.180±0.012
100	17.27±1.05	9.72±0.17	0.070±0.006	0.080±0.006
50	16.97±3.33	3.69±0.60	0.040±0.006	0.043±0.003
25	10.30±0.80	1.84±0.44	0.030±0.006	0.013±0.003
Ascorbic acid 200 µg/mL	87.57±1.98	94.97±1.90	0.153±0.007	1.51±0.032

Values represent the mean ± SEM; number of readings in each group = 3

Table 2. Effect of ethanolic extract of *L. ciliata* on serum biochemical parameters in CCl_4 induced liver damage in rats.

Treatment	Serum biochemical parameters				Liver weight (g)
	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	Bilirubin (mg/dL)	
Vehicle	22± 1.30	31.2± 1.39	161.4± 3.53	0.58± 0.04	5.96± 0.18
CCl_4	241±18.93*	294± 8.86*	456.6±20.05*	1.28± 04*	10.68±0.32*
L. ciliata (mg/kg)					
100	59.0±3.00**	40.4±1.44**	283.8±8.49**	0.72±0.06**	8.94±0.75
200	55.2±2.13**	35.6±1.86**	221.4±10.02**	0.78±0.04**	7.04±0.38**
400	32.8±2.89**	23.6± 2.54**	115.6±12.38**	0.80±0.04**	6.38±0.45**
Silymarin	43±1.82**	39.6±3.97**	180±23.73**	0.74±0.05**	6.86±0.44**

Values represent the Mean ± SEM; number of mice used in each group = 5; * P<0.001 compared with the vehicle treated group, ** P<0.001 compared with CCl_4 treated group.

In the present study four methods were used for evaluation of antioxidant activity. The DPPH radical scavenging method and nitric oxide radical scavenging method were for direct measurement of radical scavenging activity. *L. ciliata* demonstrated good radical quenching activity against both DPPH and the nitric oxide radicals.

DPPH is a relatively stable free radical which when encounters proton donors such as antioxidants, the radicals get quenched and absorbance gets reduced [13]. Results indicated definite scavenging activity of the extract towards DPPH radicals in comparison with ascorbic acid.

Nitric oxide is a free radical produced in the mammalian cells and is involved in regulation of various physiological processes. However excess production of nitric oxide is associated with several diseases [14, 15]. Ethanolic extract of *L. ciliata* leaves has demonstrated dose dependent radical scavenging activity against NO free radicals.

Reducing power method and Iron Chelation method indirectly evaluate the antioxidant activity. *O*-phenanthroline is selective chelating agent for ferrous ion. It is used for determination of extent of reduction of ferric ions to ferrous ions by antioxidants. *L. ciliata* extract showed dose dependent increase in absorbance, which indicates conversion of ferric ions to ferrous ions. In reducing power method as well, a dose dependent increase in absorbance is indicative that the extract is capable of donating hydrogen atoms.

L. ciliata demonstrated dose dependent antioxidant activity comparable with Ascorbic acid. In all the methods, maximum antioxidant activity was found at the dose of 800 µg/mL of *L. ciliata* extract.

CCl_4 is one of the most commonly used hepatotoxin in the experimental study of liver disease. The hepatotoxic effects of CCl_4 are largely due to generation of free radicals [16]. CCl_4 is biotransformed by the cytochrome P450 system to produce the trichloromethyl free radicals, which in turn covalently bind to cell membranes and organelles to elicit lipid peroxidation [17]. Several plants e.g. *Foeniculum vulgare* [18] and *Panax notoginseng* [19] have been tested for their efficacy in controlling the CCl_4 induced liver damage. Further it has been evident that several phytoconstituents have the ability to induce microsomal enzymes either by accelerating the excretion of CCl_4 or by inhibition of lipid peroxidation induced by CCl_4. Phytoconstituents like flavonoids and triterpenoids are known to possess hepatoprotective activity [20].

Damage of liver cell is reflected by an increase in the levels of hepatospecific enzymes, these are cytoplasmic and are released in to circulation after cellular damage [21]. In this study significant increase in the total bilirubin content and in the SGOT, SGPT and ALP activities in the CCl_4 treated group could be taken as an index of liver damage. Treatment with *L. ciliata* extract inhibited CCl_4 induced increase in total bilirubin and SGOT, SGPT and ALP activities as compared with CCl_4 treated group. The CCl_4 induced a significant increase in liver weight, which is due to blocking of secretion of

hepatic triglycerides in plasma [22]. Silymarin and the extract prevented increase of liver weight in rats.

In conclusion, *L. ciliata* has strong antioxidant activity and this activity may be responsible for the hepatoprotective activity against carbon tetrachloride induced liver damage.

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