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Phytochemical profiling and antioxidant activity of *Leea macrophylla* Roxb. ex Hornem.-*in vitro* study

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Leea macrophylla Roxb. ex Hornem., a traditional medicinal plant reported remedies for diseases in rural India. However, proper justification and validation of their traditional practice are lacking. Hence this study was done to explore a comprehensive account of phytochemical profiling and antioxidant activities. The presence of phenols, tannins, alkaloids, saponins and flavonoinds was confirmed through phytochemical screening. After estimation of the phytochemical, we get 104.05 mg of GAE/g of phenol, 94.78 mg of GAE/g of tannins, 21.40 mg/g of alkaloids, 05.50 mg/g of saponins and 117.98 mg of QE/g of flavonoids. Its further analysis takes place through GC-MS, get 14 bioactive compounds, which have more than 1% peak area. They had already reported a good medication value in various remedies. The antioxidant test, through DPPH assay, shows, IC_{50} values of *L. macrophylla* is 51.31 µg/mL and gallic acid (taken as standard) have 39.91 µg/mL. By comparing with gallic acid, it can be concluded that *Leea macrophylla* also has a good antioxidant activity.

Keywords: Antioxidant, GC-MS, *Hathikan, Leea macrophylla*, Phytochemicals

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Since time immemorial medicinal plants and their products have been used for the treatment of various human diseases[1]. Medicinal plants occupied a major position in the pharmaceutical companies; herbal medicines remain the major source of health care for the world population[2]. It is believed that the drug of natural origin play a vital role in healthcare without any side effects[3]. Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health[4].

The medicinal plant *Leea macrophylla* Roxb. ex Hornem, locally known as *Hathikan,* in Jharkhand belongs to the family Vitaceae. Its leaves are simple, broadly ovate, nearly as broad as long, the dark green lower leaves up to 60 cm long, the light yellowish green upper leaves 15-23 cm long, apex acute or acuminate, coarsely serrate or sub-lobed, pubescent beneath, main nerves opposite, 8-10 pairs, very prominent; petioles 5-12 cm long, deeply striate, glabrous. Inflorescence terminal, much branched, puberculous, corymbose cymes, up to 30 cm long, flower white. Berry globose, 6-8 cm in diameter, black, 3-6 celled, depressed globose, usually 3-6 lobed[5] (Fig. 1).

This plant has anticancer properties. Powder of leaves mixed with honey is given to cancer patients. Its bark powder is also given orally to cure cancer. Decoction of tuber is given to animal with drenching tube in dysentery. Tuber powder is given to cure

Fig. 1 — Morphology of the plant *Leea macrophylla* Roxb. ex Hornem.

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sexual debility in male[6]. It is used as remedy for Viper snake bite in *Pawra* Tribe of Satpura Hills, Maharashtra, India. Its seeds are crushed in water and given orally till the patient vomits[7]. Recently the plant leaf has been studied for its anti-inflammatory activity in wound healing[8]. However, its root tubers have not been scientifically validated for its various therapeutic values. Hence the present study intends to investigate the bioactive compounds and *in vitro* antioxidant activity of *Leea macrophylla* root tuber which is used for various remedies. This information can be useful in order to study the suitability of this plant as a new source of natural compounds.

Material and Methods

Collection and extraction of plant material

The root tubers of *L. macrophylla* were collected in the month of October 2016 from the well labeled germplasm conservation Garden of Birsa Agriculture University, Ranchi, Jharkhand. The plant was authenticated by Professor HP Sharma, Department of Botany, Ranchi University, Ranchi, comparing with the authentic samples at the Herbarium of Botany Department of Ranchi University, Ranchi. The voucher specimen no. of the plant is HPS/DM/150.

The tubers were separated from the plant and washed carefully. The washed tubers were cut into small pieces and shade dried. After 3 weeks, when tuber pieces are completely dried, finally it is ground into powder form. The powder was soaked with methanol (1:10 ratio) in a flask. The flask was covered with aluminum foil to avoid evaporation and then kept for 48 hours in shaker incubator. After 48 h the solution was filtered by using whattman filter paper no.1 and the filtrate was collected in a beaker. Then the filtrate was kept in incubator at 37°C to evaporate the solvent. The prepared extract was then stored at 4°C for further use[9].

Phytochemical screening

The methanolic and aqueous extract was subjected to preliminary screening for the presence of different phytoconstituents using various qualitative reagents as per standard procedures[10].

Phytochemical estimation

The phytochemicals of methanolic extract of *L. macrophylla* was estimated, based upon phytochemical screening results. Total phenolic and tannin contents were estimated by Folin-ciocalteu method[11]. Total phenolic and tannin values were expressed in terms of µg gallic acid equivalent (GAE) per mg of dry extract and the absorbance of all samples was measured at 765 nm spectrometrically. Total alkaloid and saponin content in the extract was determined on dry weight basis[12]. For estimation of total flavonoid content, Aluminium Chloride method was used[13]. In this method Quercetin was used as standard and flavonoid content were measured as quercetin equivalent (QE). The absorbance was noted at 510 nm using UV-visible spectrometer.

Chromatographic analysis

Secondary metabolites of methanolic crude extract of *L. macrophylla* were identified by GC-MS analysis. The GC-MS analysis of the extract was performed using GC-MS QP-2010 Plus SHIMADZU Company instrument equipped with Rtx-5 MS column (30×0.25 mm id, film thickness 0.25 µm). Initially, oven temperature was maintained at 80°C for 2 min and temperature was gradually increased up to 250°C at 5 min. and 1.0 µL of sample was injected for analysis. Helium was the carrier gas. The flow rate of helium gas was 1.2 mL/min. The sample injector and mass transfer line temperature were set at 250° and split ratio is 10 throughout the experiment periods. The ionization mass spectroscopic analysis was done with 70 eV. Mass spectra were recorded across the range of 40 to 600 m/z for the duration of 50 min[14]. Screening of compounds was based on comparison of their mass spectra with those of Wiley-8 and NIST-14 libraries.

Free radical scavenging activity

The total antioxidant activity was measured by the DPPH (2,2-diphenyl-2-picryl hydrazyl) radical scavenging assay method[15]. Briefly about 1 mL of DPPH solution (0.1 mmol/L) prepared in methanol was added to 3 mL of test sample and standard (gallic acid) solution at different concentration (20-200 µg/mL). After 30 min incubation at 30ºC in dark, the absorbance was measured at 517 nm and percentage inhibition was calculated. Control was also carried out to determine the absorbance of DPPH, before interacting with the extract.

Data Analysis

All the results were presented as mean±standard error (S.E.) in triplicate experiments. Significant difference was analyzed by Student t-test. A p value<0.05 was regarded as a significant difference from the corresponding control group.

Results

Phytochemical evaluation

Preliminary phytochemical screening test was done for phenol, tannins, alkaloids, saponin and flavonoids. All of these five phytoconstituents showed the positive result in methanolic extract of *L. macrophylla* (Table 1).

The mean absorbance (λ_{max}) of standard gallic acid and quercetin acid is presented in Table 2 and Table 3 respectively. Table 4 shows the contents of total phenols that were measured by Folin-ciocalteu reagent in terms of gallic acid equivalent. The result of total phenol content was calculated from the regression equation of the standard plot (y=0.008x+0.018, R^2=0.992, Fig. 2). The phenol content in 1 g methanolic extract of *Leea macrophylla* was 104.05 mg of GAE/g (gallic acid equivalent).

The tannins contents of plant extracts was also examined using the Folin-ciocalteu's reagent is expressed in terms of gallic acid equivalent from regression equation of the standard plot (y=0.008x+0.018, R^2=0.992, Fig. 2). The values obtained for the concentration of tannin contents are expressed as mg of GAE/gm of extract. The tannin content in *Leea macrophylla* was 94.78 mg of GAE/g (Table 4).

Alkaloid and saponin per gm dry sample of tuber of *Leea macrophylla* plant was found to be 21.40 mg and 05.50 mg respectively (Table 4). Alkaloids are the largest group of phytochemicals causing toxicity against cells of foreign organisms; they are also helpful in fighting with cancer[16]. Saponins have been reported as bioactive antibacterial agents of plants, which are a glycoside, have the property of precipitating and coagulating red blood cells[17].

The total flavonoid content was calculated from the regression equation of the standard plot (y=0.000x-0.002, R^2=0.993) and is expressed as quercetin equivalents (QE, Fig. 3). Total flavonoid content was recorded at 1 mg/mL methanolic plant extract was 117.98 mg of QE/g (quercetin equivalents) (Table 4). Flavonoids are the most common and widely distributed group of plant's polyphenolic compounds, characterized by a benzo-γ-pyrone structure. It is ubiquitous in fruits and vegetables.

Table 1 — Phytochemical screening of *Leea macrophylla*

Sl. no.	Phytochemical	Observation	Present/Absent
1	Phenol	Reddish black	Present
2	Tannin	Brownish black precipitation	Present
3	Alkaloid	Yellow precipitatation	Present
4	Saponin	Foam formed	Present
5	Flavonoid	Yellow color	Present

Table 2 — Absorbance of standard compound, gallic acid in 765 nm wave length

Concentration (µg/mL)	Absorbance (Mean) λ_{max} =765 nm
1	0.008
2.5	0.0320
5	0.0860
10	0.1030
15	0.1340
20	0.1750
25	0.2870
50	0.4350
100	0.8850

Table 3 — Absorbance of standard compound (Quercetin Acid)

Concentration (µg/mL)	Absorbance (Mean) λ_{max}=510 nm
1	0.0010
10	0.0020
20	0.0040
40	0.0070
60	0.0130
80	0.0200
100	0.0310
200	0.0700
300	0.0940
400	0.1200
500	0.1500
600	0.2000

Table 4 — The total phenol, flavonoids, tannins, saponins and alkoloids content present in methanolic extracts of *Leea macrophylla*

Parameter	Unit	Amount
Phenol content	mg of GAE/g of extract	104.05
Tannins content	mg of GAE/g of extract	94.78
Alkoloids content	mg/gm of dry material	21.40
Saponins content	mg/gm of dry material	05.50
Flavonoid content	mg of QE/g of extract	117.98

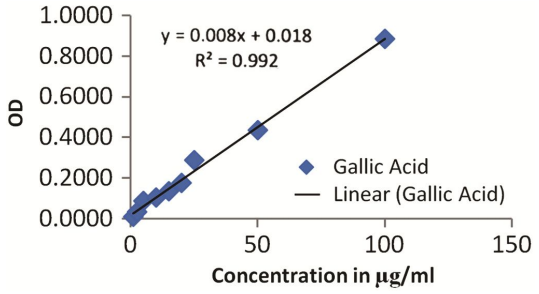

Fig. 2 — Calibration standard curve of gallic acid for determination of total phenol and tannins content in *Leea macrophylla*

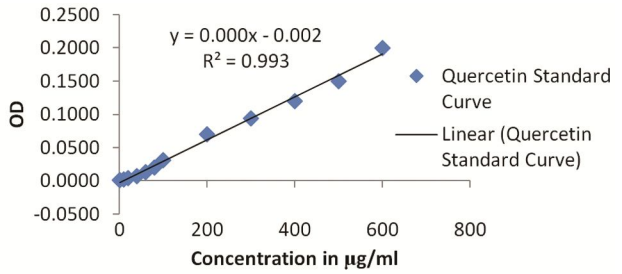

Fig. 3 — Calibration standard curve quercetin for determination of total flavonoid content in *Leea macrophylla*

GC-MS analysis

In detail, the compounds present in mathanolic extract of *Leea macrophylla* is identified by GC-MS analysis. Fig. 4 represents a typical chromatogram of GC–MS, while the useful bioactive compounds having more than 1% peak area in GC–MS analysis with their activity is given in Table 5.

The results of the GC-MS analysis showed the presence of 14 bioactive compounds have more than 1% peak area. Among them 1,3-Propanediol,

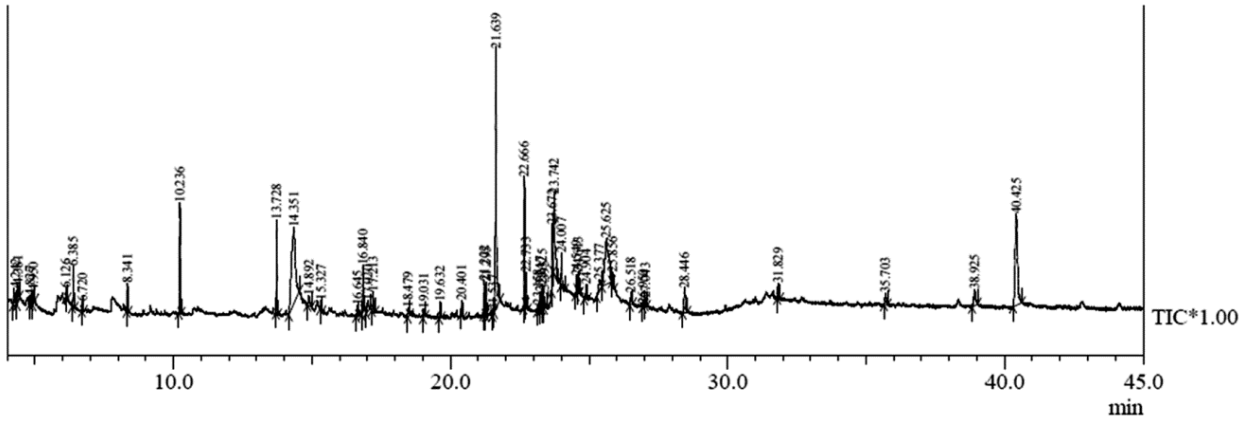

Fig. 4 — GC-MS analysis of methanolic extract of *Leea macrophylla*

Table 5 — Activities of major phyto-components identified in *Leea macrophylla* by GC-MS analysis in order of their retention time.

Sl. No	Name of compound	Peak area%	Activity
1	Cyclopent-4-ene-1,3-dione	1.13	Antifungal, antibacterial, anti-inflammatory, anti-diabetic, cytostatic and specific enzyme inhibitory activities[18].
2	Dihydroxyacetone	1.10	Antifungal, anti-pigmentation agent, delays photocarcinogenesis[19].
3	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	15.18	Microbicidal and it is used as a bacteriostat in disinfectants[20].
4	Iron, tricarbonyl[N-(phenyl-2-pyridinylmethylene	1.58	Fungicidal activity[21].
5	Hexadecanoic acid, methyl ester	1.13	Flavoring agent, Lubricant, antiandrogenic, antioxidant, 5- alpha-reductase inhibitor[19].
6	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	1.14	Antioxidant activity[22].
7	n-Hexadecanoic acid	14.59	anti-inflammatory, anti- rheumatic[23], Antifungal, Antioxidant, hypocholesterolemic, nematicide, anti-androgenic flavour, haemolytic, 5-Alpha reductase inhibitor and potent antimicrobial agent[24].
8	Palmitic Acid, TMS derivative	4.46	Anti-inflammatory[23].
9	9,12-Octadecadienoic acid (Z,Z)-	2.20	Pesticide[25].
10	cis-9-Hexadecenal	5.25	Pheromone[26].
11	Octadecanoic acid	1.63	Anti-aging, antiviral and anti-inflammatory activities[19].
12	1,2-Benzenedicarboxylic acid	1.95	Endocrine disruptors[21].
13	Stigmasterol	1.61	Antioxidant, Anti-mutagenic, Anti-inflammatory, Anti-osteoarthritic, Anti-hypercholestrolemi, Cytotoxicity, Anti-tumor, Hypoglycemic activity and effect on thyroid[27].
14	Gamma-Sitosterol	11.53	Anti-diabetic & antihyperlipidemic activity[28].

2-(hydroxymethyl)-2-nitro, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z), cis-9-Hexadecenal and gamma-Sitosterol were predominant in term of the percentage. These compounds have been reported to posses certain biological actions such as pesticide, anti-inflammatory, antibacterial, antifungal, anti diabetic, antioxidant and anti-aging[18-28].

Antioxidant activity

It is well known that there is a strong relationship between total phenol content and antioxidant activity, as phenol possess strong scavenging ability for free radical due to their hydroxyl groups. Therefore, the phenolic content of the plants may directly contribute to their antioxidant action or free radical scavengers[29]. DPPH has been widely used to evaluate the antioxidant activity of plant extracts. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH[30]. In the present study DPPH radical scavenging of *Leea macrophylla* extract was investigated and results were shown in Table 6. By solving the binomial equation ($y= -0.0022x^2+0.8129x+14.081$; $R^2 = 0.9887$) and ($y = 26.40\ln(x) - 47.33$; $R^2 = 0.988$) of the regression curve in Fig. 5, get the IC_{50} values of *L. macrophylla* and gallic acid, 51.31 µg/mL and 39.91 µg/mL respectively.

Table 6 — DPPH radical scavenging activity of *Leea macrophylla* tuber in comparison to standard gallic acid

Concentration (µg/mL)	Percentage radical scavenging activity	
	Plant extract of *L. macrophylla*	Gallic acid (standard)
20	22.38	32.24
40	41.63	46.38
60	54.38	63.81
80	63.05	69.64
100	71.19	74.38
200	86.48	91.41
IC_{50}	51.87	39.91

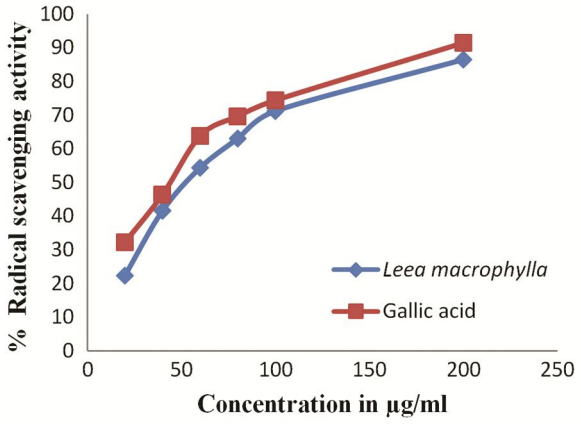

Fig. 5 — A comparative graph of % radical scavenging activity in *Leea macrophylla* with gallic acid

Discussion

The phytochemicals or secondary metabolites contribute a significant role towards the biological activity of medicinal plants[31]. Hence in the present study preliminary phytochemical screening test for significant secondary metabolites has done.

It was found in the estimation of secondary metabolites, phenols, tannins and flavanoids are in good quantity. Free radical scavenging activity of phenolics and flavonoids imparts their antioxidant potential and major phytoconstituents from plant sources responsible for antimicrobial activity includes phenolics, saponins, flavonoids, tannins, and alkaloids[32].

GS-MS analysis confirms the presence of 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z), cis-9-Hexadecenal and gamma-sitosterol in a quite considerable amount. There is no result in literatures about identification and isolation of bioactive compounds in *Leea macrophylla*. After go through the study of these individual compounds it was found that, these compounds are responsible for various pharmacological actions like pesticide, anti-inflammatory, antibacterial, antifungal, antidiabetic, antioxidant and anti-aging, etc.[18-28].

Further study explored the antioxidant potential and therapeutic values of *Leea macrophylla* root tuber trough DPPH assay. DPPH radical scavenging model is widely used method to evaluate antioxidant activity of natural compound and plant extracts[33]. In the present study DPPH radical scavenging activity in methanolic extract was found IC_{50} value 51.31 µg/mL. whereas, earlier it was reported as IC_{50} value 46.41 µg/mL in ethanolic extract[34]. Antioxidants obtained from plants are considered as important nutraceuticals on account of many health benefits. This play a pivotal role in the progression of several degenerative diseases such as aging related diseases, cancer, cardiovascular diseases, diabetes mellitus, and various neurodegenerative disease, via DNA mutation, protein oxidation, and/or lipid peroxidation[32].

Conclusion

In conclusion, the present study shows *Leea macrophylla* is an ethanomedicinal plant,

used in various remedies from ancient history to now, contains a good quantity of bioactive compounds. The phytochemical analyses of the methanolic extract the plants were carried out and found to have contained good quantity of secondary metabolites like phenolic, saponin, flavonoids, alkaloids and tannins. The study also justified the antioxidant potential of the plant extract. The identification of bioactive compound in *Leea macrophylla* was done by GC-MS analysis which shows the presence of 14 compounds in good quantity. These are very much useful in anti-bacterial, anti-fungal, anti-inflammatory, anti-diabetic, anti-oxidant, anti-mutagenic and anti-aging activities. Hence, the present study gives a scientific validation to the traditional uses of *Leea macrophylla* in various ailments and also this study will helpful to discover bioactive natural compounds that may lead to the development of new pharmaceuticals research activities. Further studies are needed to isolate these bioactive compounds for more efficacies in various remedies.

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