Journal of Plant Sciences
2015; 2(6-1): 24-29
Published online June 20, 2015 (http://www.sciencepublishinggroup.com/j/jps)
doi: 10.11648/j.jps.s.2014020601.15
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Characterization of chemical groups, and investigation of cytotoxic and antioxidant activity of *Litsea glutinosa* leaves

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To cite this article:
Rumpa Bhowmick, Md. Shahid Sarwar, Syed Masudur Rahman Dewan, Abhijit Das, Binayok Das, Md. Mohiuddin, K. M. Rahat Maruf Jitu, Mohammad Safiqul Islam. Characterization of Chemical Groups, and Investigation of Cytotoxic and Antioxidant Activity of *Litsea Glutinosa* Leaves. *Journal of Plant Sciences.* Special Issue: Pharmacological and Biological Investigation of Medicinal Plants. Vol. 2, No. 6-1, 2014, pp. 24-29. doi: 10.11648/j.jps.s.2014020601.15

Abstract: The present study was conducted to investigate possible phytochemicals, cytotoxic activity, total phenolic content and antioxidant property of different extracts of *Litsea glutinosa* leaves. To determine different pytochemicals, various standard group tests were done. For cytotoxicity test, brine shrimp lethality bioassay was carried out. To evaluate the antioxidant properties, some complementary test systems, namely DPPH free-radical scavenging assay and reducing power assay and determination of total phenolic content were conducted. The phytochemical analysis revealed the presence of alkaloids, carbohydrate, flavonoid, saponin, gum, steroid, tannins and terpinoids. In brine shrimp lethality bioassay, results showed that all the extracts possess significant ($P<0.05$) activity when compared to the standard, Colchicine. Among the extracts, the n-hexane soluble fraction showed the highest activity (LC_{50} 30.32±0.46µg/ml) which is very close to the standard (LC_{50} 30.11±0.30µg/ml) used. In DPPH free radical scavenging test, IC_{50} value of the ethyl acetate extract was found fairly significant (9.68±0.15µg/ml) while compared to that of the reference standards ascorbic acid (1.82±0.15µg/ml). In reducing power assay, the maximum reducing capacity for the methanolic extract was found 257.67±4.04 at 200µg/ml while compared to standard ascorbic acid (356.33±5.68µg/ml). The total phenolic amount was also calculated as quite high in the ethyl acetate extract (69.00±0.58mg/g of gallic acid equivalent). Presence of significant antioxidant properties of different extracts would justify its traditional use. However, it would be interesting to investigate possible causes and their mechanisms responsible for the cytotoxic and antioxidant properties of the plant *L. glutinosa*.

Keywords: Antioxidant Activities, Cytotoxicity, DPPH Free Radical Scavenging, *Litsea Glutinosa*, Total Phenolic Content

1. Introduction

Litsea glutinosa (Lour.) (Family: Lauraceae) is a well-known evergreen species growing wild in the forest of Chittagong and Sylhet districts in Bangladesh [1]. Leaves are mucilaginous and considered for antispasmodic, emollient, and poultice. The leaves are also used in diarrhea and dysentery as well as in wounds and bruises [1]. The leaves were reported for the treatment of the spontaneous and excessive flow of semen in young boys [2]. The leaf extract also shows antibacterial and cardiovascular activities [3]. The berries yield oil which is used by some tribal practitioners in the treatment of rheumatism. Tannin, β-sitosterol, and actinodaphnine are reported to be the common constituents of the species; and other constituents known are: boldine, norboldine, laurotetanine, n-methyllaurotetanine, n-methylactinodaphnine, quercetin, sebiferine, litseferine etc. [4].

Plants are potential sources of natural antioxidants. Synthetic antioxidants may have adverse biological effects on human body; therefore, much attention has been put toward natural antioxidants [5]. *L. glutinosa* was selected due to its availability in Bangladesh is huge, therefore, lots of people in the rural area use this plant for different treatments, and not such investigations have been carried out with this plant of this region. Our main goal was to evaluate the possible

chemical groups and investigate the cytotoxic and antioxidant properties of the plant leaves to validate its folkloric uses.

2. Materials and Methods

2.1. Plant Material Collection and Identification

For the investigation, the leaves of *L. glutinosa* were collected by the authors from Potia, Chittagong, Bangladesh in July 2012. The plant was identified and authenticated by an expert botanist of Bangladesh National Herbarium (DACB), Mirpur, Dhaka (Accession No. 38277) and a voucher specimen was submitted at the herbarium for future reference.

2.2. Extract Preparation

Weighed (630 g of the dried and powdered) sample was soaked in 2200 ml of 99% methanol (Merck KGaA, Germany) in clean, sterilized, and flat-bottomed glass container. Afterwards, it was sealed and maintained for 15 days accompanying occasional stirring and agitation. The complete mixture was then subjected to coarse filtration on a piece of clean, white sterilized cotton material and Whatman® filter paper. The extract was obtained by evaporation using rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at 4 rpm and 65ºc temperature. It rendered a gummy concentrate of greenish color. The gummy concentrate was designated as crude extract or methanolic extract. Then the crude methanolic extract was dried by freeze drier and preserved at +4ºC (yield 0.79%). The concentrated methanolic extract was partitioned by modified Kupchan method [6] and the resultant partitionates i.e., n-hexane (yield approx. 19.02%), ethyl acetate (yield approx. 26.54%), and chloroform (yield approx. 6.59%) soluble fractions were used for the experimental processes.

2.3. Chemicals

All the chemicals used in this study were of analytical grade, and purchased from Sigma Chemical Co. (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

2.4. Phytochemical Screening

Different phytochemicals were screened following preliminary quantitative phytochemical analysis such as alkaloids with Mayer's and Hager's reagent, carbohydrates with Benedict's test and Fehling's test, phytosterols with Salkowski's test and LibermannBurchard's test, flavonoids with alkaline reagent test and lead acetate test, tannins with gelatin test, saponins with Froth test and foam test, phenols with ferric chloride test, gums and mucilages [7-8].

2.5. Brine Shrimp Lethality Bioassay

The cytotoxic activities of the extracts were examined using brine shrimp lethality bioassay [9]. In this study colchicine was used as the positive control. Measured amount of the standard was dissolved in DMSO to get an initial concentration of 40µg/ml from which serial dilutions were made using DMSO to get 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125 µg/ml, 0.15625µg/ml and 0.78125µg/ml solution from the extracts. Then the positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.100µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups.

After 24 hours, by using a magnifying glass, the vials were inspected and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

2.6. DPPH Free Radical Scavenging Assay

The stable DPPH free-radical scavenging activity was measured using the modified method described by Chang et al. [10]. Stock solution (1mg/ml) of the extracts *L. glutinosa* was prepared in respective solvent systems from which serial dilutions were carried out to obtain the concentrations of 5, 10, 20, 40, 60, 80, and 100 µg/ml. In this assay, 2 ml of 0.1 mM methanolic DPPH solution was added to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 sec. Then the solutions were allowed to stand at dark place at room temperature for 30 min occurring chemical reaction. After 30 min, absorbance was measured against a blank at 517 nm with the double beam UV-Visible spectrophotometer. The percentage of DPPH free radical-scavenging activity of plant extract was calculated as:

$$\text{DPPH free-radical scavenging activity (I \%), } = [(A_0 - A)/A_0] \times 100,$$

Where, A_0 is the absorbance of the control solution (containing all reagents except plant extract); A is the absorbance of the DPPH solution containing plant extract.

The DPPH radical-scavenging activity (%) was plotted against the plant extract concentration (µg/ml) to determine the concentration of extract necessary to decrease DPPH radical-scavenging by 50% (called IC_{50}). The IC_{50} value of the extract was estimated by sigmoid non-linear regression, using Sigma Plot 2000 Demo (SPSS Inc., Chicago, IL, USA). All determinations were performed in triplicate. Ascorbic acid was used as positive control standard.

2.7. Reducing Power Assay

The method of Dehpour et al. [11] was followed to determine the reducing power of *L. glutinosa* leaves. 1 ml of extract solution of different concentrations (5, 10, 20, 40, 60, 80, 100 µg/ml) was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide $[K_3Fe(CN)_6]$ (1% w/v). The mixture was incubated at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of trichloroacetic

acid (10%, w/v), then the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1% w/v) solution. Then the absorbance was measured at 700 nm against a blank using UV spectrophotometer. Increased absorbance value of the reaction mixture indicates increased reducing power. Three replicates were made for each test sample and average data was noted. Here, Ascorbic acid was used as positive control standard.

2.8. Investigation of Total Phenolic Content

Using the modified Folin-ciocalteu method, total phenolic content of the extract was determined [12]. Briefly, 0.5 mL of the extract (1 mg/ml) was mixed with 5 ml Folin-ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of sodium carbonate. Then the mixture was vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. The absorbance was read at 765 nm with a spectrophotometer (UV-1800, Shimadzu, Japan). Total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve.

3. Results

3.1. Phytochemical Screening

The presence of the possible phytochemical constituents in the crude extract, and its n-hexane and ethyl acetate soluble fraction is shown in Table 1.

Table 1. *Phytochemical screening of various extracts of L. glutinosa leaves*

Plant in extract	Tannins	Flavonoids	Saponins	Gums and carbohydrates	Steriods	Alkaloids	Redu-cing sµgar	Terpiniods
Methanolic extract	_	_	+++	+++	+++	+++	+++	++
n-hexane extract	++	_	+++	++	++	+++	++	++
Ethyl acetate extract	++	++	+++	+++	++	+++	+++	++

Symbol (+++) indicates presence in high concentration, Symbol (++) indicates presence in moderate concentration, Symbol (+) indicates presence in trace concentration, and (-) indicates absence of the respective phytochemical.

3.2. Brine Shrimp Lethality Bioassay

Table 2. *Cytotoxic activity of various extracts of L. glutinosa leaves*

Test Sample	Log C	% Mortality±SEM	LC_{50} (µg/ml)
Methanol Extract	1.398	36.66±0.22*	114.70±0.26
	1.699	38.99±0.39*	
	2.000	46.85±0.16*	
	2.301	52.29±0.06*	
	2.602	61.3±0.38*	
	2.903	78.49±0.37*	
Ethyl acetate Extract	1.398	41.63±0.84	43.97±0.53
	1.699	50.23±0.17*	
	2.000	60.66±0.34*	
	2.301	81.02±0.93*	
	2.602	91.58±0.82*	
	2.903	97.9±0.50*	
n-hexane Extract	1.398	43.36±0.61*	30.32±0.46
	1.699	59.2±0.62*	
	2.000	68.77±0.33*	
	2.301	79.18±0.74*	
	2.602	89.49±0.94*	
	2.903	92.87±0.08*	
Colchicine (standard)	1.398	40±0.29	30.81±0.30
	1.699	56.58±0.32	
	2.000	75±0.29	
	2.301	100±0.0	
	2.602	100±0.0	
	2.903	100±0.0	
Control	1.398	0±0.0	188.59±1.39
	1.699	1±0.58	
	2.000	12.99±0.33	
	2.301	7.04±0.42	
	2.602	20.52±0.29	
	2.903	16.66±0.33	

*P<0.05, Statistically significant difference as compared to the standard; SEM= Standard error mean

Table 2 shows the possible cytotoxic activities of different extracts. Results showed that all the extracts possess significant activity when compared to the standard. Among the extracts, the n-hexane soluble fraction showed the highest activity which is very close to the standard used.

3.3. DPPH Free Radical Scavenging Activity

Table 3. *DPPH free radical scavenging activity of various extracts of L. glutinosa leaves*

Sample	Conc.(µg/ml)	Mean % DPPH scavenging activity ±SD	IC_{50}
Standard(Ascorbic Acid)	25	53.9±1.12**	1.82µg/ml
	50	72.7±1.94***	
	100	75.04±2.64**	
	200	80.65±1.59***	
	400	85.5±0.669***	
	800	90.0±0.990**	
Methanol Extract	25	21.67±6.66**	27.94µg/ml
	50	36.00±4.58***	
	100	51.33±3.51**	
	200	60.67±2.08**	
	400	66.00±2.65***	
	800	75.00±3.00**	
n- hexane Extract	25	8.67±0.98**	16.78 µg/ml
	50	21.14±1.03***	
	100	38.49±1.01**	
	200	54.81±1.01***	
	400	70.51±1.54**	
	800	78.71±2.07***	
Ethyl acetate Extract	25	35.17±4.40**	9.68 µg/ml
	50	48.64±2.50***	
	100	60.20±1.71**	
	200	66.59±2.10*	
	400	75.26±3.00*	
	800	83.04±2.37*	

Sample	Conc.(µg/ml)	Mean % DPPH scavenging activity ±SD	IC_{50}
Chloroform Extract	25	14.45±1.03*	45.60 µg/ml
	50	33.13±1.01*	
	100	53.12±1.01**	
	200	50.65±1.52*	
	400	57.91±2.00**	
	800	71.37±1.54**	

SEM= standard error mean (n = 5); One way ANOVA followed by Dennett's test was performed as the test of significance. The values *p < 0.05, **p < 0.01, ***p < 0.001 were considered significant as compared with control group

The investigation shows that DPPH free radical scavenging activity of crude ethanolic extract, ethyl acetate, n-hexane, and chloroform soluble fractions of *L. glutinosa* leaves were found to be increased with the increase of concentrations of the extracts (Table 3). The result revealed that ethyl acetate soluble fraction has the highest antioxidant activity than those of other extracts at concentration 800µg/ml.

3.4. Reducing Power Assay

The reducing power capacity of various extracts has been tabulated in Table 4. The results show that the reducing power capacity of the crude methanol extracts increases with increasing concentration, and shows the highest capacity at concentration of 200µg/ml.

Table 4. *Reducing power assay of methanol extract of L. glutinosa*

Sample	Final Conc. in the reaction mixture (µg/ml)	Control absorbance	Absorbance (measured at 700 nm)	Reducing capacity (Mean±SEM)	%Reducing capacity
Ascorbic acid	25	0.154	0.477	206.67±4.16***	210
	50	0.154	0.495	218.00±3.00***	221
	100	0.154	0.528	239.33±4.04**	243
	150	0.154	0.588	279.00±3.00***	282
	200	0.154	0.705	356.33±5.68***	358
Methanol extract	25	0.154	0.37	143.00±3.61***	94
	50	0.154	0.391	158.00±4.58***	142
	100	0.154	0.455	202.67±8.02**	155
	150	0.154	0.513	237.00±4.00***	166
	200	0.154	0.545	257.67±4.04***	299

SEM= standard error mean (n = 5); One way ANOVA followed by Dennett's test was performed as the test of significance. The values **p < 0.01, ***p < 0.001 were considered significant as compared with control group

3.5. Total Phenolic Content of L. Glutinosa

Based on the absorbance values of the extract solutions, the colorimetric analysis of the total phenolics of the extract was determined and compared with that of the standard solution of gallic acid equivalents. Result (Table 5) shows the total phenolic amount calculated for *L. glutinosa.* The results reported that total phenolic content of all extracts were correlated with the activity of gallic acid and showed that moderate amount of phenolics which would play an important role in the antioxidant activity of plant materials.

Table 5. *Total Phenolic Content of various extracts of L. glutinosa leaves*

Extract	Avg. absorbance at 765 nm	Total phenolic content (mg gallic acid equivalent (GAE) per gm of dry extract)
Methanol extract	0.056±0.09	51.98±1.26
n-hexane extract	0.1596±0.37	53.18±0.57
Ethyl acetate extract	0.1536±0.19	69.00±0.58
Chloroform extract	0.148±0.58	52.02±1.55

Values are expressed as mean±SD (n=3)

4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents [13]. Particularly the secondary metabolites (phytochemicals) existing in the plant extract play a key role in the pharmacological actions of any plant or plant parts. This study was conducted to make an evidential approach in ascertaining the mentioned biological functions of *L. glutinosa* leaves extract. Alkaloids, terpenoids, steroids, saponins, gums and reducing sugars were present in different amount in the studied extract. These screened results were almost similar with the previously conducted partial studies [14]. Incidentally, minor differences between the results of distinct studies could be related to differences in local climate and soil composition [15].

In our present study the cytotoxic assay was performed using brine shrimp lethality bioassay and the highest cytotoxic activity of *L. glutinosa* leaves was found in n-hexane soluble fraction. It was reported by Tiwari et al. [16] that several active compounds such as anthocyanins, saponins, tannins, flavones, and polyphenols etc. can be

easily obtained if organic solvents (methanol, n-hexane, petroleum ether, chloroform etc.) are used as solvent in the extraction technique. These compounds are known to be free radical scavenger, reactive species quencher, hydrogen donor, antioxidant enzymes activator, detoxification inducer, normal cell differentiation promoter, tumor production and proliferation cell inhibitor, and apoptosis inducer [17]. Besides, some of these bioactive compounds are shown to have inhibitory action on carcinogenesis, such as triterpenoid, saponin showed its cytotoxicity in HeLa cells through both mitochondrial dysfunction and ER stress cell death pathways [18]. It was also proved that flavonoid effectively suppressed the proliferation of a human colon carcinoma cell line (COLO 201) through apoptosis induction [19], while phenolics showed anticancer activity on cancer colon cell by arresting the cell cycle [20]. So, it may be predicted that the bioactive compounds present in the *L. glutinosa* leaves may be accountable for the possible cytotoxic effect of this plant, though the exact mechanism of action and the key compounds responsible for demonstrating the cytotoxic activity are yet to be discovered.

Free radicals and other reactive oxygen species such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide are highly reactive chemical species formed in aerobic life. They play a dual role which is both deleterious and beneficial to the living system [21]. The DPPH assay is one of the most common and relatively quick methods used for testing free radical scavenging activity of various plant extracts [22]. In the DPPH assay the antioxidants may react through donating either electron or hydrogen and here, a faster reaction indicates more potent free radical scavenging activity [23]. In our present study it is found that the ethyl acetate soluble fraction of *L. glutinosa* leaves showed better free radical scavenging activity than the others. This may be described by the facts that solvent system plays an important role in the solubility of phytochemical components of the crude extracts [24] and also the DPPH scavenging activity differs depending on used solvent and food matrix [25].

Reducing power assay is widely used to evaluate the antioxidant activity of polyphenols. The reducing power is generally associated with the presence of reductones, which exerts antioxidant action by breaking the free radical chain by donating a hydrogen atom [26]. The reducing power of a compound is related to its electron transfer ability and may, therefore, serve as a significant indicator of its antioxidant activity [27]. In the present study the reducing power assay of different extracts of *L. glutinosa* leaves along with that of ascorbic acid, at concentrations between 50–200 µg/ml, showed that high absorbance indicates high reducing power [28]. The reducing power of the plant extracts were increased as the amount of extract concentration increased. This may be because of the presence of reductants such as antioxidant substances in the samples that causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form [29]. In our study, the reducing power of extract was lower than that of ascorbic acid but the IC_{50} value of extract was close to that of ascorbic acid indicating that *L. glutinosa* has a statistically significant ($P < 0.05$) reducing power. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts [30].

Phenolic compounds are known as powerful antioxidants because of their potent chain breaking action and they may contribute directly to the anti-oxidative activity [21]. In our present study we tried to determine the amount of phenolic contents present in different extracts of *L. glutinosa* leaves and found that the highest amount of phenolic compounds is present in the ethyl acetate soluble fraction. The reason behind this may be that the solvent polarity plays a key role in increasing phenolic solubility [31]. Another reason may be different phenolic compounds may show different antioxidant activities, either synergistic or antagonistic effect, depending on their structure which are present in the crude extract [32]. Again, several studies have also showed that, the different levels of antioxidant activities in plants may be due to not only differences in their phenolic contents, but also in their phenolic acid components [33]. Thus it may be postulated that the high content of total phenolic components in the ethyl acetate soluble fraction of *L. glutinosa* leaves may have led to the better results found in the total antioxidant activity and free radical scavenging ability of the plant extract.

5. Conclusion

According to the results of the present study, it can be summarized that the plant extract possesses significant cytotoxic and antioxidant activities. Therefore, additional studies are being suggested to better understand the mechanism of such actions scientifically.

Acknowledgment

The authors are grateful to DACB. Authors are also thankful to Department of Pharmacy, Noakhali Science and Technology University for providing the laboratory facilities.

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