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In vitro antioxidant activity of methanolic leaves and barks extracts of four *Litsea* plants

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ABSTRACT

As far as our literature survey could ascertain, a very few information were available on the in vitro antioxidant activities of the Litsea (Family: Lauraceae) plant. Therefore, the aim of this current investigation is to evaluate the in vitro antioxidant capacities of leaf and bark extracts of four Litsea spp. The antioxidant activity of Litsea extracts were evaluated by various antioxidant assays such as DPPH scavenging, nitric oxide scavenging, superoxide scavenging, metal chelating activity and reducing power potency. Phytochemical screening and the total phenol and flavonoids content were also estimated. A positive correlation between the antioxidant activities and physiochemical assays was observed and the highest scavenging activity was noticed in bark of Litsea monopetala. Results obtained in the present investigation indicate clearly that the extracts of Litsea spp possesses significant antioxidant properties and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Keywords: Free radicals, Antioxidant, Phytochemicals, DPPH, *Litsea*.

INTRODUCTION

The Laurels are economically very important as sources of medicine, timber, nutritious fruits, spices and perfumes. Different parts of these plants are famous for traditional medicines [1]. The genus *Litsea* belongs to the family Lauraceae and are a potential source of biologically-active compounds, such as flavonoids (leaves of *Litsea coreana* and *Litsea japonica*) [2], butanolides (leaves of *Litsea acutivena*) [3], sesquiterpene (leaves and twigs of *Litsea verticillata*) [4], 1,3-diarylpropan-2-ol (bark of *Litsea rotundifolia*) [5], butanolide, coumarin, syringaldehyde (bark of *Litsea akoensis*) [6], and essential oils (leaves of *Litsea cubeba*, fruits, flowers and bark of *Litsea monopetala*, fruits of *Litsea glutinosa*) [7, 8, 9]. These plant-derived products can scavenge free radical species, inhibit free radical formation, and prevent oxidative damage [10]. The reactive oxygen species, such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals, are produced under oxidative stress. Reactive oxygen species play vital roles in degenerative or pathological processes like ageing [11], cancer, coronary heart disease, Alzheimer's disease [12], neurodegenerative disorders, atherosclerosis, diabetes and inflammation [13]. Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease preventing capacities. It is well known that phytochemicals are efficient in combating or inhibiting disease due to their antioxidant effect [14, 15, 16, 17, 18, 19, 20]. Antioxidant protects molecules from oxidation and they are implicated in the etiology of many diseases and in food deterioration and spoilage [21]. In food industry synthetic antioxidants have been widely used but due to the possible toxicities of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), the improvement and use of more effective antioxidants of natural origin is highly desirable [22]. Several scientific reports suggest that the genus *Litsea* is the rich source of natural antioxidants [23].

The aim of the present study was to investigate the leaf and bark of four different *Litsea* species from Terai and Duars of West Bengal as a potential antioxidant source, as an alternative to synthetic compounds. In this study we have determined the radical scavenging efficacy of leaves and stem as well as the phytonutrients of these plants.

MATERIALS AND METHODS

5.1. Plant Samples

Leaf and bark of four species of Litsea genus viz. *Litsea glutinosa* (Loureiro) Robinson, *L. monopetala* (Roxburgh) Persoon, *L. assamica* Hooker f., *L. laeta* (Nees) Hooker f. were collected from Terai and Duars of West Bengal, India. Taxonomic position was authenticated in the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material has been deposited in the 'NBU Herbarium' and recorded against the accession number 9639, 9640, 9641, 9642 dated 11-06-11.

5.2. Preparation of extracts

The leaves and barks of four species of *Litsea* were cut into small pieces and were separately crushed with mortar and pestle. Under Soxhlet extractor, the crushed samples were separately extracted with methanol for eight hours. The supernatants of refluxed samples were isolated from the residues by filtering through Whatman No. 1 filter paper. The filtrates were dried *in vacuo* by rotary evaporator and their total extractive values were calculated on dry weight basis by the formula:

$$\% \text{ extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

The samples were then kept in freeze for further use.

5.3. Chemicals

Methanol (M), 2.2-diphenyl-1-picryl hydrazyl (DPPH), nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), phenazine methosulphate (PMS), sulfanilamide, glacial acetic acid and napthylethylenediamine dihydrochloride, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), thiobarbituric acid (TBA), $FeSO_4.7H_2O$, potassium hydroxide (KOH), potassium dihydrogen phosphate (KH_2PO_4), ethylene-diamine tetra acetic acid (EDTA), 2-deoxyribose, potassium ferricyanide, ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferrozine, hydrogen peroxide (H_2O_2), sodium nitroprusside, gallic acid, Folin-Ciocaltue reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), petroleum ether, sodium hydroxide (NaOH), copper acetate, ninhydrin, chloroform, lead acetate, sulphuric acid, hydrochloric acid, Dragendroff's reagent and pyridine were either purchased from Sigma Chemicals (USA), or of Merck analytical grade.

5.4. Determination of DPPH radical scavenging assay:

Radical scavenging activity of plant extracts against stable DPPH (2,2-diphenyl-1- picrylhydrazyl) was determined spectrophotometrically. The changes in color (from deep-violet to light-yellow) were measured at 517 nm wavelength. Radical scavenging activity of extracts was measured by standard method [24]. Two microliters of each sample, prepared at various concentrations (0.5, 1, 2.5, 5, 10, 25 mg/ml), were added to 1.8 ml of 0.2 mM DPPH solution. The mixture was shaken and incubated for 30 min at 20ºC, and then the absorbance was measured at 517 nm with UV-VIS spectrophotometer. The percentage inhibition activity was calculated by the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample}) / A_{control} \times 100]$$

Where, $A_{control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{sample} is the absorbance of the remaining concentration of DPPH in the presence of methanol. IC_{50} values (mg/ml) were determined from a plotted graph of scavenging activity against the concentrations of the extracts, where IC_{50} is defined as the total amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

5.5. Determination of superoxide anions scavenging activity

Measurement of superoxide radical scavenging activity of *Litsea* spp were done by using standard method followed by Nishikimi *et al*., with minor modifications [25]. The reaction mixture contained 1 ml of NBT solution (312 µM

prepared in phosphate buffer, pH-7.4), 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4) and differentially diluted sample extracts. Finally, reaction were accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixtures were allowed at 25^{o} C for 5 min and absorbance was measured at 560 nm against methanol as control. Percentage inhibition was calculated using the same formula mentioned above.

5.6. Reducing antioxidant power

The reducing antioxidant power of plant methanolic extracts was determined by the standard method [26]. Different concentrations of 1 ml of extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^{o}C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis. Increased absorbance of the reaction mixture indicates increase in reducing power.

5.7. Metal chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al*., with slight modification [27]. To 0.4 ml of methanol extract, 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30s, 0.8 ml ferrozine (5 mM) was added. Subsequently after 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

Chelating rate (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

5.8. Determination of Nitric oxide activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction [28]. 320 µL methanol extract, 360 µL (5mM) sodium nitroprusside-PBS solution, 216 µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride) was mixed and incubated at 25^{o}C for one hour. Lastly 2 ml water was added and absorbance was taken at 546nm.

5.9. Total phenol estimation

Total phenolic compounds of leaves and bark extracts were determined by Folin-Ciocalteu method [29]. For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 0.5 ml of Folin-Ciocaltue reagent and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hr. at 20º C and the calibration curve was drawn. To the same reagent, 1 ml methanolic extracts was mixed as described above and after 1 hr. the absorbance was measured. Total phenolic content in methanolic plant extracts in Gallic Acid Equivalents (GAE) was measured by the formula:

C = c.V/m

Where, C - total content of phenolic compounds, mg/g of plant extract, in GAE; c - the concentration of gallic acid deduced from the calibration curve (mg/ml); V - the volume of extracts (ml); m - the dry weight of the plant material.

5.10. Total flavonoids estimation

Aluminum chloride spectrophotometric method was used for flavonoids determination [30]. Each methanol extracts were separately diluted with 4 ml double distilled water. Then the diluted extracts of plant were mixed with 5% (0.3 ml) $NaNO_2$. 10% aluminum chloride was then added with reaction mixture. After 6 minute 2ml (1.0 M) NaOH and 2.4 ml double distilled water was added and mixed well. Thereafter, absorbance was measured at 510 nm in spectrophotometer. Standard solution of quercetin (0-500 mg L^{-1}) was used as calibration curve.

5.11. Phytochemicals screening of the crude extracts

The methanolic crude extracts (500 mg/ml) of leaves and bark were subjected to various chemical tests in order to screening different phytochemicals like reducing sugars [31], resins [32], amino acid, anthraquinones, triterpenoids, alkaloids, glycosides [33], tannin, steroid [34], saponins and cardiac glycosides [35].

RESULTS AND DISCUSSION

Phenolic compounds are widely investigated and are naturally occurring antioxidant components of plants. These phenolic compounds are found in medicinal plants as well as fruits and vegetables and play important roles in preventing degenerative diseases, including inflammation, cancer, and arteriosclerosis [36, 37]. Figure 1 and Figure 2 presents the extractable total phenol and flavonoid contents of four different *Litsea* species of Terai and Duars region. The total phenolic contents of the leaf extracts were much higher than those of the bark extracts (except *L. assamica*). The contents of total extractable flavonoid compounds in the extracts were varied from 58.06 to 62.04 mg/100 g and showed almost similar trend to the total phenolics. In 2008, Muhammad *et al*. had worked on *Litsea monopetala* bark and they found four different phenolic compounds from the methanolic extract [38]. In several studies it was recommended that plant flavonoids, which showed antioxidant activity *in vitro*, also function as antioxidants *in vivo* [2, 39]. Naturally occurring polyphenols and flavonoids can prevent lipid peroxidation, low-density lipoprotein oxidation, and the development of atherosclerosis and heart disease [40]. According to Agrawal *et al*., [16] the genus *Litsea* contain several secondary metabolites. Our study (Table1) also proved these statements. In an earlier study, many medicinal plants contained high amounts of phenolic compounds and there was a positive linear correlation between the total phenolic content and antioxidant activity of the plants [37, 41]. This suggests that the genus *Litsea*, which contained higher levels of polyphenols might have high antioxidant properties. Figure 3, 4, 5, 6, 7 have confirmed this information. In 2009, Kshirsagar and Upadhyay found that the stem of *L. glutinosa* had high DPPH scavenging capacity than the twig of this plant [42]. In this present study the antioxidant activity of the methanolic extracts of the different parts (leaf and bark) of four *Litsea* plants were investigated by using DPPH scavenging, reducing power, metal chelating, superoxide scavenging and nitric oxide scavenging assay of the extracts. Methanolic extracts of every parts of *Litsea* plants have exhibited excellent antioxidant activity. As shown in the Figure 3, extracts from leaf had relatively strong DPPH scavenging activity (low IC_{50} value), thus exhibiting high antioxidant capacity compared to extracts from bark (except *L. monopetala*). Possible mechanism of DPPH scavenging was suggested to be through reduction of this radical by antioxidant molecule to a more stable DPPH form. Because of its unpaired or free electron, DPPH has absorption maxima at 517nm and as it gets reduced in the presence of free radical scavengers the absorbance decreases with respect to the number of electrons taken up. For the measurement of the reducing ability, Fe^{+3}-Fe^{+2} transformations in the presence of phenolic compounds of *Litsea* was found. The reducing ability of a compound may serve as a significant indicator of its potential antioxidant activity. Figure 4 shows the reducing capability of the genus. Bark of the genus of *Litsea* is more potent in reducing capacity than leaf. The bark of *L. glutinosa* has high reducing power (0.02 mg Ascorbic acid Eq/gm FWT) than other extracts. Iron is known to generate free radicals through the Fenton and Haber–Weiss reaction. Metal ion chelating activity of an antioxidant compound prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity acts as significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid peroxidation [43]. In 1990 Gordon reported that chelating agents form *s*-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential, stabilizing the oxidized form of the metal ion [44]. In the present study it was seen that all the extracts interfered with the ferrous-ferrozine complex formation, suggesting that it has chelating activity and captured ferrous ion before ferrozine. Figure 5 shows that IC_{50} of the bark extract of *L. glutinosa* and *L. laeta* for metal chelating activity are 15.25 and 16.14 mg/ml FWT respectively which is higher than the other extracts. An important messenger molecule involved in many physiological and pathological processes within the mammalian body is nitric oxide [45]. The plant products may have the property to counteract the effect of $NO^{\bullet}$ formation and in turn may be of considerable interest in preventing the ill effects of excessive $NO^{\bullet}$ generation *in vivo*. *In vitro* prevention of nitric oxide radical is a measure of antioxidant activity of plant drugs. The nitric oxide radical scavenging activity of leaf and bark extracts of four species of *Litsea* were studied and compared with each other. Figure 6 shows that *L. monopetala* plant has better nitric oxide radical scavenging activity than other plant extracts in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions. The toxicity of $NO^{\bullet}$ increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-) [46]. This superoxide radical is also very harmful to cellular components (Korycka-Dahl and Richardson, 1978). As shown in Figure 7, the superoxide radical scavenging activities of the plant extracts have significant amount of superoxide scavenging activity.

It is widely accepted that the antioxidant activity of a plant extract is correlated to its phenolic content with several authors showing this correlations by different statistic approaches [47, 48, 49]. To study the role of phenolic compounds in antioxidant or chelating properties, Pearson's correlation coefficient was performed and analyzed. High correlations were obtained between total phenol content (TPC) and IC_{50} of metal chelating (MC) activity (Table 2) [$p \leq 0.05$]; also TPC is significantly correlated with NO scavenging activity, suggesting that phenolic compounds are the major contributors of antioxidant activity. Rainha *et al*. proved the importance of phenolic compounds in the antioxidant behaviour of *Hypericum foliosum* extracts and also showed that phenolic compounds contribute significantly to the total antioxidant capacity [50].

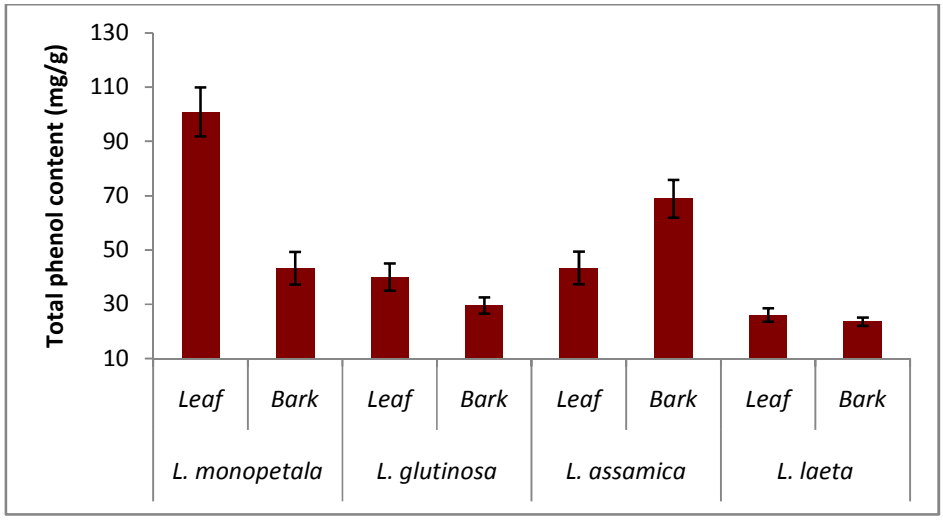

Figure 1: Total phenol content (mg/g FWT) of leaf and bark of *Litsea* spp.

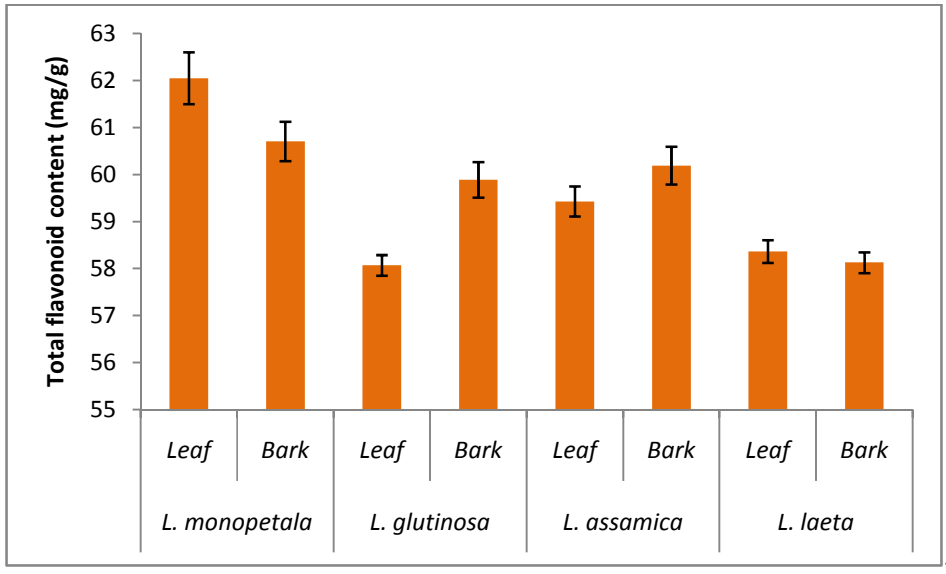

Figure 2: Total flavonoid content (mg/g FWT) of leaf and bark of *Litsea* spp.

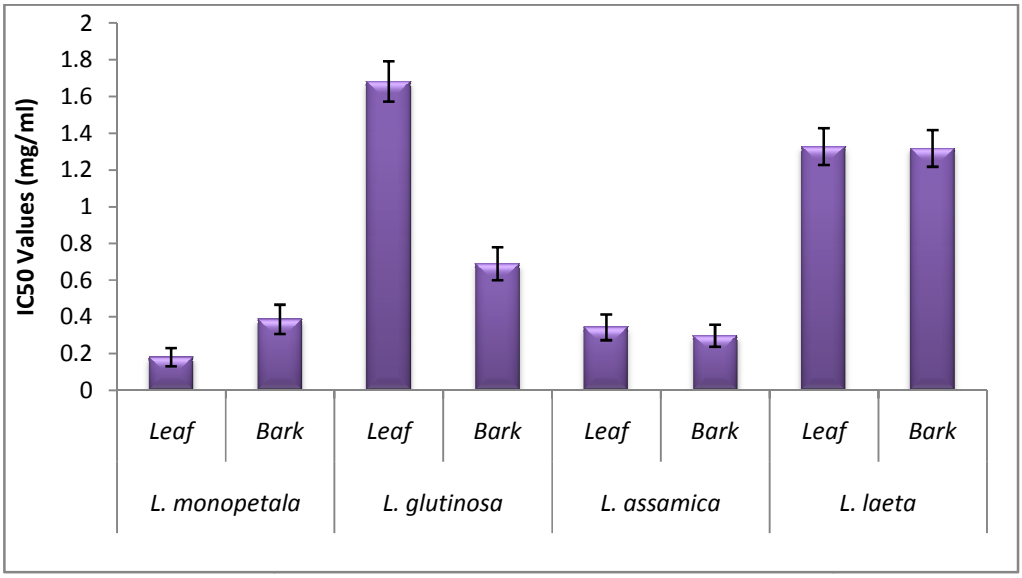

Figure 3: DPPH radical scavenging (IC_{50}) activity of leaf and bark of *Litsea* spp.

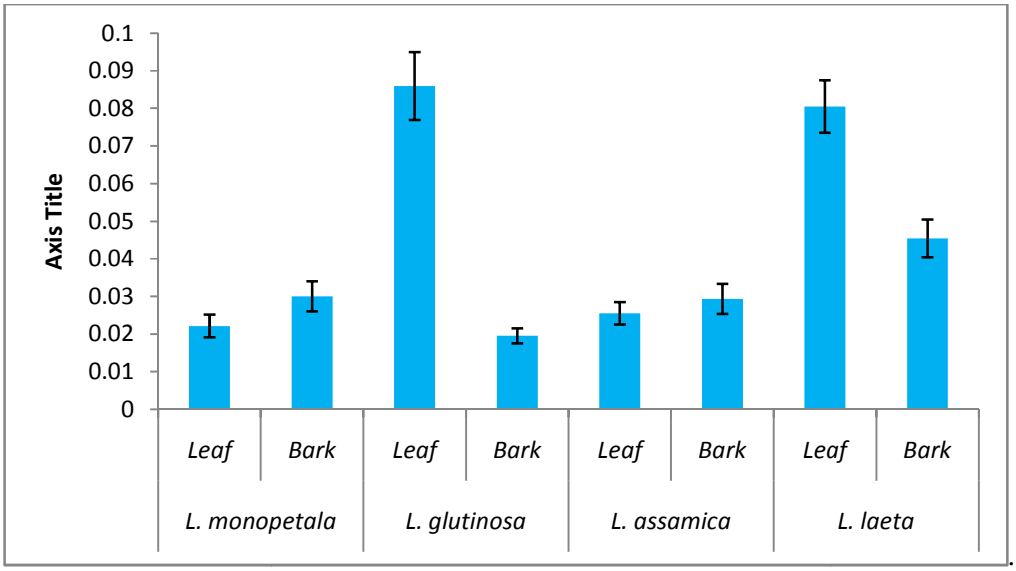

Figure 4: Reducing power of different parts leaf and bark of *Litsea* spp.

Table 1: Phytochemical profiling of *Litsea* spp (semi-quantitative)

Plants	Qualitative Phytochemical Test								
	Alkaloid	Steroids	Anthraquinones	Amino acid	Tanin	Tri terpenoids	Resin	Cardiac glycoside	Glycosides
L. glutinosa Leaf	++	++++	+	Nil	+	Nil	+++	Nil	Nil
L. glutinosa Bark	+	++	++	Nil	++++	+	+++	Nil	Nil
L. monopetala Leaf	+++	+++	+	Nil	++	Nil	++	Nil	Nil
L. monopetala Bark	+++	+++	+	+	+++	+	+	Nil	Nil
L. assamica Leaf	+++	++	++	Nil	++	+	++	Nil	Nil
L. assamica Bark	++++	+++	+++	Nil	++++	+	++	Nil	Nil
L. laeta Leaf	++++	+++	++	Nil	+++	+	+++	Nil	Nil
L. laeta Bark	+	++++	+	Nil	++	+	++	Nil	Nil

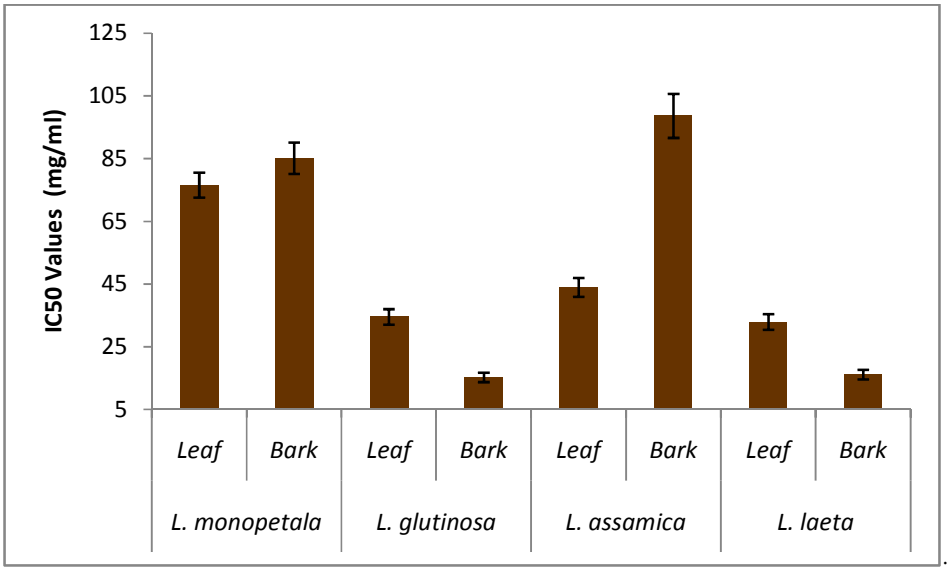

Figure 5: Metal chelating (IC_{50}) activity leaf and bark of *Litsea* spp.

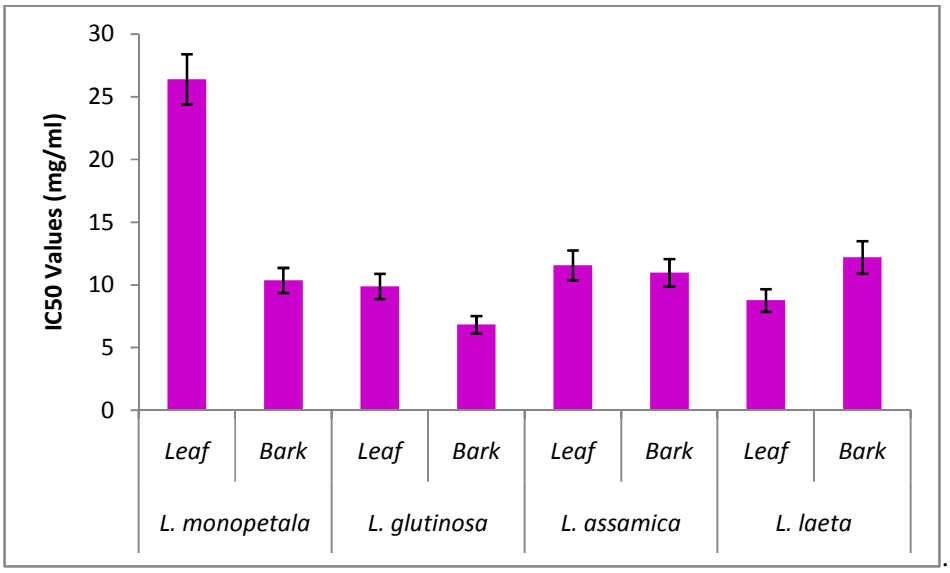

Figure 6: Nitric oxide scavenging (IC_{50}) activity leaf and bark of *Litsea* spp.

Table 2: Correlation Matrix of antioxidant activity and phytochemicals

	DPPH	SO	NO	RP	MC	TFC
SO	-0.120					
NO	-0.426	-0.322				
RP	**0.899(**)**	0.117	-0.341			
MC	-0.685	0.203	0.399	-0.395		
TFC	-0.649	0.181	0.271	-0.402	0.613	
TPC	-0.648	-0.039	**0.848(**)**	-0.462	**0.726(*)**	0.612

*** Correlation is significant at the 0.01 level (2-tailed).*
** Correlation is significant at the 0.05 level (2-tailed).*

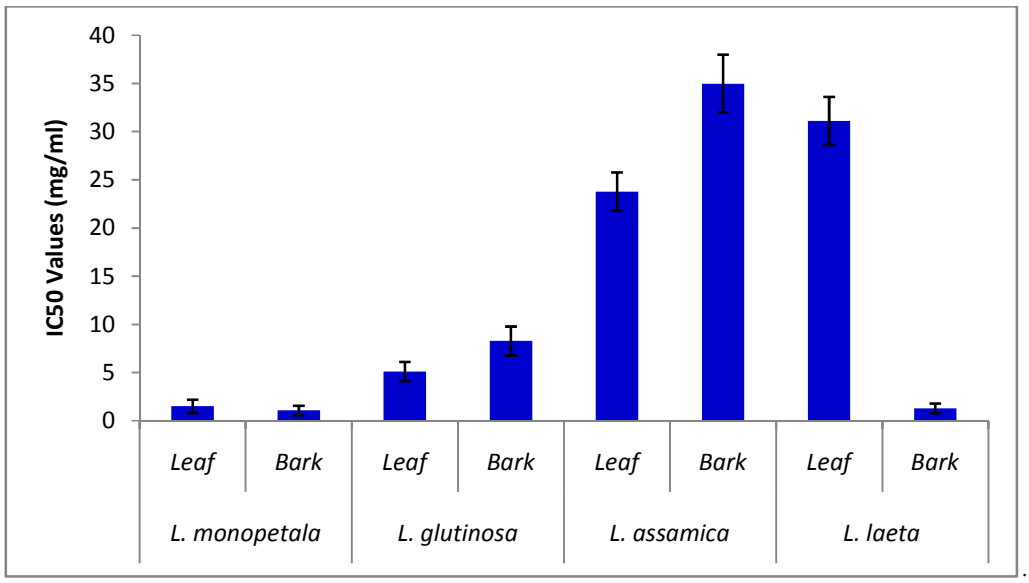

Figure 7: Superoxide scavenging (ic_{50}) activity leaf and bark of *Litsea* spp.

CONCLUSION

In conclusion, the data obtained from the present study showed that the leaf and bark extracts of *Litsea* spp are the potential sources of natural antioxidant which might help in preventing the progress of various oxidative stresses. It can be assumed that these plants possesses the significant antioxidant activity compared to other well characterized, standard antioxidant systems *in vitro* and could serve as free radical inhibitors which might be due to the presence of phenol, flavonoids, alkaloids, steroids, anthraquinones, tannins, resin. These finding suggests that these plants are the potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases.

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