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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL PROPERTY OF DIFFERENT EXTRACTS OF *LITSEA SALICIFOLIA* (ROXB.EX NEES) LEAF.

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ABSTRACT

A lot of research works are going on worldwide to find out natural antioxidants of plants origins as various oxidative stress related diseases result due to accumulation of free radicals in the body. The aims of this study were to evaluate in vitro antioxidant activities and antimicrobial property of *Litseasalicifolia*(ROXB.EX NEES) leaf.In total phenol content determination methanolic extract showed highest value of 102±1.414 mg/gm as Gallic Acid Equivalent (GAE). Ethyl acetate extract showed highest total flavonoid content with 285.5±6.364 mg/gm as Quarcetin Equivalent (QE) and total anti-oxidant capacity with 264.5 ±10.607 mg/gm as Ascorbic Acid Equivalent (AAE). Methanolic extract showed highest tannin content with 253.5714 ±5.051mg/gm as Tannic Acid Equivalent (TAE) and good DPPH free radical scavenging activity with IC_{50} of 251.227 µg/mL. In antimicrobial assay extracts showed poor activity. Further studies are needed to isolate active compounds.

Keywords: Plant extracts; natural antioxidants; total phenolics; antioxidant evaluation; antimicrobial

INTRODUCTION

The plant kingdom can be said as a reservoir of biologically active compounds with various chemical structures. Since the journey of human civilization, herbal medications have been used as a reliable source for the treatment of symptoms of disease [1]. Emergence of much interest on medicinal plant is due to their long use in folk medicines as well as their prophylactic properties, especially in developing countries. A large number of medicinal plants for their antioxidant properties have been investigated. Antioxidants from natural sources are very effective to prevent the destructive processes caused by oxidative stress either in the form of raw extracts or their chemical constituents [2]. It is generally accepted that medicines derived from plant sources are safer than their synthetic counterparts, although the toxicity profile of most medicinal plants have not been thoroughly evaluated [3,4].

The living cells of human body due to physiological and biological processes generates free radicals and other reactive oxygen species by products. These free radicals are responsible for creating oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging and other degenerative diseases in humans [5].there is a substantial evidence that indicates key roles for reactive oxygen species (ROS) and other oxidants which are responsible for

numerous disorders and diseases. The evidence has brought the attention of researcher at a greater extend to antioxidants for prevention and treatment of diseases, and maintenance of human health [6]. There is an inherent antioxidative mechanism in human body and many of the biological functions such as the antimutagenic, anti-carcinogenic, and anti-aging responses originate from this property [7, 8]. Antioxidants are molecules that stabilize or deactivate free radicals, often before they attack targets in biological cells [9]. In recent years, interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, because they possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance [10,11].In developing countries, there is a crisis for new infection-fighting agents to control microbial infections as the synthetic drugs are expensive and inadequate for the treatment of disease [12].

The present study was designed to investigate the Total phenol content, total flavonoid content, total tannin content, total antioxidant content, DPPH free radical scavenging activities and cupric reducing power for antioxidant capacities determination and antimicrobial activities through disc diffusion method.

MATERIALS AND METHODS

Chemicals: Ammonium molybdate, Methanol, Sodium Phosphate (Na_3PO_4) and Folin-Ciocalteu reagent were purchased from Merck, Germany. Sodium carbonate (Na_2CO_3), Potassium Acetate and H_2SO_4 (98%) were purchased from Merck (India) Limited. Gallic acid, Quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Aluminium Chloride and Ascorbic acid were purchased from SD Fine Chem. Ltd., Biosar, India. All chemicals and reagents used were of analytical grade.

Collection of Plant Material: The leaf of the plant was collected from Gazipur, Bangladesh and identified by the taxonomist of the Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh.

Preparation of Plant material & Extraction procedure: Leaf of the plant were first washed with water to remove adhering dirt and then cut into small pieces and sun-dried for few days and then dried in a hot air oven (Size 1, Gallenkamp) at reduced temperature (not more than 50°C). Dried leafs were grinded into coarse powder using high capacity grinding mill. The powdered leafs were used for serial extraction by Soxhlet apparatus at elevated temperature (65°C) using n-Hexane, Ethyl Acetate and Methanol consecutively (500 mL of each solvent). After each extraction the plant material was dried and used again for the next extraction. Extraction was considered to be completed when the plant materials become exhausted of their constituents that were confirmed from cycles of colorless liquid siphoning in the Soxhlet apparatus. The filtrates obtained were dried at temperature of 40±2°C to have gummy concentrate of the crude extract. The extract was kept in a suitable container with proper labeling and then stored in cold and dry place for further use [13].

Phytochemical Screening: The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents i.e. carbohydrates through molisch's test and fehling's test, flavonoids, glucosides through general test for glycoside and glucoside, steroids through liebermann-burchard's test, saponins through frothing test, tannins through Ferric chloride and Potassium dichromate test, alkaloids through mayer's test, hager's test, wagner test and dragendorff's test. These phytochemicals were identified from their respective characteristic color changes as stated in the standard procedures [14].

Antioxidant Activity Evaluation

Total phenol content determination: Total phenolic content of the prepared n-hexane, ethyl acetate and methanol extracts was determined by using Folin–Ciocalteu Reagent (FCR) [15]. One (1.0) mL of leaf extract (200 µg/mL) and the standard (gallic acid) of different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) were taken into marked six test tubes. All test tubes were marked accordingly. Five (5) mL of Folin–Ciocalteu reagent solution (diluted to 10 fold) were taken in the test tubes followed by the addition of 4 mL of 7.5% sodium carbonate solution in each. The test tubes were incubated at 20°C (30 minutes for standard solutions, and 1 hour for extract solution). Absorbance at 765 nm was measured using a UV-Vis spectrophotometer (Shimadzu UV PC-1600) against a blank. Total phenol contents of the fractions were expressed as Gallic acid equivalents (GAE) [16, 17].

Determination of Total Flavonoids Content: Total Flavonoid was determined using the Aluminum chloride colorimetric method described by Wang and Jiao [18]. One (1.0) mL of leaf extract (200µg/mL) and standard (Quercetin) of six different concentrations (6.25, 12.5, 25, 50, 100 and 200

µg/mL) were taken into different marked test tubes. Then 3 mL of methanol was added to each of the test tubes followed by 200 µL of 10% aluminium chloride solution and 200 µL of 1 M potassium acetate solution. Finally, 5.6 mL of distilled water was added to the test tubes. After this the test tubes were incubated for 30 minutes at room temperature to complete the reaction. Absorbance of the solution was measured at 415 nm using UV-Vis spectrophotometer (Shimadzu UV PC-1600) against a blank. Total Flavonoid content of the extract was expressed as Quercetin equivalents (QE).

Determination of Total Tannin Content: The tannins were determined by slightly modified Folin and Ciocalteu method [15]. The standard (Tannic Acid) solution of six different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) and the leaf extract (200µg/mL) of 0.1 mL were taken in different marked test tubes. Then 7.5mL of distilled water, 0.5 mL of Folin Phenol reagent, 1 mL of 35% sodium carbonate solution were added and the volume was finally adjusted upto 10 mL with distilled water. The mixture was shaken well, kept at room temperature for 30 minutes and absorbance was measured using UV-Vis spectrophotometer (Shimadzu UV PC-1600) at 725 nm against a blank. Total Tannin content of the extracts was expressed as Tannic Acid Equivalent (TAE).

Determination of Total Antioxidant Capacity: Total antioxidant capacity of the leaf extracts was determined by following the method described by Prieto P et al., 1999 [19]. Three hundred micro-liters (300µL) of extract (200µg/mL) and standard (ascorbic acid) of different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) were taken in different marked test tubes and then 3 mL of reagent solution (a mixture of 3.3mL of concentrated 98 % H_2SO_4, 0.381gm sodium phosphate and 0.494gm of ammonium molybdate prepared in a 100mL volumetric flask adjusting the volume to 100 mL with distilled water) was added to each test tubes. These test tubes were then incubated at 95°C for 90 minutes to complete the reaction. Absorbance of each of the incubated solutions, after cooling to room temperature, was measured at 695 nm using a UV-Vis spectrophotometer (Shimadzu UV PC-1600) against a blank. Total antioxidant capacity of the extract was expressed as ascorbic acid equivalent (AAE).

DPPH Free Radical Scavenging Assay: DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity of the plant extract was determined following the method described by Braca *et al*. [20]. One (1.0) mL leaf extract of different concentrations (12.5, 25, 50, 100, 200, 400 and 800 µg/mL) and 1.0 mL standard (ascorbic acid) of different concentrations (2.5, 5, 10, 20, 40 and 80 µg/mL) were taken in different pre-marked test tubes. Then, 2 mL of 0.004% methanolic DPPH solution was added to each test tube. All the prepared test tubes with their contents were then incubated for 30 minutes at room temperature. Absorbance of each of the incubated solutions was determined at 517 nm using UV-Vis spectrophotometer (Shimadzu UV PC-1600) against a blank and IC_{50} value was calculated from the curve. (Fig.: 5).

Cupric Reducing Antioxidant Capacity: Cupric Reducing Antioxidant Capacity of the leaf extracts was determined following the method described by Resat A.et. al. [21]. Five hundred (500) µL solution of each plant extract and standard (Ascorbic Acid) having different concentrations (12.5, 25, 50, 100, 200, 400 and 800 µg/mL) were taken in different marked test tubes. One (1.0) mL of 0.01M $CuCl_2.2H_2O$ solution, 1.0 mL of ammonium acetate buffer (pH 7.0), 1.0 mL of 0.0075 mL of neocaproin solution and 600 µL of distilled water were added to each test tubes and the final volume of the mixture was adjusted to 4.1 mL. The total mixtures were incubated for 1 hour at room temperature and the absorbance of the solutions were measured at 450 nm using UV-Vis spectrophotometer (Shimadzu UV PC-1600) against a blank.

Determination of Antibacterial Activity by Disc Diffusion Method: The antimicrobial activity of different extracts was determined by the disc diffusion method [22]. The bacterial strains used for the experiment were collected as pure cultures from Department of Microbiology of Jahangirnagar University. Both Gram positive and gram negative organisms were taken for the test and they are listed in the following Table 1. Solutions of known concentration (40µg/mL) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs were then impregnated with 10µL of the test samples (400µg/ Disc) using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Chloramphenicol 50 µg/disc) and blank discs (impregnated with respective solvents 10µL) were used as a positive and negative control. These plates were then incubated at 37ºC for 24 h allowing maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct

zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter.

RESULTS AND DISCUSSION

Phytochemical screening: The preliminary phytochemical screening was done to detect the presence of different phytochemical compounds in the methanolic, ethyl acetate and n-hexane extracts of the leaf of *Litsea salicifolia(Roxb. Ex Nees) Hook*. The results of the phytochemical testing are given in Table 1.

Different crude extracts of leaf of *L. salicifolia* have been shown to possess various phytoconstituents including carbohydrates (monosaccharides, reducing sugars), alkaloids, tannins, flavonoids and saponin. There is absence of glycosides, glucosides, steroids among all the extracts. Ethyl Acetate Extract of leaf of *L. salicifolia* possesses least phytochemical constituents. These phytoconstituents present in the extracts may account for their various pharmacological activities shown in other investigations [14].

Antioxidant activity evaluation

Total phenol content determination: Phenolic compounds of plants have been said to account for most of the antioxidant activities of plant extracts [23]. They are said to account for most of the antioxidant activities of plant extracts [24]. The total phenolic compounds content of the test solutions were calculated using the calibration curve of the standard (Fig. 1)of Gallic acid ($y = 0.0106x + 0.0507$, $R^2 = 0.9998$)**.** The results were expressed as gallic acid equivalents (GAE) per gram of the extract. Methanolic extract of leaf of *L. salicifolia* was found to contain the highest amount of phenols 102 ± 1.414 mg/gm GAE (Table 2). Phenol contents of the extracts were found to the following order: methanol> ethyl acetate> n-hexane (Table 2).

Determination of Total Flavonoids Content: The total Flavonoid content of the extracts were calculated using the standard line (Fig. 2) of Quercetin ($y=0.0055x+0.0142$, $R^2 = 0.9973$). Flavonoid content of the extracts was expressed as mg/gm Quercetin equivalent (QE). The Ethyl Acetateextract was found to have the highest total flavonoid content 285.5±6.364 mg QE/gm of the extract and the methanolic extract was lowest 54 ± 1.414 mg QE/gm (Table 2). The flavonoid content in plant extracts depend on the polarity of solvent used in extract preparation[25]. The flavonoid contents of the extracts were found to the following order: ethyl acetate>n-hexane >methanol (Table 2).

Determination of Total Tannin Content: Total tannin content of the different extracts of leaf of *L. salicifolia* was evaluated by the Folin method and was expressed as tannic acid equivalents (TAE) per gram of plant extract. Total tannin capacity of the test samples was calculated using the standard curve of tannic acid ($y = 0.0014x + 0.023$; $R^2 = 0.9987$) (Fig. 3). Methanolic extract was found to possess the highest Total tannin content (253.571 ± 5.051 mg/gm TAE); ethyl acetate extract also possesses good 208.929 ± 7.576 mg/gm TAE (table 2). Total tannin content of the extracts was found to the following order: Methanol> Ethyl Acetate > n-Hexane (Table 2).

Total antioxidant capacity assessment: Total antioxidant capacity of *L. salicifolia*leaf extracts was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of leaf extracts. Total antioxidant capacity of the test samples was calculated using the standard line (Fig. 4) of ascorbic acid ($y=0.0052x+0.0164$, $R^2 = 0.9908$). Ethyl Acetate extract of*L. salicifolia*leaf was found to possess the highest total antioxidant capacity with 264.50 ±10.607 mg/gm AAE (Table 2). Total antioxidant capacity of the extracts was found to decrease in the following order: Ethyl Acetate > n-Hexane> Methanol (Table 2).

DPPH Free Radical Scavenging Assay: The DPPH (1, 1-diphenyl-2-picrylhydrazyl) free Radical Scavenging test showed the ability of the test compound to act as a free radical scavenger. DPPH assay method is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants [26].The stable free radical DPPH has been widely used to test the free radical-scavenging ability of various dietary antioxidants [27].As the radical compound is stable and need not be generated, the DPPH free radical scavenging assay is considered as a valid accurate, easy and economic method to evaluate radical scavenging activity of antioxidants[28]. The DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance at 517 nm. The IC_{50} values of the different extractsof*L. Salicifolia*leaf are presented in the Table 2. The methanolic fraction exhibited highest antioxidant activity with an IC_{50} value of 251.227 μg/mL compared to other fractions. The IC_{50} value of the standard ascorbic acid is16.654μg/mL. DPPH radical scavenging capacity of the extracts was found to decrease in the following order: Methanol >Ethyl Acetate>n-Hexane (Fig. 5).

The lower the IC_{50} the higher the antioxidant property of a plant [29]. In the present study, extracts showed DPPH radical scavenging activity in a similar manner to that of the reference antioxidant ascorbic acid-increasing activity with the increase in concentration (Fig. 5). This free radical scavenging activity might be due to the presence of phenols and flavonoids in the extracts.

Determination of Antibacterial Activity by Disc Diffusion Method: The result of antimicrobial screening of different extracts of leaf of *L. salicifolia* has been presented in Table 3. Among the extracts the methanol extracts of leaf showed a slight activity against *Bacillus subtilis* and *Salmonella typhi*. The standard, chloramphenicol, exhibited significant zone of inhibition against all the test organisms.

The methanol extract of leaf exhibited slight antimicrobial activity and it is probably attributed to the presence of saponins, flavonoids and total tannin content [30, 31] which were detected in phytochemical screening (Table 1).

CONCLUSION

This study affirms the in vitro antioxidant potential of methanol, ethyl acetate and n-hexane extracts of *L. salicifolia*leaf. We might say that our results further support to research of natural antioxidants.

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Table 1: Results of Phytochemical Screening of the extracts.

Phytochemical Group Of Compounds	n-Hexane	Ethyl Acetate	Methanol
Alkaloids	+	-	+
Carbohydrates	+	+	+
Flavonoids	–	-	+
Glucosides	-	-	-
Glycosides	–	-	-
Saponins	–	-	+
Steroid	–	–	–
Tannin	+	-	+

['++' sign indicates strongly presence & '+' sign indicates presence of phytochemical group of compounds while the '-' sign indicates absence of phytochemical group of compounds tested for]

Table 2: Total phenolic, flavonoid contents, Total Tannin Contents (mean ± SD) of extracts from leaf extracts of *L. salicifolia*

Plant Extracts	Total Phenolic Content (mg/gm GAE)	Total Flavonoid Content (mg/gm QE)	Total Tannin Content (mg/gm TAE)	Total Antioxidant Capacity (mg/gm AAE)	DPPH Scavenging Assay IC_{50} (µg/mL)
Methanol	102.00 ± 1.414	54 ± 1.414	253.571 ± 5.051	174.50 ± 1.414	251.227
Ethyl Acetate	47.25 ±3.182	285.5±6.364	208.929 ± 7.576	264.50 ±10.607	645.574
n-Hexane	12.25 ±2.475	110.5±9.192	39.286 ± 5.051	221.50 ± 4.243	945.287
Ascobic Acid	NA	NA	NA	NA	16.655

Table 3: **Antimicrobial screening of different extracts of *L. salicifolia***

Test Organisms	**Inhibition Zone Diameter (mm)**			
	Chloranphenicol (50µg/Disc)	Methanol (400 µg/Disc)	Ethyl Acetate (400 µg/Disc)	n-Hexane (400 µg/Disc)
Bacillus subtilis	23	10	7	_
Escherichia coli	32	_	_	_
Salmonella typhi	39	9	_	_
Pseudomonusaeruginosa	23	_	_	_

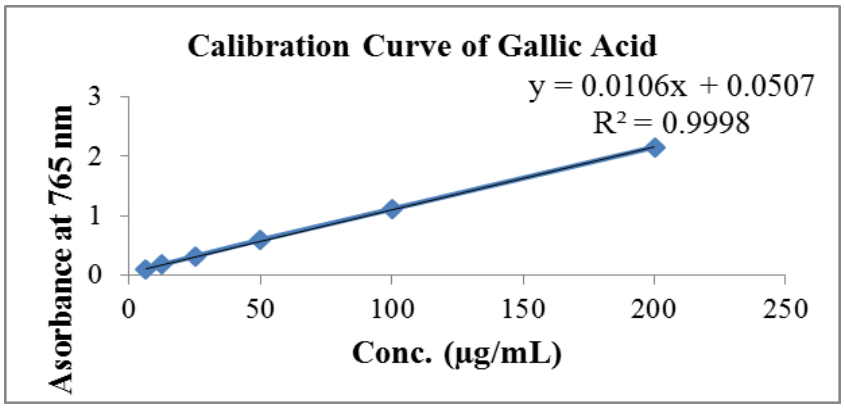

Figure 1 Calibration Curve of Gallic Acid

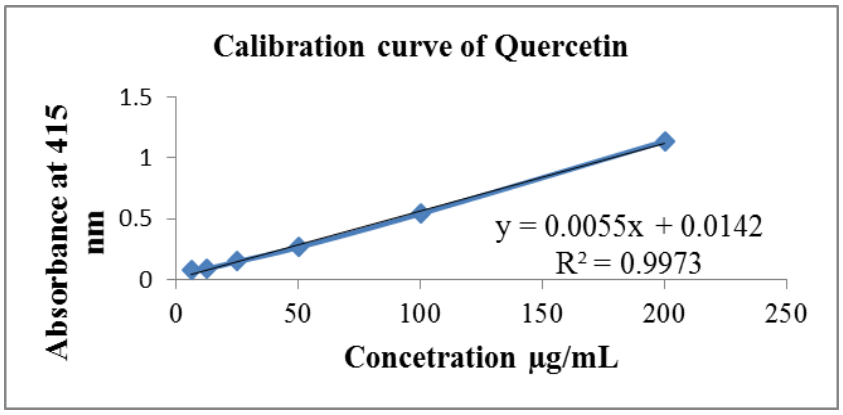

Figure 2 Calibration Curve of Quercetin

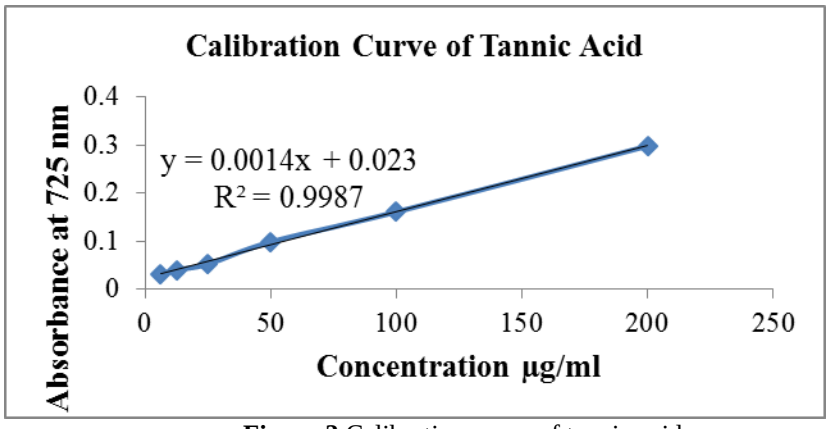

Figure 3 Calibration curve of tannic acid

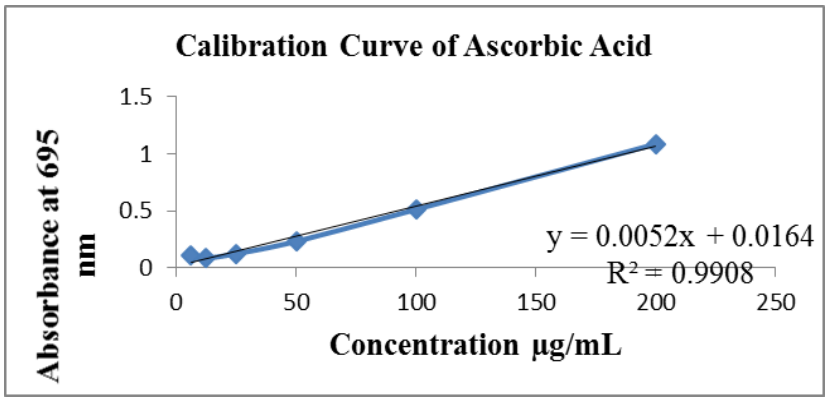

Figure 4 Calibration curve of ascorbic acid

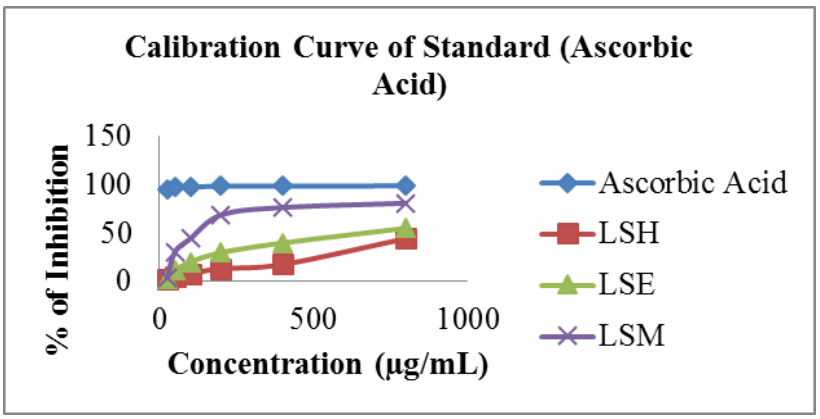

Figure 5:DPPH radical scavenging activity of the different extracts of *L. salicifolia*

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