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Comparative phytochemical and antioxidant analysis in leaf and bark of *Symplocos cochinchinensis* (Lour.) Moore ssp. *laurina* (Retz.) Nooteb.

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ABSTRACT

Medicinal plants are widely used for therapeutic purposes as their side effects are comparatively less. The genus *Symplocos* belonging to the family Symplocaceae comprises of 300-500 species. The present study was to evaluate phytochemical constitution and antioxidant activity of ethanolic leaf and bark extracts of *S. laurina* (L.) for understanding, therapeutic effects of the plant for the first time. Phytochemical constituents like total phenols, flavonoids and alkaloids were also determined in this study. Our results demonstrated that the ethanolic extracts of *S. laurina* (L.) had more flavonoid content in leaf (360 mg) than bark (70 mg) and also the total phenolics content in the ethanolic leaf extract of *S. laurina* (L.) showed more than bark (720 mg and 180 mg respectively). Also the free radical scavenging properties of these extracts were compared by DPPH assay and found significant inhibition were exhibited depending on the concentration, IC_{50} value in both the leaf and bark were observed 500 µg/mL. With increasing concentrations ethanolic leaf extracts of *S. laurina* (L.) showed more reducing activity compared to bark at 10 µg/mL. The HPLC analysis showed the presence of β-sitosterol in ethanolic leaf and bark extracts of *S. laurina* (L.).

Keywords: *S. laurina* (L.), Flavonoids, Phenols, Alkaloids, Antioxidants

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INTRODUCTION

For the past thousands of years, plants have been used to treat human diseases. The medicinal properties of the plants are attributed by the presence of their chemical [1]. These are essential for their existence. Plants synthesize secondary metabolites which are shown to have many pharmacological properties. These metabolites include terpenoids, phenolics, etc., [2]. Plants have always been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defences against predation or infection. Recently there has been a considerable increase in using medicinal plants in western medicine. These days many drugs are made from plants [3].

Symplocos laurina belonging to the family Symploceaceae is a small evergreen tree which is approximately 7 -15 meters in height. The leaves of this plant is thick, glossy, lanceolate, elliptic and serrate whereas the bark of this plant is light grey, thin and smooth [4, 5]. The plant usually grows at an altitude of 800 metres above the sea level. The distribution of this plant varies from South India, usually Western Ghats and Eastern Ghats to Northeast India and extends up to China, Burma, Korea and Japan [6, 7].

S. laurina (L.) is known for its medicinal properties. In the ancient Ayurveda system, it is used for the conditions of kapha and pittha, dropsy, ulcers, arthritis, bronchitis, leprosy, asthma, diarrhoea, dysentery and skin diseases [8]. The bark is usually used to treat the diseases of raktha pittha and diseases of eyes. The leaves are mainly used in the treatment of scalp diseases [9].

In our present study we compare the phytochemical and antioxidant properties of the leaf and bark of *S. laurina* (L.) for the first time. The phytochemical properties of the leaf and bark are compared using the total flavonoids, total phenolics and test for alkaloids. The free radical scavenging properties of the leaf and bark are compared using the DPPH assay and reducing power assay. An attempt is also made to find the sterols present in *S. laurina* (L.) through HPLC.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picryl hydrazyl), TCA (Trichloro acetic acid), ferric cyanide and ferric chloride were obtained from Himedia Laboratories, India. All other chemicals used were of analytical grade.

Plant materials

The leaves and bark of *Symplocos cochinchinensis* (Lour.) Moore ssp. *laurina* (Retz.) Nooteb. was collected from Konni, Pathanamthitta. It was identified by Dr. P. Sujanapal and a voucher specimen number KFRI 28015 was deposited at Kerala Forest Research Institute, Thrissur, Kerala, India.

Method of extraction

The leaves and bark were chopped into small pieces, air-dried and powdered. About 25 gms of the powder were extracted in soxhlet assembly with ethanol for leaves and bark respectively. Both the extracts were concentrated using rotary vacuum evaporator. The extracts obtained were weighed and the yield percentage was calculated in terms of dry weight of the plant material. The colour and consistency of the extracts were also noted. The extracts were dissolved in DMSO prior to analysis depending on the solubility of the extracts. The extracts were subjected to further analysis and they were all performed in triplicates [10].

Determination of *in vitro* antioxidant assays

Test for alkaloids

From both the extracts 200 mg were taken in 10 mL methanol and filtered. From this 2 mL each from the filtrates were taken and 1% HCl was added to each filtrate and steamed for 10 minutes. After steaming 6

drops of Drangendroff's reagent were added to both the filtrates. Reddish brown precipitate indicates the presence of alkaloids [11].
Drangendroff's reagent

Solution A: 0.08 gm of Bismuth nitrate pentahydrate is dissolved in 40 mL distilled water and 10 mL of glacial acetic acid is added to this. Solution B: 8 gm of Potassium iodide is dissolved in 20 mL distilled water. Mix solution A and solution B to get the Drangendroff's reagent.

Determination of total flavonoids

The total flavonoid content (TFC) of ethanolic extract of leaves and bark of *S. laurina* (L.) was determined by a slightly modified method [12]. Potassium acetate and aluminium nitrate was added to the extracts and incubated at room temperature for 40 minutes. The absorbance was measured at 415 nm using Quercetin as the standard.

Determination of total phenolics

The total phenolic content (TPC) of both extracts was determined by the method of Folin–Ciocalteu reaction [13], Gallic acid was used as a standard. Folin–Ciocalteu reagent and sodium carbonate were added to the extracts, and kept for incubation for 20 minutes in room temperature. The absorbance was measured at 730 nm.

DPPH radical scavenging assay

DPPH quenching ability of the extracts was measured according to Ohinishi *et al* [14]. The stock was prepared as 0.1M DPPH in ethanol. Different concentrations (10, 50, 100, 250, 500) of each extract was taken and added 3 mL of freshly prepared ethanolic DPPH solution and kept for incubation in dark for 30 minutes. Then the absorbance was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation:

% DPPH radical scavenging = [(Absorbance of control - Absorbance of sample/Absorbance of control)] X 100

The percentage of radical scavenging was plotted against the sample extract concentration in order to calculate the IC_{50} values of concentration (µg/mL) of extract that causes 50% loss of DPPH activity. Results were compared with quercetin as the positive control.

Reducing power assay

The reductive capacity of iron (III) was assessed as described by Oyaizu [15]. Briefly, different concentrations (10, 20, 30, 40, and 50) of 1 mg of extract dissolved in 1 mL DMSO was mixed with phosphate buffer and potassium ferricyanide solution. It was then incubated at 50°C for 20 minutes and 10 % TCA was added and the mixture was centrifuged at 2000 rpm for 10 min. Then 1.5 mL of the supernatant was taken and was mixed with 1.5 mL of distilled water and 300 µL of 0.1 % ferric chloride. Absorbance was recorded at 700 nm with ascorbic acid as the standard.

High performance liquid chromatography (HPLC) analysis of ethanolic leaf and bark extract of *S. laurina* (L.)

The high-performance liquid chromatography (HPLC) analysis of the ethanolic extract of *S. laurina* (L.) was carried out by the methodology of Ye *et al* [16]. YungLin HPLC system equipped with Phenomenex Luna C18, 5mm (4.6 x 250 mm) column, LC10AT VP pumps, SCL-10AVP system controller, SIL-10 AD VP auto injector, SPD-M10 AVP photodiode array detector and class VP software was used with a flow rate of 1 mL/min and a sample size of 20 µL. The mobile phase used was 15% ethanol and 85% acetonitrile. The sample was monitored with UV detection at 210 nm at the flow rate of 1 mL/min at ambient temperature.

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Statistical Analysis

All experiments were repeated at least three times. Results were reported as Mean ± SE. Statistical differences were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test when required. Differences were considered statistically significant when $p < 0.05$. Correlation analyses between different antioxidant assays and between the TPC and TFC were carried out using Graphpad Prism 5.0 version.

RESULTS AND DISCUSSION

***In Vitro* antioxidant assays**

Test for alkaloids

Both the leaf and bark extracts gave brisk effervescence with reddish brown precipitate. Hence both gave positive results for alkaloid test.

TFC and TPC

The total flavonoid content in the ethanolic leaf and bark extracts of *S. laurina* (L.) was found be 360 mg and 70 mg of Quercetin equivalent per gram of *S. laurina* (L.) respectively (Table 1) (Standard curve equation: y = 0.0051 x, r^2 = 0.9961). Values are representatives of mean ± S.E (n=3). Phenolics play a vital role in the free-radical scavenging properties of plants. The total phenolics content in the ethanolic leaf and bark extracts of *S. laurina* (L.) was found to be 720 and 180 mg of Gallic acid equivalent per gram of *S. laurina* (L.) respectively (Table 1) (Standard curve equation: y = 0.0242 x, r^2 = 0.9754). Values are representatives of mean ± S.E (n=3).

Table 1: Total Flavonoids and Total Phenolics of the leaf and bark extracts of *S. laurina*

Ethanol Extracts	TFC (mg of gallic acid equivalents/g)of extract	TPC(mg of quercetin equivalents/gm)of extract
LEAF	360 ± 0.0463	720 ± 0.0085
BARK	70 ± 0.0136	180 ± 0.0055

Values are representatives of mean ± S.E (n=3).

Many properties like anticancer, antidiabetic, antiageing, etc., have been attributed to the antioxidant activity which is due to the phenolic substances and flavonoids [17, 18]. Therefore the total phenolic and total flavonoid contents of ethanolic leaf and bark extract of *S. laurina* (L.) were investigated in the present study. Poly phenolic compounds like flavonoids contain hydroxyl groups and conjugated ring structures which has the potential to function as antioxidants by scavenging superoxide anion, singlet oxygen etc. It was found that the leaf had more phenolics and flavonoid content than the bark. Hence, the presence of flavonoids and phenols in the ethanolic leaf and bark extracts of *S. laurina* (L.) confirm the antioxidant activity of the plant [19]. In addition to phenolics and flavonoids we also observed the presence of alkaloids in ethanolic leaf and bark extract of *S. laurina* (L.). Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their antioxidant activity [20].

HPLC

The presence of sterols at 225 nm by HPLC was determined in *S. laurina* (L.) and compared with published data [16]. By comparing the retention times of the standards from the published data, following compound (Table 2, Figure 1) is identified in *S. laurina* (L.) *viz*, (β- sitosterol). β-Sitosterol, a promising antidiabetic agent with well known antioxidant effect, is currently used in clinical studies for drug development [21].

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Table 2: Compound and retention time of ethanolic extract of *S. laurina* (L.)

Peak No.	Compound Name	Retention time (Minutes)	Retention time (Minutes) of standards	Reference
1	β-Sitosterol	9.60	9.63	Ye et al. (2010)

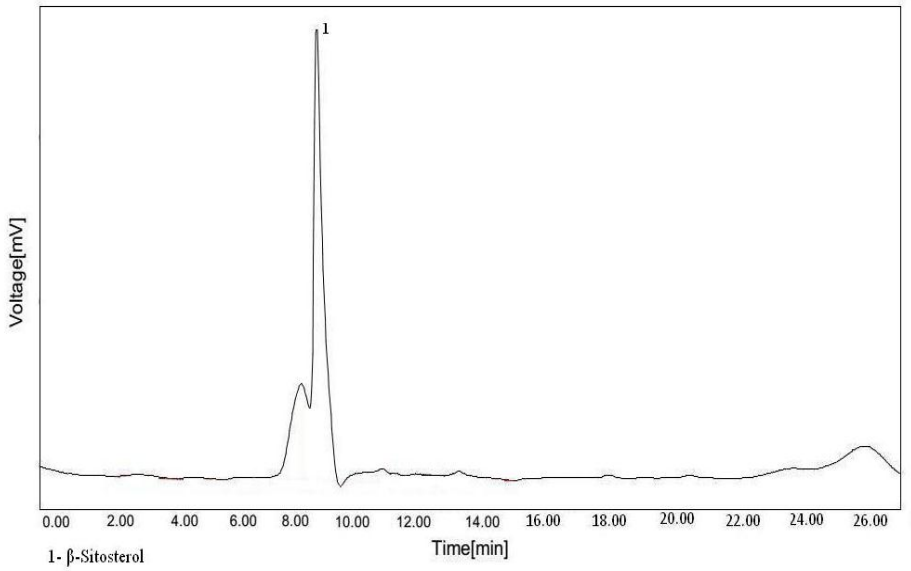
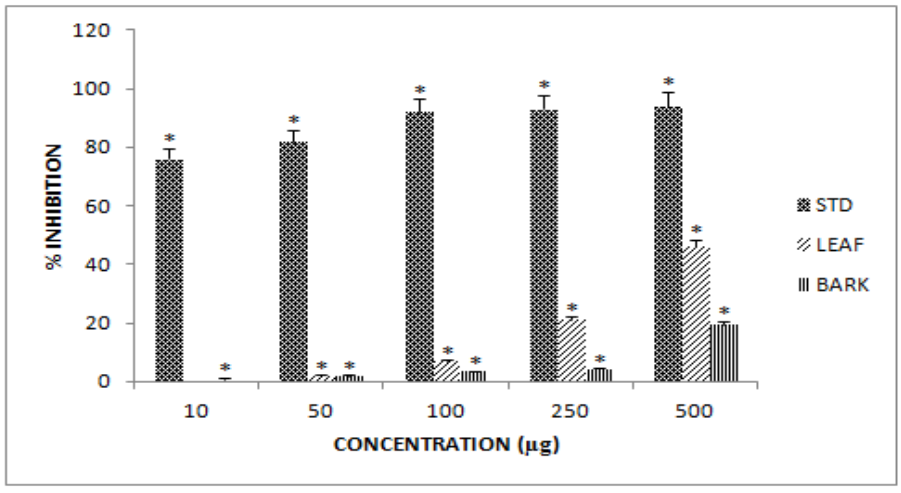


Figure 1: HPLC Chromatogram of ethanolic extract of *S. laurina* (L.)

DPPH radical scavenging assay

The ethanolic leaf and bark extracts of *S. laurina* (L.) exhibited a significant concentration dependant inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration 500 µg/mL. At higher concentration leaf was showing more scavenging activity than bark (Figure 2). There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases such as cancer, neurological disorders etc. Many synthetic antioxidant components have shown toxic and/or mutagenic effects. Hence attention has been given to naturally occurring antioxidants. Therefore, in the present study, different methods such as reducing power, DPPH were performed to evaluate the antioxidant property of ethanolic leaf and bark extracts of *S. laurina* (L.).

Figure 2: DPPH radical scavenging activity of ethanolic leaf and bark extracts of *S. laurina* (L.)



Values are representatives of mean ± S.E (n=3). * indicates statistically significant from control P<0.05

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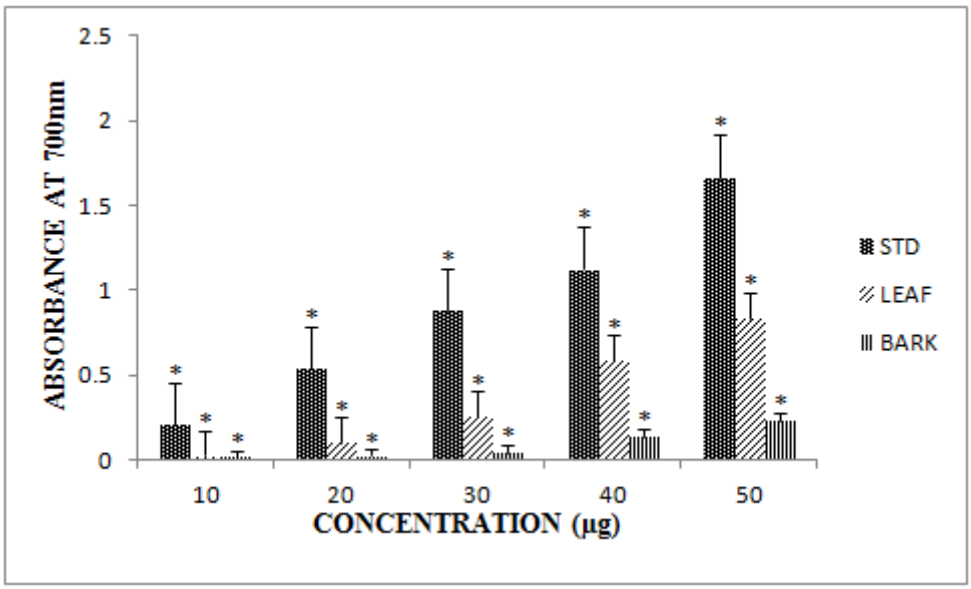
Reducing power Assay

With increasing concentration leaf showed more reducing activity compared to bark. At 10 µg/mL ethanolic leaf and bark extracts of *S. laurina* (L.) showed an absorbance of 0.024 and 0.016 whereas at 50 µg/mL concentration, it was 0.832 and 0.232 respectively (Figure 3). The reducing power assay is based on its hydrogen donating ability which will terminate the free radical chain reaction [22, 23]. As the concentration increases, we found that the reducing power is also increased in both the extracts and comparatively ethanolic leaf extract showed more reducing power than the bark. The reducing capacity of the extracts may serve as an indicator of its potential antioxidant activity [24, 25]. DPPH is stable nitrogen centered violet colored free radical which upon reduction is converted to yellow color giving diphenylpicrylhydrazine [26, 27]. This can be due to the reduction of alcoholic DPPH solution in the presence of electron or hydrogen donating ability of the antioxidant compound found in the extract [28]. The results of present study confirmed the antioxidant activity of ethanolic leaf and bark extracts of *S. laurina* (L.) by its scavenging effects of different types of reactive species such as DPPH radicals through their electron donating properties.

Figure 3: Reducing power activity of ethanolic leaf and bark extracts of *S. laurina* (L.)



Values are representatives of mean ± S.E (n=3). * indicates statistically significant from control P<0.05

Comparison between different antioxidant assays by correlation analysis

Correlation between the results of different antioxidant assays is represented in Table 3. TPC showed a good correlation with DPPH in both the ethanolic leaf (r^2 = 0.778) and bark (r^2 = 0.871) extracts of *S. laurina* (L.) and moderate correlation with Reducing power assay in ethanolic leaf extracts of *S. laurina* (L.) (r^2 = 0.778) where as poor correlation in bark (r^2 = 0.395). The TFC showed a poor correlation with antioxidant assays like DPPH radical scavenging assay (r^2 = 0.380, 0.0219) and reducing power assay (r^2 =0.380, 0.387). There was also good correlation among different antioxidant assays.

Table 3: Comparison between different antioxidant assays as represented by correlation coefficient (r^2) n = 3.

	TPC		TFC		DPPH		RP	
	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark
TPC	-	-	0.836	0.047	0.778	0.871	0.778	0.395
TFC	0.836	0.047	-	-	0.380	0.021	0.380	0.387
DPPH	0.778	0.871	0.380	0.021	-	-	0.380	0.750
RP	0.778	0.395	0.380	0.387	0.380	0.750	-	-

The results from the antioxidant assays showed that ethanolic leaf and bark extracts of *S. laurina* (L.) may act as radical scavengers. There was also good correlation among different antioxidant assays. The difference in correlation may be due to the diverse mechanisms tested by the chosen assays. This has been frequently reported in antioxidant studies [28]. Therefore, single test may not be sufficient to assess the Total antioxidant activity.

CONCLUSION

In our present study, we found from the estimation of total flavonoid content and total phenolics content that the ethanolic extracts of both leaf and bark of *S. laurina* (L.) exhibited antioxidant activity, but leaf showed more activity than bark. From this we can infer that the ethanolic extracts of the leaves and bark of *S. laurina* is a natural source of antioxidants like flavonoids, this can be used for further purification and identification of an effective antioxidant compound that can protect from oxidative stress thereby guard the body against various degenerative diseases and also prevent deterioration of food ingredients. The results of the present study confirmed the antioxidant action of *S. laurina* (L.) by its scavenging effect such as DPPH radicals, and hydroxy radicals through their hydrogen or electron donation properties. HPLC was also carried out to know the presence of sterols and a peak was obtained at 225nm that confirmed the presence of sterols. However, the complete understanding of the role of meticulous compounds in the different antioxidant mechanisms requires systematic phytochemical and pharmacological investigations.

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