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Phytochemical Screening, Antioxidant and Anti-Inflammatory Properties of *Litsea floribunda* Gamble, Leaf and Bark Extract

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ABSTRACT

The *Litsea floribunda* Gamble was widely used for various medicinal purposes. The present study was aimed to screening the phytochemicals presentin theleaf and bark extract of *L. floribunda* and also assess their antioxidant and anti-inflammatory properties. The results revealed that the leaf and bark extract showed the extracts are rich in phytomoleculeslikephenolics and tannin. The leaf extract showed more antioxidant activitiessuch as total antioxidant, ABTS, superoxide radical scavenging, FRAP, and DPPH reducing power activities were done respectively. Better radical activities Albumin denaturation and Anti-proteinase were observed in leaf extractthan in the bark extract, also it showed significant anti-inflammatory activities. Finally, *L. floribunda* could be considered as the most valuablealternative medicinalsource for many diseases.

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Introduction

Natural products are important sources of biologically active drugs. There has been an increasing interest in the study of medicinal plants as natural products in different parts of the world (Sevindik et al., 2017; Koparde et al., 2019; Uysal et al., 2021). The plant kingdom is an excellent source of potential drugs, in recent years there has been an increasing awareness about the importance of medicinal plants, which are a rich source of secondary metabolites with interesting biological activities (Kına et al., 2021; Pehlivan et al., 2021). These secondary metabolites are an important source with a variety of structural arrangements and properties (Doss, 2009; de-Fatima et al., 2006; Korkmaz et al., 2021). From the ancient time plants had been utilized as a wide source for discovering novel drugs or compounds. Now a day's medicines obtained from different parts of the plant have made huge contributions towards human health and well-being (Rout et al., 2013). The medicinal plants are rich sources of secondary metabolites which are chemically and taxonomically extremely diverse compounds with obscure functions. A large number of phytochemicals are widely used in human therapy, agriculture, veterinary, various scientific researches, and in different areas (Vasu et al., 2009; Akgül et al., 2020). Secondary metabolites are synthesized in all parts of the plant body such as bark, leaves, stem, root, flower, fruits, seeds, etc. i. e. any part of the plant body may contain active components (Tiwari et al., 2011).

Litsea floribunda Gamble is a dioecious tree species endemic to India and comprising roughly 200–400 tree species distributed abundantly across tropical and Subtropical regions of India, China, and Australia. It is a medium-sized tree with smooth, greyish-brown bark, leaves simple, alternate, existing variation in leaf size, leaf blade sweet to taste smell slightly of crushed mango leaves. Inflorescence isracemose with 6-8 flowers, fruit ovoid berries seated on copular perianth tube (Saldanha, 1996;

Gamble and Fischer, 1998). *L. floribunda* Gamble leaves are used as one of the ingredients in the preparation of herbal shampoo, in Southern India. In health traditions, the local inhabitants use *L. floribunda* to treat certain gastrointestinal and respiratory disorders. The leaves of *L. floribunda* are used as one of the main ingredients in the preparation of herbal shampoo, in southern India (Girish et al., 2014). In the health traditions, the local inhabitants use *L. floribunda* to treat certain gastrointestinal and respiratory disorders (personal observation). *Litsea* species also contain structurally diverse and biologically active phytochemicals with a broadspectrum of biological activities (Agarwal et al., 2011). Ghani (2003) reported that The bark paste is applied to facilitate the wound healing process. Rout and Thatoi (2009) demonstrated that leaf juice used to relieve respiratory disorders, roots are used for poulticing sprains, bruises, in wounds and the essential oil of berries used by some tribal practitioners in the treatment of rheumatism. The leaf juice was used for the treatment of the spontaneous and excessive flow of semen in young boys (Kirtikar and Basu, 1981). Hence the present study was carried out to screen to evaluate the presence of phytochemicals, i*n vitro* antioxidant, and anti-inflammatory propertiesof leaf and bark extract *L. floribunda.*

Materials and Methods

Collection of Plant Material

The fresh leaves and bark of *Litsea floribunda* was collected from the Nilgiri district, (Western Ghats) Tamil Nadu. The samples were then rigorously washed with freshwater, sponged up, and shade dried for 2 weeks, and ground to a fine powder then it sstored at 4°C in sterilized containers for further usage.

Extract Preparation

The dried leaf and bark samples were extracted with methanol, using maceration extraction method using orbital shaker 90 rpm at 24 h. The solvents were evaporated by a rotary vacuumevaporator at 45°C. The freeze-dried extracts were used for biochemical and further studies.

Estimation of Primary Metabolites

Total carbohydrate

The estimation of carbohydrate was measured according to the method described by Hodge and Hofreiter, 1962. The 100 mg of plant sample was weighed and hydrolyzed by keeping it in a boiling water bath for 3 h with 2.5 N HCl. Neutralized with solid sodium carbonate until the effervescences ceased. Volume was made up to 50 ml and centrifuged. The supernatant was collected and used for the estimation of carbohydrates. To the 50 µL of supernatant, 4 ml of anthrone reagent (Dissolve 100 mg of Anthrone in 50 ml of ice-cold 95% sulphuric acid) was added and heated for 8 min in a boiling water bath. The absorbance was read at 630 nm. The amount of carbohydrate (mg\g of the sample) present in the material was calculated by using a standard graph. Glucose was used as the standard.

Total starch

The protein was measured according to the method described by Hodge and Hofreiter, 1962. 100 mg of plant material was taken in a mortar and pestle homogenized with 10mL of 80% ethanol, centrifuged, and collected the residue. The supernatant was washed repeatedly with hot 80% ethanol until it becomes colorless with anthrone reagent and dried using a water bath. To the residue add 52% perchloric acid and centrifuge, the supernatant was saved and used for estimation. To the 1mL of supernatant, 4 ml of anthrone reagent was added and heated for 8 mins in a boiling water bath. The absorption was read at 630 nm. The amount of starch (mg/g of sample) present in the material was calculated by using a standard graph. Glucose was used as standard.

Total protein

The protein was measured according to the method described by Lowry et al., 1951. 100 mg of plant powder and ground well with a mortar and pestle using 10 mL of phosphate buffer (0.2 M, pH-7.4). Centrifuged and used the supernatant for protein estimation. To the 50 µL of supernatant, 5ml of alkaline copper solution (2% sodium carbonate in 0.1 N sodium hydroxide (Reagent-A), 0.5% copper sulphate in 1%potassium sodium tartrate (Reagent-B), and Mix 50 mL of A and 1 ml of B (Reagent C)) were added and incubated for 10 mins. To that, 0.5 mL of 1N folin-ciocalteau reagent was added and incubated in dark for 30 mins and absorbance read at 660 nm.The protein present in the extract was determined from a standard graph using Bovine Serum Albumin (BSA) as standard. The results are expressed as amounts of proteins in mg\g of the sample.

Total free amino acids

The total free amino acids were measured according to the method described byMoore and Stein, 1948. 100 mg of plant powder was weighed and macerated with a mortar and pestle in 10 mL of 80% ethanol. The homogenate was centrifuged for 10 mins at 3000 rpm. The supernatant was saved. The extract was used for the estimation of amino acids. To the 200 µL of supernatant, add 1 mL of ninhydrin solution (Dissolve 0.2 g of stannous chloride in 125 mL of 0.2 M citrate buffer (pH-5.0) were added. To this solution, 5 mg of ninhydrin in 125 mL of methyl cellosolve (2-methoxy ethanol) was added and made up to 2 mL. Then, it was heated for 20 mins in a boiling water bath. To it, an equal volume of water and n-propanol (1:1) was added. The test tubes were incubated for 15 mins and the absorbance was measured at 570 nm. The number of amino acids (mg/g of sample) present in the material was calculated by using a standard graph. Leucine was used as the standard.

Qualitative Phytochemical Screening

Test for alkaloids

Wagner's test: About 10 mg of extract was taken and few drops of Wagner's reagent were added. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Test for saponins and steroids

Foam test: 0.5 mg of extract was diluted with 20 mL distilled water and shaken well in a graduated cylinder for 15 mins. The formation of foam to a length of 1cm indicated the presence of saponins and steroids.

Test for phenols

Sodium hydroxide test: 0.5 mg of extract was dissolved in 0.5 mL of 20% sulphuric acid solution. Followed by the

addition of few drops of aqueous sodium hydroxide solution, it turns blue which indicates the presence of phenols.

Test for flavonoids

Shinoda Test: To 0.5 mg of extract a pinch of magnesium turnings and 1-2 drops of conc. HCl acid was added. The formation of pink color indicates the presence of flavonoids.

Test for tannins

Lead acetate test: 0.5 mg of extract was taken and 0.5 ml of 1% lead acetate solution was added and the formation of a precipitate indicates the presence of tannins compounds.

Quantitative Phytochemical Screening

Total Phenol

The quantification of total phenols was determined as described method (Makkar, 2003). The 200 µL of extracts were taken into test tubes and 500 µL of folin-ciocalteau phenol reagent (1N) was added to all the test tubes including the blank. After 5 mins, 2.5 ml of sodium carbonate (20%) was added to all the test tubes. The test tubes were mixed well and incubated in dark for 40 mins. The formation of blue color in the incubated test tubes indicated the presence of phenolics. Soon after incubation, the absorbance was read at 725 nm against the reagent blank. The results were expressed as Gallic acid equivalents (GAE). The analyses were performed in triplicates.

Total Tannin

The tannin content of the sample was determined according to the method described by Makkar, 2003. The 200 µL of each plant sample was incubated with 100 mg of polyvinyl polypyrrolidone (PVPP) and 500 µL of distilled water for 4 h at 4ºC. After incubation, the Eppendorf tubes were centrifuged at 3000 rpm for 10 mins at 4ºC. The supernatant contains only the non-tannin phenolics since the tannins would have been precipitated along with PVPP. The supernatant was collected and phenolics were determined by the same method described for the quantification. The 100 µL of extracts were taken in a test tube and 500 µL of folin-ciocalteau phenol reagent (1 N) were added to all the test tubes including the blank. After 5 mins, 2.5 ml of sodium carbonate (20%) was added to all the test tubes. The test tubes were vortexed well to blendthe contents and incubated in dark for 40 min. The formation of blue color in the incubated test tubes indicated the presence of phenolics. Soon after incubation, the absorbance was read at 725 nm against the reagent blank. The analyses were also performed in triplicates and the results were expressed in Gallic acid equivalents. From these two results, the tannin content of the plant samples was calculated as follows,

Tannins= total phenolics - non phenolics

Total saponin

The total saponin content of the sample was determined by the method (Makkar et al., 2007). The 0.2 mL of extract was taken to which 0.25 mL vanillin reagent (8% vanillin in ethanol) and 2.5 mL of 72% aqueous H_2SO_4 were added. The reaction mixtures in the tubes were heated in a water bath at 60°C for 10 mins. After those tubes were cooled in ice for 4 min and then allowed to cool at room temperature, the absorbance was read at 544 nm. Diosgenin was used as a standard and the results obtained were expressed as mg diosgenin equivalent per g of sample dry matter.

Total flavonoids

The total flavonoidwasdetermined by aluminium trichlorideméthoddescribed by Zhishen et al., 1999. A volume of 200 µL of the extract was taken in test tubes and 2 mL of distilled water was added to each test tube. A tube contained 2.5 mL of distilled water served as a blank. To this 150 µL, 5% of $NaNO_2$ was added to all the test tubes and incubated the whole mixture for 6 mins at room temperature. After incubation, 150 µL of 10% $AlCl_3$ was added to all the test tubes including the blank. Then, the test tubes were incubated for 6 mins at room temperature. Then 2 mL of 4% NaOH was added and made upto 5mL using distilled water. The contents in all the test tubes were mixed well and allowed to stand for 15 mins at room temperature. The development of pink color due to the presence of flavonoids was read spectrophotometrically against prepared reagent blank at 510 nm. Samples were analyzed in triplicates and the amounts of flavonoids were expressed in Rutin equivalents.

Antioxidant Activity

Determination of DPPH assay

The free radical scavenging activity of the extracts was doneusingthe 1,1-diphenyl-2-picrylhydrazyl test according to the method described by (Braca et al., 2001). The 0.2 mL extract was added to 3 mL of 0.004% methanol solution of DPPH. Absorbance was taken at 517 nm after 30 mins and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]$ x100, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard.

Determination of total antioxidant assay

The antioxidant power of the extracts has been assayed with the phosphomolybdenumreduction assay (Prieto et al., 1999). It was based on the reduction of the extract and subsequent formation of a complex (green color) at acidic pH, 200 µL of an extract with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 mins at 695 nm using spectrophotometer against blank.

Determination of superoxide radical scavenging activity

Superoxide scavenging was determined by the nitrobluetetrazolium reduction methodNishikimi et al., (1972). The reaction mixture consisted of 200 µL of nitrobluetetrazolium (NBT) solution (l M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (l M NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of different fractions and ascorbic acid (50 mM phosphate buffer, pH 7.4) was mixed. The reaction was started by adding 100 µL of (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) in the mixture. The tubes were uniformly illuminated with an incandescent visible light for 15 mins and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. The abilities to scavenge the superoxide radical were calculated by using the following formula:

$\%\ \text{scavenging} = (1 - Ae/Ao) \times 100$

where

Ao is the absorbance without sample, and Ae is absorbance with the sample.

Determination of FRAP assay

The FRAP assay was done according to Benzie and Strain (1996) with some modifications, stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mMHCl, and 20 mM $FeCl_3$ $6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ $6H_2O$ solution and then warmed at 37°C before use. 200 µL of extracts were allowed to react with 2850 µL of the FRAP solution for 30 mins in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The result was expressed in mM TE/g fresh mass.

Determination of $ABTS^{\bullet+}$radical scavenging activity

Free radical scavenging activity $ABTS^{\bullet+}$ assay was determined followed by Arnao et al. (2011) method with some modifications. The stock solutions included 7.4 mM $ABTS^{\bullet+}$solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$solution with 60 mL methanol to obtain an absorbance of 1.170±0.2 units at 734 nm using the spectrophotometer. The Fresh $ABTS^{\bullet+}$solution were prepared for each assay. The 0.2 mL of extract was allowed to react with 2850 µL of the $ABTS^{\bullet+}$solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. Results are expressed in mM Trolox equivalents (TE)/g fresh mass. Additional dilution was needed if the $ABTS^{\bullet+}$value measured was over the linear range of the standard curve.

Determination of Anti-Inflammatory Assay

Inhibition of albumin denaturation

The anti-inflammatory activity was studied by using the inhibition of albumin denaturation technique according to the method described by Sakat et al., 2010. The reaction mixture consisted of 200 µL test extracts and a 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using a small amount of 1N HCl. The sample extracts were incubated at 37ºC for 20 mins and then heated to 51ºC for 20 mins, after cooling the samples the turbidity was measured at 660 nm The experiment was performed in triplicate. The percentage inhibition (PI) of protein denaturation was calculated as:

PI = (Abs Control –Abs Sample) × 100/ Abs control.

Determination of anti-proteinase activity

The test was performed according to the modified method Sakat et al., (2010). The reaction mixture (2 mL) containing 0.06 mg trypsin, 1 mL 20 mMTrisHCl buffer (pH 7.4) and 200 µL test sample andthemixture was incubated at 37ºC for 5 mins and then 1 mL of 0.8% (w/v) casein was added. The mixture was incubated furtherfor 20 min. 2 mL of 70% perchloric acid was added to arrest the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer the blank. The experiment was performed in triplicate. The percentage inhibition (PI) of proteinase inhibitory activity was calculated.

PI = (Abs control –Abs sample) × 100/ Abs control

Statistical Analysis

The results were analyzed by one-way ANOVA to calculate the significance of difference among the treatment means compared using Duncan's Multiple Range Test (DMRT) at $P < 0.05\%$ level. Data were analyzed statistically using IBM, SPSS 20 software.

Result and Discussion

Estimation of primary metabolites

In this study methanolicextract of leaf and bark of *L. floribunda*, showedthe maximum level of total carbohydrate, starch, protein, and amino acids levels were observed (Figure 1) inleaf extractthan in the barkextract sample. A high level of starch and protein was observed inthe leaf sample because of the photosynthesis that occurred in the leaf and has been an abundance of rubisco protein in the leaf.

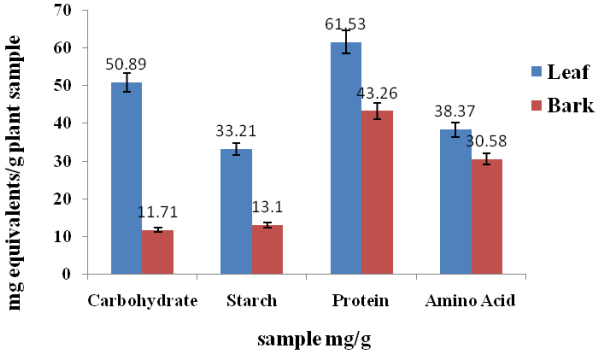

Figure 1. Primary metabolites ofleaf and bark ethanolic extracts of *L. floribunda*

Table 1. Qualitative phytochemical screening of *L. floribunda* leaf and bark methanolic extract

Phytochemicals	Leaf	Bark
Phenolic	+++	+++
Tannins	+	++
Alkaloids	-	-
Flavonoids	++	++
Saponins	++	++
Steroids	+	+

(+)- Positive result of the test, (-)-Negative result of the test

Qualitative and Quantitative Phytochemical Screening

The qualitative phytochemical the presence of phenol, flavonoids, tannin, saponin steroids and alkaloids were analyzedfor *L. floribunda* leaf and bark extract (Table 1). They arerich inhigher amountof phenolic compounds. The previous studies of *L. floribunda* support this investigation qualitative (Angel and Mary, 2019). Also leaf and stem extract (Devika and Nalini, 2018). *L. glutinosa* phytochemical screening of bark extracts showedthe presence of a high level of flavonoid, alkaloids, saponins, and tannins (Pradeepa et al., 2011).

The quantity of secondary metaboliteslike total phenol, flavonoids, tannin, and saponinwas analyzed (Table 2). A high level of total phenol and tannin were observed in leaf and bark than total saponin and total flavonoids, this result wasequivalents to the standard. The high amount of phenolic compound tested from *L. floribunda* leaf and bark

extract (Devika et al., 2016). Angel and Mary, (2020) studied *L. floribunda* flower extract for the presence of volatile compounds using GC-MS. The phenolic compounds are more soluble in polar organic solvents, due to the presence of hydroxyl groups, therefore methanol extracts showed a maximum amount of phenolic compounds from the plant source (Aryal et al., 2019).

Table 2. Quantitative phytochemical screening of *L. floribunda* leaf and bark methanolic extract

Secondary metabolites	Leaf	Bark
Total Phenol[1]	2.996±0.002[a]	2.910±0.012[b]
Tannin[1]	2.873±0.006[ab]	3.012±0.002[a]
Total Flavonoid[2]	1.919±0.002[c]	1.846±0.005[c]
Total Saponin[3]	0.818±0.050[d]	0.729±0.027[d]

1: (mg Gallic acid Equivalents /g); 2: (mg RutinEquivalents /g); 3: (mg DiogenineEquivalents /g); Values are presented as means ± SEM of triplicate determinations and expressed per g of plant extracts. Means significantly different between the extract (P<0.05).

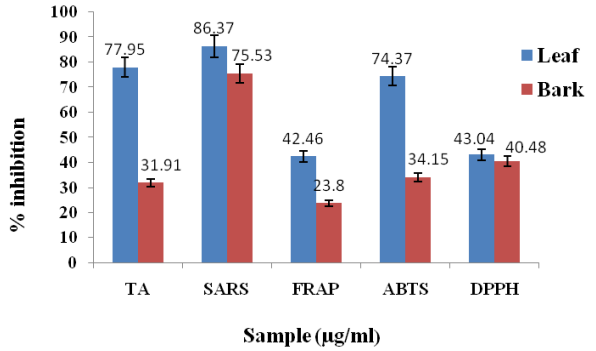

Figure 2. Antioxidant activity ofleaf and barkmethanolic extracts *L. floribunda*
TA-Total Antioxidant activity, SARS-Superoxide Radial Scavenging Activity, FRAP-Ferric Reducing Antioxidant Power, ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, DPPH-2,2-diphenyl-1-picryl-hydrazyl-hydrate,

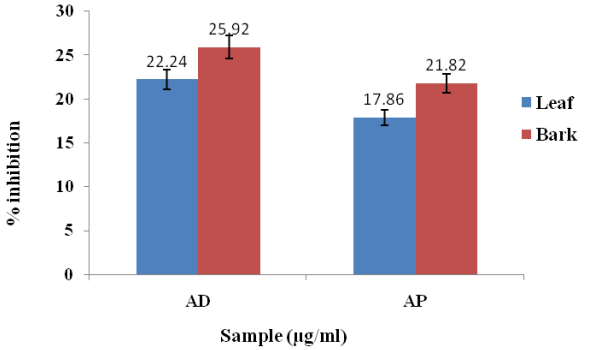

Figure 3. Anti-inflammatory Activity of *L. floribunda* Leaf and Bark Extracts
AD-Albumin denaturation, AP-Anti-proteinase

Antioxidant Activities

The antioxidant activities of *L. floribunda* methanol extract were estimated using various assays. Free radical scavenging action may bedue to the presence of flavonoids, tannin, and phenolic in *L. floribunda* plant extracts. The result was showed (Figure 2), the highest antioxidant activities were showed in leaf extract than bark extract followed by DPPH, FRAP, total antioxidant, ABTS, and superoxide radical scavenging activities. The leaf extract showed significant superoxide radical scavenging activity. The plant based phenolics and flavonoids are naturally strong antioxidant properties that prevent various diseases (Balamurugan et al., 2020). The ABTS cation radical ($ABTS^{\bullet+}$), which (giving a bluish-green color) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid)). In the presence of phenolics (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. ABTS can be oxidized by potassium persulphate or manganese dioxide, giving rise to the ABTS cation radical (Pisoschi and Negulescu, 2011). Ferric reducing antioxidant power assay the formation of a blue-colored Fe (2)-tripyridyltriazine compound from colorless oxidized Fe (3) is formed by theaction of electron-donating antioxidants. The *L. glaucescens* extract showed strong antioxidant activity (Lopez-Romero et al., 2018). The high free scavenging property was attributed to hydroxyl groups existing in the chemical structure of phenolic compounds that can provide the necessary components as a radical scavenger (Nahak and Sahu, 2010).

Anti-inflammatory Activities

The anti-inflammatory drug makes half of the analgesics, suppressing pain by reducing inflammation at a particular body part of an organism (Karthik Madhayan et al., 2021). The protein denaturation and antiproteinase activities were done in leaf and bark methanol extracts. The better protein denaturation and antiproteinase activities were found in bark extract ompared with leaf extract (Figure 3). Inflammation is one of the most impertinence physiological defense mechanisms against different factors such as burn, toxic, infections, and allergens. Denaturation of the protein involved the change or disruption of the secondary, tertiary, and quaternary structure of the molecules and finally leads to cell death, it occurs due to stress like a high level of salt, temperature, and high level of acidity. The mechanism of denaturation probably involved alteration in electrostatic, hydrogen, hydrophobic, and disulphide bonding. Denaturation of proteins was well documented as contributing to inflammatory conditions like arthritis, diabetes, and cancer (Shilpa et al., 2018). Among the ethanol and methanol extracts of *Ceropegia juncea* Roxb. *in vivo* and *in vitro* anti-inflammatory activity was reported they showed better inhibition percentage against albumin denaturation (Saraswathy et al., 2017). The anthocyanins, flavonoids, and phenolic acids could be responsible for the anti-inflammatory activities (Cespedes et al., 2008).

Conclusion

The present studyis concluded that the leaf and bark extract possessesa high level of antioxidant and anti-inflammatory properties. We have observed that all the extract has good sources of secondary metabolites like total phenol, tannin, total saponin, and total flavonoid. This study highlighted that the suitable *in vitro* antioxidant activities of the methanol extract act as a preliminary oxidant scavenger and they are valuable in secondary

metabolic components as well as good anti-inflammatory activity. Hence the leaf extract could be active and used to treat for many diseases.

Conflict of Interest

Authors declare no conflict of interest.

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