SCREENING OF *AEGIALITIS ROTUNDIFOLIA* ROXB. LEAVES FOR THEIR ANTIOXIDANT POTENTIAL, POLYPHENOL CONTENT AND FLAVONOID CONTENT

Usha Rani N.[a]*, Pavani P.[a], Lakshmi Tulasi S.[a] and T. S. R. K. Prasad Rao P.[b]

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ABSTRACT

The present research is focused on investigating the phytochemical analysis and antioxidant activity of *Aegialitis rotundifolia* Roxb. leaf. Different solvent extracts of *A. rotundifolia* Roxb. leaves are screened for secondary metabolites by qualitative and quantitative analysis. The resultant phytochemical screening proves that the plant leaves consist of various secondary metabolites. The quantitative analysis proves that phenolic compounds and flavonoids are dominant among them. Four different antioxidant activity methods, namely, ABTS radical scavenging activity, phosphomolybdate assay, peroxide activity and FRAP (Ferric Reducing Antioxidant Power), are used for assessing the activity of the leaf extract. Ethanolic and aqueous extracts of the leaf have high potency of antioxidant activity and reported dose-dependent activity. Similar promising antioxidant results are also reported for ABTS radical scavenging activity (82.4% reduction at 200 µg mL^{-1} of ethanol and 90.0% reduction at 200 µg mL^{-1} of aqueous extract), phosphomolybdate assay (82.4% reduction at 200 µg mL^{-1} of ethanol and 74.5 % reduction at 200 µg mL^{-1} of aqueous extract) and peroxide activity (92.1 % reduction at 200 µg mL^{-1} of ethanol and 93.1 % reduction at 200 µg mL^{-1} of aqueous extract). The antioxidant activity reported with ethanol (157.4 mmol Fe^{2+}/g) and aqueous extract (129.8 mmol Fe^{2+}/g) was also studied by analyzing with FRAP reduction. The obtained findings confirmed the high antioxidant activity of plant leaf extract and its application for pharmacological activity.

Keywords: *Aegialitis rotundifolia* Roxb., ABTS, FRAP, Phosphomolybdate, Peroxide activity, antioxidant activity

INTRODUCTION

Two shrubs by mangrove species belong to the genus *A. rotundifolia* Roxb., one indigenous to Southeast Asia and the other to Papua New Guinea and Australia[1]. *A. rotundifolia* Roxb. is an evergreen shrub with a height ranging from 30 – 300 cm. Aerial roots are absent in members of the Aegialitis species, in contrast to other mangrove species[2,3]. Compared to other mangrove species in its region, this species has a relatively limited and patchy distribution[4]. The plant is sometimes harvested in the wild as a source of tannins for local use. The bark contains around 11 % tannin on a dry weight basis and is used for preserving grope.

Antioxidants are strong chemicals that can shield the body from oxidative stress created by free radicals. Antioxidant properties of various medicinal plants have been studied for pharmacological application. Natural antioxidants are potent in stopping the harmful effects of oxidative stress, whether in the form of crude extracts or as their chemical components[5]. Antioxidant content could contribute to disease protection offered by medicinal plants. Investigations on vegetables, fruits, and herbal plants have shown the existence of antioxidants like proanthocyanins, tannins, flavonoids, and phenolics. The present study was aimed to determine phytochemicals like phenolic, flavonoids, and tannins and their antioxidant activity.

MATERIALS AND METHODS

List of chemicals: The solvents utilized for leaf extraction were hexane, ether, ethanol and water. The laboratory reagent-grade chemicals were used for phytochemical screening and estimation. They were purchased for Merck Chemicals Pvt. Ltd., Mumbai. Distilled water was employed for aqueous extraction. Ascorbic acid, ABTS+ ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-

[a] Department of Freshman Engineering, PVP Siddhartha Institute of Technology, Kanuru, Krishna Vijayawada - 520 007, Andhra Pradesh, India
[b] Department of Chemistry, P B Siddhartha College of Arts & Science, Vijayawada- 520 010, Andhra Pradesh, India
*For Correspondence: E-mail: nannapaneniusharani73@gmail.com

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sulfonic acid)) and ferric chloride hexahydrate standard were obtained from Sigma Aldrich Chemicals, Bangalore, India.

List of apparatus: Soxhlet extraction apparatus, heating mantle, incubator, Denver electronic balance, autoclave, and TECHCOMP–UV2301 "Double Bean UV Visible Spectrophotometer" having HITACHI 2.2 software.

Sample collection: *A. rotundifolia* Roxb. plant leaves were collected from the areas at Machilipatnam, Krishna district, Andhra Pradesh, India. The plant authentication was done by Dr. Ch. Srinivasa Reddy, Assistant professor, Dept. of Botany, P. B. Siddhartha College of Arts and Science, Vijayawada, Andhra Pradesh, with voucher specimen number BO6435. The leaves were separated and allowed to dry. The dry leaf sample was grounded, powdered, and subjected to solvent extraction.

Solvent extraction: The leaf extracts of *A. rotundifolia* Roxb. were prepared with solvents such as ether, hexane, water and ethanol in a sequence of extraction techniques from low to high polarity by the Soxhlet extraction technique. The crude extracts were maintained in a desiccator after the solvents were evaporated using a rotary evaporator.

Preliminary phytochemical screening

The crude extracts of the leaf were examined for the existence of glycosides, saponins, tannins, steroids, and alkaloids. The qualitative finding is reported as positive for the presence and harmful for the non-existence of phytochemicals[6,7].

Test for terpenes

5 mL leaf extract was added to a mixture of 2 mL chloroform and 3 mL of conc. H_2SO_4. The reddish-brown ring formation was observed and confirmed the existence of terpenes.

Test for flavonoids

The extract of the plant material was combined with a small amount of strong hydrochloric acid. The rapid development of red color confirmed the flavonoids' presence.

Test for saponins

Frothing test: In a test tube, distilled water was used to dissolve around 0.5 g of the extract. The existence of saponins in the leaf extract was confirmed by the presence of frothing that remained after heating.

Test for steroids

Liebermann–Burchard reaction: 2 mL of acetic anhydride and 2 mL of conc. H_2SO_4 were added to 5 mL of the extract. The mixture solution color was changed from violet to blue, and the existence of steroids in the leaf extract was confirmed.

Test for cardiac glycosides

About 2 mL of glacial acetic acid (one drop of ferric chloride solution) and 1 mL of Conc. H_2SO_4 were added to the leaf extract. The brown ring appeared and confirmed the presence of cardiac glycosides in the leaf extract.

Test for tannins and phenolic compounds

The plant extract was mixed with to acetic acid, and red-colored solution formation was observed, which confirmed the existence of tannins and phenolic compounds in the leaf extract.

Test for alkaloids

In a steam bath, about 0.5 g of the leaf extract was mixed with 5 mL of the 1 % aqueous HCl. A few drops of Dragendorff's reagent were added to 1 mL of the filtrate. A reddish-brown precipitate was observed and confirmed the presence of alkaloids.

Test for carbohydrates

Barfoed's reagent was added to the extract and boiled in the water bath for a few minutes; a reddish precipitate was observed, which confirmed the presence of carbohydrates.

QUANTITATIVE ANALYSIS

Quantitative analysis of phenolic compounds

FCR (Folin-Ciocalteu's Reagent) calculates the total phenolic concentration of numerous solvent extracts. The technique combines 1 mL of an extract with 0.4 mL FCR diluted 1:10 V/V. 4 mL of 20% w/V sodium carbonate solution is added after five minutes. The solution is then allowed to stand for 90 minutes at room temperature after being diluted to a final volume of 10 mL in the tubes. The sample's absorbance is measured using a spectrophotometer at 765 nm compared to the blank. Gallic acid solutions are applied as the standard to generate a calibration curve. The total amount of phenol in the extract is represented as a milligram of gallic acid/gram of dry weight[8-11].

Total flavonoid content determination

Using quercetin as a standard, the total flavonoid concentration was assessed using aluminum chloride ($AlCl_3$) following a well-established procedure. 3 mL of

distilled water and 5% $NaNO_2$ were combined with 1 mL of the plant extract 0. 3 mL. $AlCl_3$ 0.3 mL, 10% was added after 5 min at 25 °C. The reaction mixture was diluted with 2.0 mL of 1 M NaOH after 5 minutes. The reaction mixture was then diluted in 10 mL of water, and the absorbance was evaluated at 510 nm. Using quercetin solutions as the standard, a calibration curve was created, and the total phenolic extract contents were quantified in mg of quercetin per mg of dry weight.

MEASUREMENT OF ANTIOXIDANT ACTIVITY

ABTS radical's scavenging activity

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was evaluated by estimating the ABTS radical cation disappearance in Re et al.'s[12] technique. Potassium per sulfate 2.4 mm and ABTS 7 mm were combined to create the stock solution, which was then maintained in the dark for 12 h to 16 h at room temperature. To get an absorbance of 0.708±0.001 units at 734 nm from the spectrophotometer, the solution was then diluted by combining 1 mL of ABTS solution with 60 % methanol. Fresh ABTS solutions were created for each test. The absorbance was then determined at 734 nm after 1 mL of plant extracts has been added to react with 1 mL of the ABTS solution. The absorbance decline was assessed every minute for up to six minutes. The ultimate absorbance was then recorded. The formula below was used to compute the percentage inhibition.

"Percentage inhibiting activity= (1–Abs. of sample/ Abs. of control) ×100"

Phosphomolybdate assay (Total antioxidant capacity)

This technique assesses samples' total antioxidant capability[13]. A vigorous stirrer was used to combine a 0.1 mL aliquot of the sample solution with 1mL of the reagent solution 4M ammonium molybdate, 28M sodium phosphate, and 0.6M sulfuric acid. At 95 °C, the water bath was covered, and the test tubes were incubated for 90 minutes. The mixture's absorbance at 765 nm was determined after the samples had been cooled. The standard was ascorbic acid. The following formula was used to assess the antioxidant capacity.

"Total anti-oxidant capacity (%)=[(Abs. of control–Abs. of the sample)/(Abs. of control]×100"

H_2O_2-scavenging activity

The extract's H_2O_2 activity was measured using Ruch et al's[14] technique. 50 mm phosphate buffer pH 7.4 was used to create a two mm H_2O_2 solution. Test tubes were filled to a capacity of 0.4 mL with 50 mm phosphate buffer pH 7.4 after an aliquot 0.1 mL of the extracted sample was added. A 0.6 mL H_2O_2 solution was added to the tubes, which were then mixed, and the H_2O at 230nm absorbance was measured.

"Percentage inhibiting activity= (1–Abs. of sample/ Abs. of control) ×100"

The value of EC is the effective content needed to scavenge 50 % of H_2O_2 radicals. The 50 % inhibition of DPPH radicals is obtained using EC values. Standards include rutin and ascorbic acid.

FRAP

Following Benzie and Strain's approach, the spectrophotometer is used to examine the antioxidant capacity of plant leaf extracts[15]. The process depends on reducing the Fe^{3+} TPTZ complex, a color less complex, into Fe^{2+}- TPTZ, a blue complex, created when antioxidants donate electrons at low pH. The change in absorbance at 593 nm was used to track this process. The FRAP reagent combines 20mm $FeCl_3.6H_2O$, 10mm TPTZ in 40 mm HCl, and 300 mm acetate buffer in a 10:1:1 ratio at 37 °C. Using a 1-5 mL variable micropipette 3.995 mL, freshly made functioning FRAP reagent was pipetted into a mixture containing 5 µL of the correctly diluted plant material, and the mixtures were well mixed. A solid blue color complex was generated when Fe TPTZ (Ferric tripyridyltriazine) complex was reduced to ferrous form as well as absorbance at 593 nm was determined against a reagent blank (3.995 mL FRAP reagent + 5 µL distilled water) after 30 minutes incubation at 37 °C. All measurements were conducted in triplicate. The absorbance at 593 nm vs. various $FeSO_4$ concentrations plot results in the calibration curve and by plotting the amounts of $FeSO_4$ vs the content of the common antioxidant Trolox. The FRAP values were determined by evaluating the absorbance shift in the test mixture with that acquired from the rising range of Fe, and they were given as mg of Trolox comparable per gram of the sample.

RESULTS AND DISCUSSION

In the present study, the leaf of *A. rotundifolia* Roxb. was screened for determining its phytochemicals in three extracts. In the aqueous extract, steroids, alkaloids, flavonoids, glycosides, tannins and phenolic compounds were detected Tannins, flavonoids, alkaloids, phenolic compounds, tri terpenoidal saponin and carbohydrates were detected in the ethanolic extract. In the ether extract,

only saponins and steroidal saponin were identified. All the qualitative results are presented in Table I. To assess phenolic chemicals and flavonoids, a quantitative analysis of the extracts has also been performed (Table II and Fig. 1). Total phenolic compounds were observed in ethanolic as well as water extracts with estimates as 59.6 mg g^{-1} and 41.6 mg g^{-1} extract equivalent to gallic acid, respectively. Flavonoids were reported in ethanolic and water extracts and were quantified as 19.8 mg g^{-1}, and 36.7 mg g^{-1}, equal to quercetin were found. The results indicate a high number of phenolic compounds and flavonoids in leaf material. Phenolic compounds also confirm the local usage of plants as a source of tannins. Previously, Debjit Ghosh et al (2019)[16] reported 25 phyto constituents in the extract, the majority of which belonged to alkaloids and flavonoids by "LC-Q-TOF-MS" analysis in ethanolic leaves extract of *A. rotundifolia* Roxb. Seven *in vitro* models also reported dose-dependent antioxidant activity. Hence, it is proved that the plant leaf consists of various secondary metabolites.

Numerous phytochemicals were discovered to offer a wide variety of therapeutic characteristics that may aid in preventing different illnesses. Hence the plant leaf extracts were further studied for antioxidant property by using antioxidant methods. These three leaf extracts have been tested for their antioxidant capacity to investigate the plant's biological activities. Anti-oxidant activity of the extracts was performed by four antioxidant activity methods i.e, ABTS radical scavenging activity, phosphormolybdate assay, peroxide activity, and FRAP reduction. Results reveal that the aqueous and ethanolic extracts, which consist of flavonoids and phenolic compounds in high amounts, have shown high antioxidant

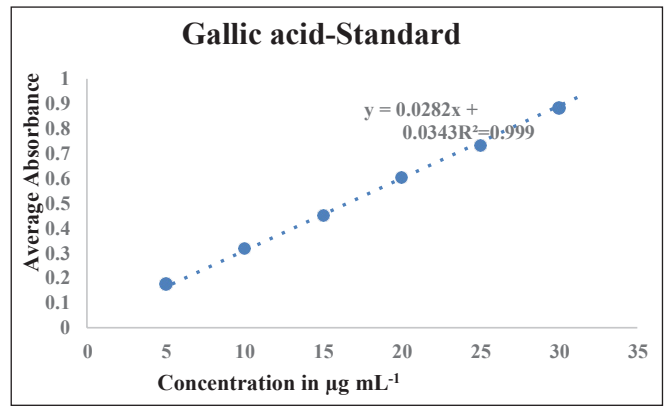

Fig. 1: Calibration curve of gallic acid (phenolic compounds)

activity (Fig. 2) compared to the ether extracts in all four studied methods. Almost similar dose-dependent antioxidant activity has been noted with both ethanolic extract and aqueous extract i.e., ABTS radical scavenging activity (82.4% reduction at 200 µg mL^{-1} of ethanol and 90.0 % reduction at 200 µg mL^{-1} of aqueous extract), phosphomolybdate assay (82.4% reduction at 200 µg mL^{-1} of ethanol and 74.5% reduction at 200 µg mL^{-1} of aqueous extract) and peroxide activity (92.1 % reduction at 200 µg mL^{-1} of ethanol and 93.1% reduction at 200 µg mL^{-1} for of aqueous extract). The IC_{50} value with ABTS radical scavenging activity includes 86.91 µg mL^{-1} for ascorbic acid, 197.9 µg mL^{-1} for ether extract, 144.3 µg mL^{-1} for ethanol extract, and 116.8 µg mL^{-1} for aqueous extract, respectively. The IC_{50} value with phosphomolybdate assay includes 246.3µg mL^{-1} for ascorbic acid, 577.0 µg mL^{-1} for ether extract, 339.9 µg mL^{-1} for ethanol extract, and 326.5 µg mL^{1} for aqueous extract, respectively. The IC_{50} value with peroxide activity includes µg mL^{-1} for

Table I: Results of phytochemical screening

Compounds	Hexane	Ether	Ethanol	Water
Steroids	Negative	Negative	Positive	Positive
Triterpenoids	Negative	Negative	Negative	Negative
Saponins	Negative	Positive	Negative	Negative
Steroidal saponin	Negative	Positive	Negative	Negative
Triterpenoidal saponin	Negative	Negative	Positive	Negative
Alkaloids	Negative	Negative	Positive	Positive
Carbohydrates	Negative	Positive	Positive	Negative
Flavonoids	Negative	Negative	Positive	Positive
Glycosides	Negative	Negative	Negative	Positive
Phenolic Compounds	Negative	Negative	Positive	Positive
Tannins	Negative	Negative	Positive	Positive

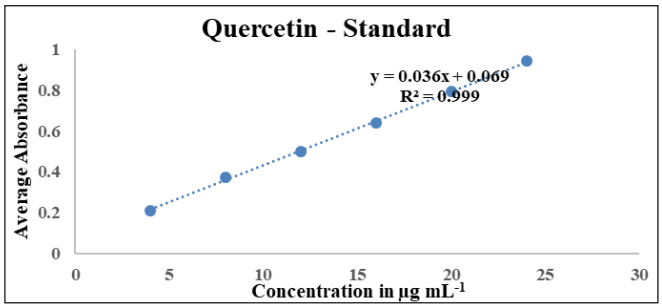

Fig. 2: Calibration curve of querecetin (flavonoid)

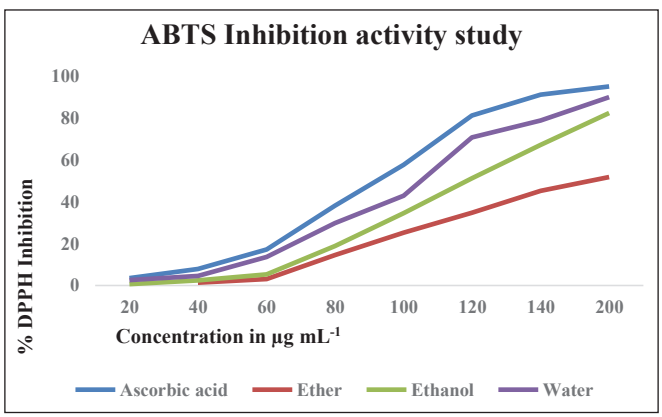

Fig. 3: Comparative graph of ABTS radical scavenging activity

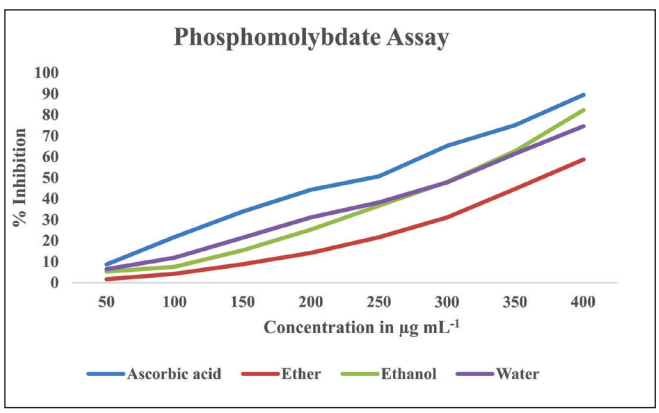

Fig. 4: Comparative graph of phosphomolybdate assay

ascorbic acid, 206.1 µg mL^{-1} for ether extract, 160.8 µg mL^{-1} for ethanol extract, and 151.8 µg mL^{-1} for aqueous extract, respectively. Similar antioxidant activity is reported with ethanol (157.4mmol Fe^{2+}/g) and aqueous extract (129.8 mmol Fe^{2+}/g) by analyzing with Ferric reducing antioxidant power (FRAP) reduction. All the results are presented in Figs. 3-6 and Table III.

Various authors have reported similar antioxidant studies with leaf and other parts of *A. rotundifolia* Roxb.Reddy ARK et al (2016)[17] reported the potential antioxidant activity of leaf extracts of *A. rotundifolia* Roxb.

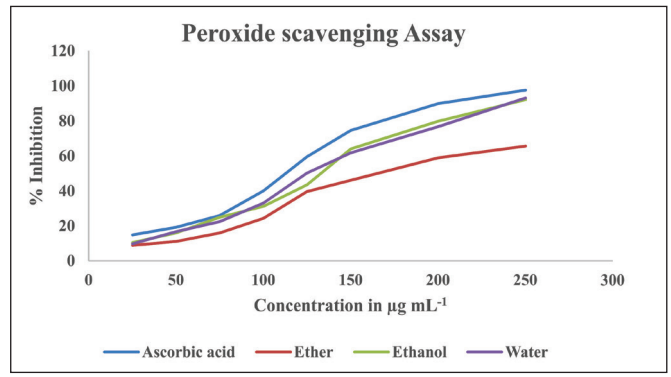

Fig. 5: Comparative graph of peroxide activity

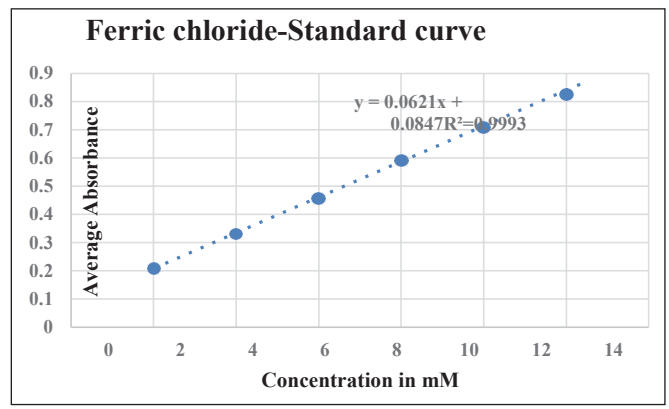

Fig. 6: Comparative graph of ferric reducing antioxidant power (FRAP) reduction

Table II: Quantitative analysis of phenolic compounds and flavonoids *A. rotundifolia* Roxb leaf extracts

Solvent extract	Phenolic compounds	Flavonoids
Ethanol	59.6 mg g^{-1}	19.8 mg g^{-1}
Water	41.6 mg g^{-1}	36.7 mg g^{-1}

Table III: Results of ferric reducing antioxidant power (FRAP) reduction

Solvent extract	Concentration µg mL^{-1}
Ether	24.6mmol Fe2+/g
Ethanol	157.4mmol Fe2+/g
Water	129.8mmol Fe2+/g

along with *Brugeiera gymnorrhiza* in chloroform, acetone, ethyl acetate and methanol. Results prove that methanol infusions have the highest antioxidant activity. R. K. Reddy et al (2017)[18] reported the anti-cancer activity of *A. rotundifolia* Roxb. ethanolic extract along with three other mangrove plants with MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Saurav Sett et al

(2014)[19] reported the antifungal activity of *A. rotundifolia* Roxb. acetone extract of leaf against pathogenic fungi *Mycovellosiella* which is found in greyish brown spot on the leaves of *Syzygium samarangenseisis.* Imam Hasan et al[20] reported the antibacterial activity of methanolic extract of *Staphylococcus aureus* as a Gram-positive (+ve) as well as *Pseudomonas aeruginosa, Escherichia coli*, and *Salmonella typhi* as Gram-negative (–ve) bacteria by disc diffusion technique. Methanolic extracts of whole plants of *A. rotundifolia* and *A. neriifolia* were used and dose-dependent antibacterial activity was reported.

The present investigation reports better comparative antioxidant activity results and their relation with the presence of secondary metabolites like phenolic compounds and flavonoids. The treatment of many diseases, like diabetes, atherosclerosis, aging, immune suppression and neuro- degeneration, might be supported by medicinal plants' antioxidant content. It is generally recognized that free radical reactions play a considerable role in the pathology of numerous acute and chronic human illnesses. Natural antioxidants have drawn attention due to their capacity to scavenge free radicals. Hepatic damage was linked to medicinal plants, including high antioxidant content as a therapeutic strategy.

CONCLUSION

In the current investigation, qualitative analysis of secondary metabolites of the leaf was analyzed and the antioxidant activity was studied. Qualitative results prove that the plant consists of various secondary metabolites. Quantitative analysis of phenolic compounds and flavonoids demonstrates the presence of a high amount of antioxidant activity. Four different methods have been studied for evaluating the antioxidant activity to confirm that the ethanolic and aqueous extracts have high potency for activity. Further assessment of phytochemicals and their activity is necessary for recognizing the nutritional potential and potential to manufacture new drugs.

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