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PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF METHANOLIC EXTRACT OF SELECTED MEDICINAL PLANTS OF FAMILY *PHYLLANTHACEAE*

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ABSTRACT

Phytochemical screening and antioxidant activities in methanolic extracts of *Phyllanthus madraspatensis* and *Breynia vitis idaea*, was carried out. The extracts were subjected to various chemical test for phytochemical constituents, total phenolic contents were evaluated using Folin Ciocalteu method and their antioxidant activity was assayed through (*in vitro*) radical scavenging activity using DPPH assay, FRAP and reducing power. The phytochemical screening of this study indicated the presence alkaloids, terpenoids, tannis, reducing sugar, saponins, flavonoids, Quinine, protein and steroids in whole plant parts. Results obtained in this investigation indicate that *P. madraspatensis* extract, rich in phenolics and flavonoids exhibited highest antioxidant. It was observed that the whole plant parts extracts contained high level of phenolic and flavonoid contents that might have accounted for the strong activity observed against DPPH radicals, FRAP and Reducing power. This shows that *P. madraspatensis* methanolic extract had positive correlation when compared to *Breynia vitis idaea* methanolic extract may be a potent source of natural antioxidant and its use in the management of diseases associated with oxidative stress is justified.

INTRODUCTION

Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectivity. These are the reservoirs of potentially useful chemical compounds which could serve as newer leads and clues for modern drug design (Vijyalakshmi and Ravindran, 2012). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Doss, 2009). Correlation between the phytoconstituents and the bioactivity of plant is desirable to know for the synthesis of compounds with specific activities to treat various health ailments and chronic diseases as well (Pandey *et al.,* 2013).

For ages nature has gifted us plenty of herbs and plants which form the main source of traditional medicines used to help in relief from illness and are still widely used all over the world. Herbal treatment is still used for many health problems. Herbs are safe, less toxic, economical and a reliable key natural resource of drugs all over the world. Use of traditional medicine among the tribal local people and medicinal healers (Hakim) is a significant part of Indian's tradition and it is widely practiced till date (Al-Essa *et al.,* 1998).

Secondary metabolites are produced by plants mainly as products of primary metabolism and as part of the defence mechanisms of plants. Phytochemicals such as, alkaloids, tannins and flavonoids are examples of secondary metabolites produced by plants, from which the plants are thought to get their healing properties (Bhandary *et al.,* 2012). Phenolic compounds have been associated with antioxidant activity due to their free radical scavenging activities (Maria John *et al.,* 2015; Rezaie *et al.,* 2015).

The genus *Phyllanthus* L. (*Phyllanthaceae*, formerly *Euphorbiaceae*) consist of about 800 species of trees, shrubs and annual or biennial herbs distributed throughout the tropical and subtropical regions of both hemispheres (Govaerts *et al*., 2000). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There is growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity (Kumar *et al*., 2009). *Phyllanthus madrespatensis* is an erect or spreading sub shrub, growing to only 50 cm tall, well branched and hairless. It is also called as Madras Leaf flower as it is

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originated from the Madras region of India. *P. madrespatensis* occurs in deciduous wood land, wooded savanna and grass land, on beaches and dunes, and also along streams and ponds in cultivated and distributed localities, from sea level up to 1400 m altitude. It grows on a wide variety of soils, usually on heavy clay and alluvials soils of low altitude river valleys on river banks and in flood plains.

The active constituents of *P. madrespatensis* are essential oil, Maderin, mucilage, β-sitosterol. The clear deep yellow oil can be extracted from the seeds of *P. madrespatnsis.* The seeds contain myristic, palmitic, stearic, oleic and linolenic acids and β-sitosterol. The deffated seed cake contains fibrous mucilage which can be hydrolysed to galactose, arabinose, rhamnose, and aldobionic acid.

Breynia vitis idaea (Burm.f.) is a perennial tree-like species of *Phyllanthaceae* (Euphorbiaceae s.l.), found from India east to Taiwan and Okinawa and south to Indonesia. It is a shrub or tree let with egg-shaped leaves that can reach up to 3 m tall. It has staminate flowers and spherical, red fruit. The seeds are black and have a very hard seed coat. In this plant in roots contain β-sitosterol. Leaves contain triacontane, ceryl alcohol, lanosterol, pentatriacontanoic acid. New-fangled sulphur containing spiroketol glycoside, breynin I and a new terpenic glycoside, breyniaionoside E together with 10 known compounds were isolated from the aerial parts of *Breynia vitis-idaea.* The decotion of the root is employed as mouthwash for toothache. Leaves applied as poultice to hasten suppuration. The leaf juice given after parturition to prevent the haemorrhage. Dried leaves are smoked like tobacco to relief in tonsillitis. Astringent bark used to guard against haemorrhage (Pullaiah and Moulali, 1997).

The presence of an excess of oxygen in the human body has some negative effects as it can trigger radical chain reactions in the presence of reactive species. This can cause health problems, such as aging and cell destruction (Ye *et al.,* 2015). Antioxidants have been found to be the solution to this problem as they interrupt these chain reactions to form radicals that can easily be removed from the human body, thereby generally improving health, assisting cell rejuvenation, cancer prevention and cardiovascular diseases prevention (Li *et al.,* 2015). Thus it is important to investigation was carried out to screen the phytochemical and antioxidant potential of *P.madrespatensis* and *Breynia vitis idaea* medicinal plants.

MATERIALS AND METHODS

Collection and Preparation of Plant

The medicinal plants were collected from the Agricultural field of Thiruvarur District of Tamil Nadu and were identified and authenticated by the experts of Botanical Survey of India, Agricultural University at Coimbatore. A herbarium was deposited in the Department of Botany as BSI/SRC/5/ 23/2016/Tech.-1831 and BSI/SRC/5/23/2016/ Tech.-1830 in M.R. Govt. Arts College, Mannargudi. The whole plants were selected for phytochemical analysis. Fresh plant of medicinal plant namely *P. madraspatensis* and *B. vitis idaea* were collected in sterile polythene bags. The whole plant were collected and dried for 8 days and pulverized into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis was commenced.

Preparation of the Plant Extracts

The selected medicinal plants were dried powder (500g) in an electric oven at constant temperature and were extracted with polar and Non polar solvents such as Methanol, n-Butyl alcohol, Acetone, Diethyl ether and Aqueous extract using the Soxhlet apparatus. 1gm of powdered material was taken in a clean conical flask and soaked in each 20 ml of Aqueous, Methanol, n-Butanol, Acetone and Diethyl ether into a conical flask, closed with rubber corks and left for 3 days with occasional shaking. The extracts were filtered through Whatman no.1 filter paper, with respectively. The preparation of the extract was carried out by hot distillation using Soxhlet apparatus. The water extract was obtained at 100ºC and reduced to near dryness by freeze-drying.

Phytochemical Screening

The phytochemical experiments were carried out for all extracts as per the standard methods described by Harborne (1998).

Detection of Alkaloids

(Mayer's Test): One ml of extract was treated with 3-5drops of Iodine solution and added 1ml of Mayer's reagent and observed for the formation of yellow color precipitate.

Test for Terpenoids

Salkowski Test: One ml of extract was added to 1ml of concentrated H_2SO_4 and heated for 2 min. formation of greyish color immediately indicated the presence of terpenoids.

Test for Tannins

Ferric chloride test: One ml of extract was mixed with 2-3 ml of % ferric chloride solution and observed for formation of blue or green colour indicates presence of Tannins.

Test for Reducing Sugar

Fehling's Test: One ml of plant extract was mixed with equal volume of Fehling's solutions A and B and boiled for few minutes. The formation of a red or brick red color precipitate was indicates the presence of a reducing sugar.

Test for Saponins (Foam test)

One ml of extract was added to 4-5ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Test for Flavonoids

Shinoda Test: One ml of crude extract was treated with few fragment of magnesium ribbon and added two-Three drops of concentrated HCl and appearance of pink scarlet colour, confirm to presence of flavonoids.

Test for Quinones

One ml of extract was treated with One ml of 1% NaOH and observed for the formation of blue green or red indicates the presence of Quinones.

Test for Protein

One ml of extract was treated with few drop of Nitric acid, observed for the formation of yellow colour indicates the presence of protein.

Test for Sterols (Liebermann-Burchard test)

One ml of extract was treated with One ml of chloroform with One ml of conc. H_2SO_4 and observed for the formation of red colour indicates the presence of sterols.

Determination of Total Phenolic Content

One ml of extract was transferred to a test tube, then 0.5 ml 10% of the Folin-Ciocalteu reagent mixed well and add 2 ml of 20% of Na_2CO_3 solution was added and followed by incubate 45°Cin shaking incubator for 15 min. after which the absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid (Mc Donald *et al*., 2001).

Determination of Total Flavonoids Content

The method is based on the formation of the flavonoids - aluminium complex which has an absorptivity maximum at 415nm. The 100µl of the plant extracts in methanol (10 mg/ml) was mixed with 100 µl of 20 % aluminum trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5ml. The absorption at 415 nm was read after 40 minutes. Blank samples were prepared from 100 ml of plant extracts and a drop of acetic acid, and then diluted to 5ml with methanol. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates.

Antioxidant activity

The antioxidant activity carried out the plant extracts were; Ferric Reducing Antioxidant Power (FRAP), Diphenyl-2-Picryl-Hydroxyl (DPPH) Radical Scavenging Activity.

Ferric-reducing/antioxidant power (FRAP) assay

The Fe^{3+} reducing power of the extracts were determined by the method of Brand-Williams *et al.* (1995). Three different concentrations of plant extracts (100, 200 and 300 µg) and standard (ascorbic acid) were mixed with 250 µl of 0.2 M phosphate buffer (pH 6.6) and 250 µl of potassium ferricyanide (1 %), and then was incubated at 50 °C for 30min. Later, 250 µl of trichloroacetic acid (10%) was added to the mixture and then immediately centrifuged at 5000 rpm for 10 min. 500 µl of the upper layer solution was taken into fresh tubes in which 400 µl of 0.2 M phosphate buffer already present and followed by the addition of 100 µl of $FeCl_3$ (0.1%). The absorbance was read against blank at 700 nm.

Diphenyl-2-Picryl-Hydroxyl (DPPH) radical scavenging activity (Sharma and Bhat, 2009)

One ml of extract was transferred to a test tube, then 100µl of1 molar DPPH solution mixed well and ad 400 µl of 50 milli molar Tris HCl and then incubate room temperature for 30 minutes. Read the measurement at 517 nm.

Reducing Power (Oyaizu, 1986)

One ml extract of the plant material is added 1 ml of a Phosphate buffer solution and add 1ml of Pottassium Ferric cyanide mixed well and incubate 50°C for 20 min. and then add 1ml of 10% trichloro acetic acid mixed well add 1 ml of distilled water and 0.5 ml of 0.1% Ferric chloride solution. Read the measurement at 700 nm.

Statistical analysis

The data are expressed as the mean ± SEM analyzed by one-way analysis of variance (ANOVA) and Tukey's t-test was used as the test of significance. P value<0.05 was considered as the minimum level of significance. All statistical tests were carried out using SPSS statistical software (Snecdecor and Cochran, 1980).

RESULTS

Phytochemical screening

Phytochemicals are the core of phytomedicines; their therapeutic efficiency directly correlates with the presence of various phytochemicals (Somit *et al.,* 2014). The screening of whole plant parts of selected plants namely *P. maderaspatensis* and *Breynia vitis idaea* for phytochemical constituents was performed using generally accepted technique for qualitative determination. This study indicated the presence of Alkaloids, Terpenoids, Tannin Flavonoids, Quinine and Protein in all plant parts and the characters summarized in the (Table 1&2). Identification of plant chemical constituents is desirable because such information will be value for synthesis of complex chemical substances. Previous reports about species of Liliaceae family demonstrate the presence as phytochemicals in plants of current study is correlated with specific biological activity as immunological adjuvant, human platelet anti-aggregation, anti-inflammatory, adaptogenic, antibacterial and antioxidant activities (Ebrahimzadeh *et al.,* 2010; Karamian and Hosseini, 2014; Maja *et al.,* 2014; Turgut and Leyla, 2016). The plant products over synthetic compound in the treatment of diseases are needed, because it does not have a deleterious effect in higher plants and animals including man. The results obtained in this study thus suggest the identified phytochemical compounds may be the bioactive constituents and these plants are proving to be an increasingly valuable reservoir of bioactive compounds of substantial medicinal merit.

Total phenolic content

Phenolic compounds are high level antioxidants because they have the ability to absorb and neutralize free radicals. The mechanism of action of phenol compounds as antioxidants is based on scavenging and chelating process (Mitic *et al.,* 2014). The levels of total phenols content in methanol extracts of whole plant parts of selected plants according to the Folin–Ciocalteu method. The total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation ($y = 6.6104x + 0.0108$), $R2 = 0.9939$). In this study, the absorbance of series concentrations of gallic acid was plotted to their concentration to yield a linear calibration curve of gallic acid. According to the results data are present in (Figure 1&2). The amount of total phenols varied from 1.255 to 1.805 and 0.080 to 1.706 mg GAE/g DW for whole plant parts extracts of *P.*

maderaspatensis and *Breynia vitis idaea*, repectively. The methanolic *P. maderaspatensis* extracts possessed higher concentration of total phenols (1.805 mg GAE/g DW) than the other tested extracts. Results of this study showed different TPC between *Breynia vitis idaea* extracts. Several researchers also reported in this study (Ebrahimzadeh *et al.,* 2010; Karamian and Hosseini, 2014; Turgut and Leyla, 2016).

The difference in amounts of phenols is probably related to Folin assay which gives a crude estimate of the amount of phenolic compounds present in an extract. It is not specific to polyphenols but many interfering compounds may react with the reagent, giving elevated apparent phenolic concentrations. Moreover, various phenolics compounds respond differently in this assay, depending on the number of phenolic groups they have and total phenolics content does not incorporate necessarily (Moussa *et al.,* 2011). The total phenolic content in whole plant parts extracts is higher than 0.850 mg GAE/g dry weight could be considered as very high (Tawaha *et al.,* 2007). On the basis of this, the methanol extracts of whole plant parts must be considered as good sources of phenolic compounds. Also, all tested methanolic extracts exhibit highest phenolic content and highset antioxidant activity, this suggest that the effectiveness of the antioxidant activity of plant extract is probably related to their phenolic contents which have hydroxyl groups in phenolics (Karamian and Hosseini, 2014).

Table 1 Phytochemical screening of Whole plant of *P. madraspatensis*

Phytochemical Screening Test	Aqueous	Methanol	n-Butyl alcohol	Acetone	Diethyl ether
Alkaloids	+	+	+	+	+
Terpenoids	+	+	+	_	+
Tannins	+	+	+	+	+
Reducing Sugar	_	_	_	_	_
Saponins	+	_	_	_	_
Flavonoids	_	+	_	_	_
Quinine	+	+	+	+	+
Protein	+	+	+	+	+
Steroids	+	–	–	–	–

+ = present; - = absent

Table 2 Phytochemical screening of Whole plant of *Breynia vitis idaea*

Phytochemical Screening Test	Aqueous	Methanol	n-Butyl alcohol	Acetone	Diethyl ether
Alkaloids	+	+	+	+	+
Terpenoids	+	_	+	+	_
Tannins	+	+	+	+	+
Reducing Sugar	_	+	_	_	_
Saponins	+	_	_	_	_
Flavonoids	+	_	_	_	_
Quinine	+	+	+	+	+
Protein	+	+	+	+	+
Steroids	–	–	–	–	–

+ = present; - = absent

Total flavonoid content

Flavonoids are the most common group of plant polyphenols. Different studies have shown that these compounds are used for prevention and cure of many diseases. Flavonoids transfer hydrogen atom to free radicals, leading to interruption of free radical reactions (Mitic *et al.,* 2014). The content of flavonoids whole plant parts extracts of *P. maderaspatensis* and *Breynia vitis idaea* was determined and the data was shown in (Fig. 1&2). As in the case of total phenolics, the concentration of flavonoids in whole plant parts extracts (ranged from from 0.051 to 0.956 mg RUE/g DW) were higher than the extracts (ranged from 0.150 to 0.358 mg RUE/g DW). This method has also showed increases of antioxidant activity with concentration increasing. The similar results were observed in some medicinal plants (Sharififar *et al.,* 2009; Stankovic, 2011; Fariba *et al.,* 2012)

Studies have shown that formation of flavonoids has been shown to be light dependent. In addition, higher amounts of flavonoids may be required in the leaf for protection against environmental stresses (Liu *et al.,* 2008). The variation in total phenols and flavonoids content among species could be due to various intrinsic and extrinsic factors, one of such factors may be the genetic potential of individual species for polyphenol biosynthesis. Apart from the genetic background, the environment and maturation stage may also be critical in this respect this may due to or a various intrinsic and extrinsic factors, one of such factors may be the genetic potential of individual species for polyphenol biosynthesis.

Antioxidant activity

DPPH radical scavenging assay

The high DPPH activity could be correlated with high phenolic content. Literature survey revealed high level of phenolic content showed fast decrease in absorbance of DPPH radical (Rattanachitthawat *et al.,* 2010). DPPH is a stable, nitrogen centered free radical which produces violet colur in methanol solution. It was reduced to yellow coloured product, diphenylpicryl hydrazine, with the addition of the methanolic whole plant extract in a concentration dependent manner.

Scavenging of nitric oxide radical is based on the generation of nitric oxide. Sodium nitroprusside in buffered saline, reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. *Phyllanthus madraspatensis* and *Breynia vitis idaea* are decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*.

Superoxide anions are a precursor to active free radicals, which is normally formed first in cellular oxidation reactions.

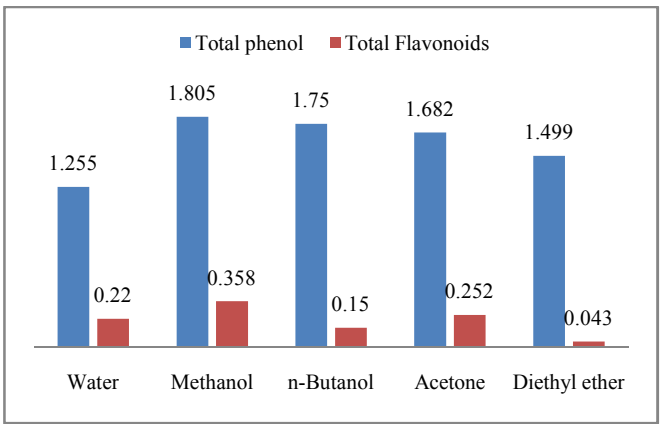

Figure 1 Total Phenolic and Total Flavonoids content of *P. madrespatensis*

Although, it is not highly reactive, it can produce other ROS such as hydrogen peroxide, hydroxyl radical and single oxygen. Furthermore, superoxide anion radical and its derivatives can cause damage in lipids, proteins and DNA. Therefore, it is of great important to scavenge superoxide anion radical (Xie *et al.,* 2008). The methanolic whole plant extract of *P. madraspatensis* exhibited significant superoxide dismutase activity (Table -3&4). This results also confirmed by the result of Sumczynski *et al.* (2015) and Kayini Chigayo *et al.* (2016).

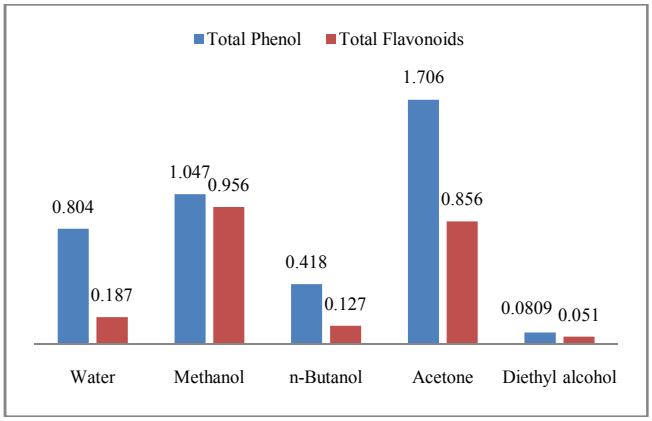

Figure 2 Total Phenolic and Total Flavonoids content of *Breynia- vitis idaea*

Table 3 Antioxidant activity of Uv-Spectrophotometry Analysis

Antioxidant activity	*Phyllanthus madraspatensis*				
	Water	Methanol	n-Butanol	Acetone	Diethyl ether
DPPH activity	0147±0.11	0.775±0.18	0.614±0.12	0.241±0.12	0.430±0.27
FRAP	0.019±0.13	0.111±0.15	0.023±0.17	0.046±0.22	0.028±0.16
Reducing Power	0.305±0.16	1.235±0.19	1.006±0.18	1.342±0.20	0.448±0.21

Table 4 Antioxidant activity of Uv-Spectrophotometry Analysis

Antioxidant activity	*Breynia vitis idaea*				
	Water	Methanol	n-Butanol	Acetone	Diethyl ether
DPPH activity	0.279±0.22	1.124±0.23	0.314±0.19	0.258±0.15	0.007±0.17
FRAP	0.064±0.23	0.098±0.17	0.0350±0.14	0.031±0.17	0.0790.16
Reducing Power	0.512±0.16	0.701±0.17	0.416±0.18	0.790±0.20	0.598±0.18

Ferric-reducing/antioxidant power (FRAP) assay

The literature survey showed that the FRAP assay is sensitive method for the measurement of total antioxidant power of the fresh biological fluids such as plant homogenates and pharmacological plant products (Vasco *et al.,* 2008). The methanolic whole plant extract of *P. madraspatensis* exhibited high FRAP value even at the very lower concentration (Table-3&4). Several researchers also reported by the antioxidant activity in some plants (Dhanani *et al.,* 2013; Xie *et al.,* 2015; Kayini Chigayo *et al.,* 2016).

Reducing power assay

The antioxidant activities of natural components may have a reciprocal correlation with their reducing capacity (Duh and Yen, 1997).Thus the reducing capacity of these compounds may serve as a significant indicator of its potential antioxidant activity (Liu *et al.,* 2013). High reducing power of flavonoids suggested their remarkable potency to donate electrons to reactive free radicals, thus converting them into morestable non-reactive species and finally terminate the free radical chain reaction (Zha *et al.,* 2009). In our present study, the transformation of Fe^{3+} to Fe^{2+} was determined as reducing capacity. The data was presented in table-3&4. Considering the poverty of methanolic extract in term of compounds responsible for reducing power (flavonoids), the results obtained were justified. Adding to that statement, interferences due to other compounds are present in the extract for reducing power assay which is often a limiting factor. These results were consistent with previous tests concerning flavonoid amountand radical scavenging activity. This results confirmed by the results of Zhang *et al.* (2015), Alkhawalidy and Hossain (2015) and Sumczynski *et al.* (2015).

CONCLUSION

In the present study clearly indicated that phytochemical analysis and *in vitro* antioxidant activity were studied in selected medicinal plants from *Phyllanthaceae* family. *Phyllanthus madraspatensis* and *Breynia vitis idaea*, had revealed the presence alkaloids, terpenoids, tannis, reducing sugar, saponins, flavonoids, Quinine, protein and steroids in whole plant parts by positives reaction with the respective test reagent. Results obtained in this investigation indicate that *Phyllanthus madraspatensis* extract, rich in phenolics and flavonoids exhibited highest antioxidant. It was observed that the whole plant parts extracts contained high level of phenolic and flavonoid contents that might have accounted for the strong activity observed against DPPH radicals, FRAP and Reducing power. Our data indicate that the whole parts of studied plants are potential sources of secondary metabolites and their methanolic extracts possess good antioxidant activity. However, further studies are needed to evaluate the *in vivo* potential of these extracts in animal models and also isolation and characterization of the active antioxidant compounds.

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