In vitro antioxidant potential of eleven medicinal herbs in Sri Lanka: Correlation with phenols and flavonoids

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Abstract: Antioxidants play a crucial role in preventing and treating non-communicable diseases (NCDs) by scavenging free radicals. Medicinal herbs, used for centuries in traditional healthcare systems, have been gaining attention recently due to the negative effects of synthetic medicines. This study assessed the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of aqueous extracts from eleven commonly used Sri Lankan Ayurvedic plants and determined the relationship between their phenolic and flavonoid content with antioxidant activities. TPC and TFC were measured using the Folin-Ciocalteu and Aluminum chloride methods, respectively. Antioxidant activity was evaluated using ABTS, FRAP, and DPPH assays. Pearson correlation analysis assessed the relationship between TPC and TFC with antioxidant activity. *Phyllanthus emblica* (PE) showed the highest TPC, TFC, and antioxidant activity ($p \leq 0.05$) significantly. TPC and TFC exhibited significantly positive correlations with FRAP and ABTS assays while the DPPH assay showed a negative correlation. Phenols and flavonoids in the selected extracts may significantly contribute to the antioxidant activity measured by ABTS and FRAP assays, while other secondary metabolites and their synergism effect may influence the DPPH assay. The significant antioxidant properties of PE, highlight its potential to treat various NCDs. Further studies are essential to determine their bioactivities, effective doses, and toxicity levels.

1. INTRODUCTION

Non-communicable diseases (NCDs), also known as chronic diseases, have emerged as a global pandemic, leading to millions of deaths and disabilities worldwide. According to the World Health Organization (WHO), in 2023, NCDs accounted for 74% of all annual deaths globally, affecting over 41 million people, with more than 15 million falling within the age group of 30 to 69 years (World Health Organization, 2023). Various physiological processes in the human body produce free radicals, and their overproduction causes oxidative damage to biomolecules, which eventually leads to numerous chronic diseases including cardiovascular diseases, cancers, and diabetes mellitus (Fu et al., 2011).

Antioxidants play an important role in the prevention and treatment of chronic diseases by scavenging free radicals. Due to the adverse side effects associated with synthetic antioxidants, there is a growing trend to substitute them with naturally occurring antioxidants (Dudonné et al., 2009). Plants are reported to contain a wide range of free radical scavenging molecules like phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins), nitrogen compounds (alkaloids, amines, and betalains), vitamins, terpenoids (including carotenoids), and some other metabolites that exhibit potent antioxidant properties (Choi et al., 2002; Fu et al., 2011).

Medicinal herbs are increasingly recognized as rich sources of natural antioxidants and play a significant role in traditional healthcare systems. The World Health Organization (WHO) reports that nearly 80% of the population in many developing countries, representing

approximately 60% of the world's population, relies on alternative medicine derived from medicinal plants (Krishnaraju et al., 2005; Tugume & Nyakoojo, 2019). Recognizing the significance of medicinal plants in traditional medicine, there is a growing global interest in medicinal plants and their healthcare practices for promoting health and preventing and curing diseases.

Sri Lanka, a tropical island abundant in numerous medicinal herbs, has the majority of them not yet to be scientifically evaluated for their antioxidant properties. Therefore, this study aims to assess the *in vitro* TPC, TFC, and antioxidant activity of eleven medicinal herbs highly consumed in the Ayurvedic system of Sri Lanka and determine the relationship between their phenolic and flavonoid content with antioxidant potentials.

2. MATERIAL and METHODS

2.1. Plant Materials

Eleven plant materials representing ten Sri Lankan medicinal plant species were purchased from an Ayurvedic Shop in Kandy, Sri Lanka (

Table **1.**). All the samples were authenticated by the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. The plant samples were identified by the author (Ruvini Liyanage) and the descriptions given in the book “Medicinal Plants (Indigenous and Exotic) used in Ceylon” D.M.A. Jayaweera” were used to confirm its identity. Voucher specimens were deposited in the Laboratory of Nutritional Biochemistry, National Institute of Fundamental Studies, Kandy, Sri Lanka for future reference.

Table 1. The botanical name, vernacular (Sinhala) name, abbreviation, plant material, voucher number of the medicinal plants selected for the study, and their images in natural form.

Botanical name	Vernacular name	Abbreviation	Plant material	Voucher number	Image of the natural plant material
Aegle marmelos	Beligeta	AM-Fr	Fruits	RL_AM-Fr_001	
Aegle marmelos	Belimal	AM-Fl	Flowers	RL_AM-Fl_002	
Cassia auriculata	Ranawara	CA	Flowers	RL_AM-Fl_003	
Phyllanthus emblica	Nelli	PE	Fruits	RL_AM-Fl_004	
Hemidesmus indicus	Iramusu	HI	Roots	RL_AM-Fl_005	

Scoparia dulcis	Walkoththamalli	SD	Aerial parts	RL_AM-Fl_006	
Sida rhombifolia	Babila	SR	Aerial parts	RL_AM-Fl_007	
Asteracantha longifolia L	Neeramulliya	AS	Aerial parts	RL_AM-Fl_008	
Aerva lanata	Polpala	AL	Aerial parts	RL_AM-Fl_009	
Tinospora cordifolia	Rasakinda	TC	Stem parts	RL_AM-Fl_010	
Coscinium fenestratum	Venivel	CF	Stem parts	RL_AM-Fl_011	

2.2. Preparation of Water Extracts

The dried plant samples of each 10 g were boiled with 100 mL of distilled water for 20 minutes at 100 ^{0}C on a stirring hot plate (IKA WERKE, Japan) and then filtered. The filtrate was adjusted to a volume of 100 mL by adding distilled water and subsequently centrifuged at 3000 rpm for 10 minutes using a CP Centrifuge (Beckman, USA). The water extracts were stored in the refrigerator (LIEBHERR, CBNP5156-20B 001, Netherland) at -20 ^{0}C until further analysis.

2.3. Bioactivity Studies

2.3.1. *Determination of TPC*

Total phenolic content (TPC) was determined by the Folin-Ciocalteu method adapted from (Blainski et al., 2013) with minor modifications. The sample extract (50 µL), distilled water (15 µL), and 10% of 1N Folin-Ciocalteu's reagent (105 µL) were added into the 96 well plate and kept for 3 minutes. Then 7.5% Na_2CO_3 (80 µL) was added into the wells and incubated for 30 minutes at room temperature. The absorbance was measured at 760 nm using a Microplate Spectrophotometer (Synergy, HTX, multi-mode reader). A blank sample was prepared using plant extract (50 µL) and 200 µL of distilled water. Results were calculated using a standard curve prepared with Gallic acid using different concentrations of 0-10 µg/mL and the content of phenolic in the extract was expressed in mg gallic acid equivalent (mg of GAE/g).

2.3.2. *Determination of TFC*

Total flavonoid content (TFC) was determined by the Aluminum Chloride colorimetric assay method described by (Baba & Malik, 2015) with some modifications. Catechin (0.2 mg/mL) was used as a reference standard for plotting the calibration curve. The sample extract (50 µL) and 20 µL of sodium nitrate (5%) solution were added into the 96 well plate and kept for 6 minutes. Subsequently, 20 µL of 10% aluminum chloride was added into the wells and kept for another 6 minutes. Then, 200 µL of 4% sodium hydroxide solution was added into the wells

and incubated for 30 minutes at room temperature. The absorbance was measured at 510 nm using a Microplate Spectrophotometer (Synergy, HTX, multi-mode reader). Three replicates were used for each sample, and the blank sample was prepared using plant extract (50 µL) and 240 µL of distilled water. The content of flavonoids in the extract was expressed in terms of catechin equivalent (mg of CE/g).

2.3.3. *Determination of ferric reducing antioxidant power (FRAP)*

The FRAP assay was done according to the method described by (Fadlinizal Abd Ghafar et al., 2010) with minor modifications. The FRAP reagent was generated by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM (tripyridyl triazine) TPTZ solution, and 10 mM $FeCl_3.6H_2O$ solution at a ratio of 10:1:1. 50 µL from each sample and 200 µL from the FRAP reagent were added to the 96-well plate and the reaction mixture was incubated at room temperature for 4 minutes. The absorbance was measured at 593 nm using a Microplate reader. Three replicates were used from each sample and the blank sample was prepared using plant extract (50 µL) and 200 µL of distilled water. A different concentration of $FeSO_4$ (1.5 mM) was used to generate the standard curve. The antioxidant potential based on the ability to reduce ferric ions of the sample was expressed as mM $FeSO_4$ equivalents per gram of dry weight of the sample.

2.3.4. *Determination of ABTS radical scavenging capacity*

The ABTS free radical-scavenging activity was determined according to the method described by (Tupe et al., 2013) with minor modifications. A mixture of ABTS (2 mM) and potassium persulfate (70 mM) was allowed to stand at room temperature in the dark to form the radical cation of ABTS, 16 hours prior to use. The volume of 50 µL of each sample stock was added to 96 well plates followed by 200 µL $ABTS^+$ solution. Then absorbance was measured at 734 nm using a Microplate reader after 6 minutes of incubation at room temperature. A different concentration of (0.25 mM) 6-hydroxy-2-5-7-8- 15 tetramethyl chroman-2 carboxylic acid (Trolox) was used as the standard curve. Results were expressed as mM Trolox equivalents per gram of dry weight of the sample.

2.3.5. *Determination of DPPH radical scavenging capacity*

The ability of the extracts to annihilate the DPPH radical (1, 1-diphenil-2- picrylhydrazyl) was investigated by the method described (Brand-Williams et al., 1995) with minor modifications. Different sample volumes (30 µL, 60 µL, 90 µL, 120 µL, and 160 µL) of sample stocks were added into 96 well plates and followed by 100 µL (0.38 mM) DPPH solution and different volumes of distilled water. The reaction mixture was incubated for 30 min at room temperature and the absorbance was recorded at 517 nm using a Microplate reader. The percentage of radical scavenging activity at each concentration of the sample was calculated (5 different concentrations with three replicates). Control samples of DPPH were used with 150 µL of distilled water and 100 µL of DPPH solution.

2.4. Statistical Analysis

All the experiments were performed in triplicates, and a complete randomized designs (CRD) model was used. Data were analyzed using the SAS statistical software version 9.1 (SAS Institute Inc. NC. USA). Results were expressed as mean ± standard deviation (SD). Probability (p) values of < 0.05 were considered to be significant.

3. RESULTS

3.1. Total Phenolic Content

In the present study, the total phenolic contents of selected plants were investigated using the simple and widely utilized Folin–Ciocalteu method, and the findings are presented in (**Error! Reference source not found.**). There was a wide range of phenolic concentrations in selected plant extracts and, the values varied from 4.65 ± 1.42 to 271.23 ± 4.60 mg gallic acid equivalent

(GAE)/g dry weight. According to the statistical analysis ($p \leq 0.05$) the highest total phenolic content was recorded in the PE extract followed by CA and AM-Fr extracts.

3.2. Total Flavonoid Content

In the current study, there was a wide range of flavonoid concentrations in the medicinal plants as the values varied from 1.86 ± 0.08 to 85.51 ± 0.43 mg of catechin equivalent/g dry weight. According to the results, significantly higher ($p \leq 0.05$) total flavonoid content was recorded in the PE extract, while no significant differences ($p \leq 0.05$) were observed among the CF, SR, HI, and SD extracts, which exhibited the least activity (Error! Reference source not found.).

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of selected plant extracts.

Medicinal Plants	Plant parts	TPC (mg GAE/g)	TFC (mg CE/g)	FRAP (mM Fe^{2+}/g)	ABTS (mM TE/g)	DPPH IC_{50} value (mg/mL)
P. emblica	Fruit	271.23± 4.06^{a}	85.51±0.43^{a}	7147.9±289.03^{a}	8406.06±98.31^{a}	0.03±0.00^{g}
C. auriculata	Flower	52.29±1.23^{b}	26.07±1.03^{b}	414.3±6.68^{b}	5521.93±45.37^{b}	0.383±0.16^{g}
H. indicus	Root	7.82±0.22^{g}	1.86±0.08^{h}	82.23±2.91^{de}	79.46±1.47^{d}	66.81±4.05^{a}
A. marmelos	Fruit	42.68±1.34^{c}	18.28±0.09^{d}	170.29±2.63^{cd}	613.51±7.56^{c}	0.593±0.07^{fg}
A. marmelos	Flower	31.92±0.93^{d}	21.47±0.15^{c}	215.91±4.64^{c}	729.38±15.87^{c}	0.407±0.00^{g}
T. cordifolia	Stem	8.28±0.07^{g}	6.11±0.10^{f}	45.68±2.05^{e}	73.47±0.45^{d}	23.28±0.97^{d}
C. fenestratum	Stem	4.65±0.06^{h}	2.08±0.01^{h}	20.57±1.94^{e}	43.99±0.90^{d}	34.78±2.20^{b}
A. lanata	Aerial parts	16.75±0.75^{e}	5.05±0.21^{g}	35.46±1.89^{e}	71.19±0.52^{d}	6.2±0.30^{e}
S. rhombifolia	Aerial parts	12.34±1.07^{f}	2.72±0.21^{h}	82.23±2.70^{de}	79.25±1.28^{d}	30.93±3.48^{e}
S. dulcis	Aerial parts	10.92± 0.15^{f}	2.24±0.15^{h}	75.42±2.06^{de}	92.76±0.22^{d}	3.702±0.65^{fe}
A. longifolia L	Aerial parts	33.17±0.37^{d}	7.23±0.08^{e}	75.90±1.10^{de}	32.06±1.10^{d}	0.61±0.01^{fg}

*Data are presented as mean ± standard deviation (n=3). Mean values with different superscript letters within a column are significantly different ($p \leq 0.05$).

3.3. Evaluation of the Antioxidant Potentials

In this study, the antioxidant activity of eleven plant extracts was evaluated using DPPH radical scavenging, ABTS radical scavenging, and ferric reducing antioxidant power (FRAP) assays, which are the most widely utilized techniques for determining total antioxidant activity. For the ABTS assay, PE extract exhibited significantly the highest ($p \leq 0.05$) antioxidant activity as (8406.06±98.31 mM TE/g) followed by CA (5521.93±45.37 mM TE/g) and AM-Fl (729.38±15.87 mM TE/g).

There was a wide range of Fe (II) ions reducing antioxidant activity in the selected medicinal plants as the values varied from 21.21 ± 1.10 to 7147.9 ± 289.03 mM Fe^{2+} equivalents per gram of dry weight (**Error! Reference source not found.**). According to the statistical analysis

(p≤0.05), significantly the highest Fe (II) ions reducing ability was recorded in the PE extract followed by CA and AM-Fl.

According to the DPPH assay, there was a wide range of antioxidant activity in the medicinal plants as the IC_{50} values varied from 0.033 ± 0.001 to 66.81 ± 4.05 mg/mL. Furthermore, no significant difference (p≤0.05) was observed in PE, CA, and AM-Fl extracts which exhibited the highest activity, while the least activity was recorded in the CF extract (p≤0.05).

3.4. Correlation between Phenolic and Flavonoid Content with Antioxidant Capacities

In the current study, a Pearson correlation analysis was employed to assess the relationship between TPC and TFC with selected antioxidant assays (Figure 1.). The results revealed that the TPC exhibited significantly positive correlations with the FRAP (r=0.99) and ABTS (r=0.88) assays, while the DPPH assay showed a negative correlation (r=-0.37) with the TPC. Similarly, the TFC demonstrated significantly positive correlations with the FRAP (r=0.88) and ABTS (r=0.91) assays, whereas a negative correlation (r=-0.52) was observed with the DPPH assay for the TFC.

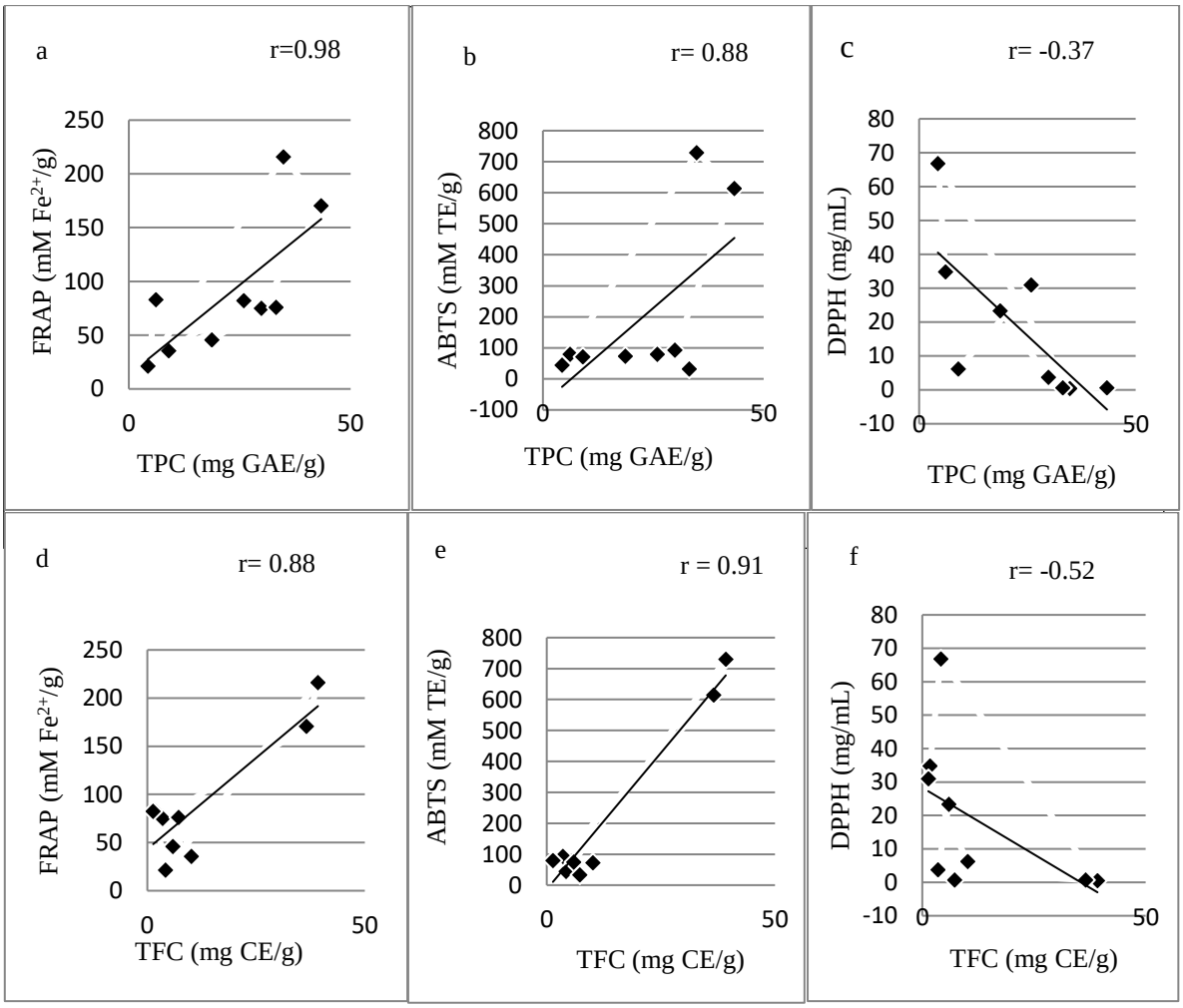

Figure 1. Correlation analysis between TPC and TFC with antioxidant activity. Correlation analysis between a: TPC and FRAP; b: TPC and ABTS; c: TPC and DPPH; d: TFC and FRAP; e: TFC and ABTS; f: TFC and DPPH. r: Pearson correlation coefficient.

4. DISCUSSION and CONCLUSION

In the present study, aqueous extracts were utilized to assess the antioxidant potential of eleven herbal plants commonly used and readily available in the Sri Lankan Ayurvedic system. These plants were selected because they are widely consumed by the local population, often prepared as herbal teas to address their daily healthcare needs. Traditionally, medicinal plants in Sri Lanka are boiled in water, and the resulting extracts are consumed to obtain health benefits. To align with these traditional preparation practices, the aqueous extraction method was chosen. Additionally, this method offers a safer and more practical approach for extracting antioxidants compared to organic solvents like methanol, due to its non-toxic nature.

Phenolic compounds are one of the most prominent groups of phytochemicals in medicinal plants and they exhibit a variety of physiological features, including antioxidant activity. Numerous studies have investigated the total phenolic content of medicinal plant extracts, both *in vitro* and *in vivo* (Cai et al., 2004; Li et al., 2008; Verma et al., 2009). Flavonoids, lignans, phenolic acids, tannins, coumarins, and stilbenes are reported as the major groups of natural phenolic compounds present in plants (Ali et al., 2008).

According to (Charoenteeraboon et al., 2010) reported the total phenolic content of water extract of dried fruit of *P. emblica* was about 342.22 ± 1.74 mg GAE/g of dry weight, which was found to be 271.23 ± 4.60 mg GAE/ g in the current study.

Flavonoids are one of the hydroxylated phenolic substances mostly found in medicinal plants. The main classes of flavonoids that are found naturally in plants are flavones, flavanol, isoflavones, flavanones, flavanonol, and anthocyanidin (Shahidi & Ambigaipalan, 2015). The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their potential antioxidant and free radical scavenging activities (Atanassova & Georgieva, 2011; Saxena et al., 2013).

In a previous study, (Sapkota et al., 2022) the flavonoid content of the aqueous extract of *P. emblica*, ranged within 20.98 ± 3.36 mg QE/g, dry basis, which was found to be 85.51 ± 0.43 mg QE/g, dry weight in our study. This difference could be due to environmental factors, the maturity level of the fruit, extraction methods, and other factors.

The antioxidant activity of medicinal plant extracts is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, singlet oxygen quenchers, and potential metal chelators (Adebiyi et al., 2017). Due to the diverse nature of antioxidants present in plant extracts, obtaining an accurate measurement of total antioxidant activity with a single assay is challenging; therefore, conducting a combination of at least two assays is necessary for a realistic assessment of antioxidant capacity (Maneetong, 2019; Sadeer et al., 2020).

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2, 4, 6-tri(2-pyridyl)- s-triazine (TPTZ), forming an intense blue color Fe^{2+} -TPTZ complex with absorption at 593 nm (Gupta et al., 2009). DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts. DPPH is a stable, nitrogen-centered free radical that creates a violet color in ethanol solution. Antioxidants present in plant extracts donate protons to these radicals and the absorbance decreases and this is taken as a measurement of the radical scavenging to assess the antioxidant activity of the selected plants (Lobo et al., 2010). According to (Charoenteeraboon et al., 2010), reported the DPPH radical scavenging activity of water extract of dried fruit of *P. emblica* was about 0.051 ± 0.01 mg/mL which was found to be 0.033 ± 0.001 mg/mL in the current study.

It was previously thought that the high amount of vitamin C content found in *P. emblica* fruit was the primary cause of its antioxidant effect. However, some studies previously have shown that in *P. emblica,* antioxidant activity is not solely dependent on ascorbic acid content; other constituents may also play significant roles. More studies have indicated that the vitamin C like activity of *P. emblica* was mainly due to the presence of low molecular weight hydrolyzable tannins, emblicanin A and emblicanin B (Bhattacharya et al., 2002; Poltanov et al., 2009;

Suryanarayana et al., 2004). According to, (Pozharitskaya et al., 2007) the DPPH radical scavenging activity of emblicanins A and B was 7.86 and 11.20 times higher than that of ascorbic acid, and 1.25 and 1.78 times higher than that of gallic acid, respectively (Pozharitskaya et al., 2007).

Correlation analysis is used to assess the relationship between multiple variables, where changes in one variable are linked to corresponding changes in another variable, either in a positive direction (positive correlation) or a negative direction (negative correlation) (Sapkota et al., 2022).

A strong correlation was observed between the TPC and TFC with antioxidant activity determined by ABTS and FRAP assays, suggesting that the phenolic and flavonoid compounds may be the major compounds that affect the ABTS free radical scavenging activity and Fe (II) reducing ability of selected medicinal plant parts. A poor correlation was observed between the TPC and TFC with the DPPH radical scavenging assay, suggesting that not only phenolic and flavonoid compounds, but the presence of other secondary metabolites and their synergism effect may be responsible for the DPPH radical scavenging activity of selected medicinal plants.

Several studies have reported the strong relationships between phenolic content and flavonoid content with antioxidant activity determined through DPPH, ABTS, and FRAP assays (Fu et al., 2011; Seo et al., 2023; Ulewicz-Magulska & Wesolowski, 2023). These findings reveal that phenolic acids and flavonoids may be key compounds that contribute to the antioxidant properties of selected plant extracts. However, it's important to note that there is some contradictory information in the literature regarding the relationship between TPC and TFC with antioxidant activity. Some previous studies have found that negative relationship between TPC and TFC with antioxidant activity in their selected samples (Amin et al., 2006; Deepa et al., 2006; Ikram et al., 2009). These conflicting findings indicate that besides phenolic compounds, there might be other secondary metabolites such as alkaloids, terpenes, and glycosides that may also contribute to the antioxidant activity of selected samples. Furthermore, the synergism among the secondary metabolites in the mixture makes the antioxidant activity dependent not only on the concentration but also on the structure and interaction between the secondary metabolites (Djeridane et al., 2006). Moreover, other chemical components contained in the extracts, including sugars, ascorbic acids, and other substances, may cause interference, and different methods of extraction may change the antioxidant activity of selected plant parts (Ghasemi et al., 2009).

In conclusion, the present study found that PE exhibited the highest TPC, TFC, and antioxidant activity ($p \leq 0.05$) out of eleven selected medicinal herbal extracts. A strong correlation was observed between the TPC and TFC with antioxidant activity determined by ABTS and FRAP assays, suggesting that phenolic and flavonoid compounds may be the key compounds that affect the ABTS free radical scavenging activity and Fe (II) reducing ability of selected medicinal plant parts. A poor correlation was observed between the TPC and TFC with DPPH radical scavenging assay, suggesting that not only phenolic and flavonoid compounds, but the presence of other secondary metabolites and their synergism effect may be responsible for the DPPH radical scavenging activity of selected plants. The significant antioxidant properties of PE, underscore its potential as a promising source for developing nutraceuticals to treat cancers and diabetes like various NCDs. However, further *in vitro, in vivo* and clinical studies are necessary to determine their bioactivities, effective doses, and toxicity levels.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Pathumi Ariyarathna: Experimental studies, data acquisition, data analysis, and writing the original draft. **Rizliya Visvanathan**: Experimental studies, data acquisition, data analysis, manuscript writing - review and editing. **Isuri Rathnayaka**: Experimental studies and data acquisition. **Terrence Madhujith:** Supervision, manuscript writing - review & editing. **Ruvini Liyanage**: Conceptualization, supervision, manuscript writing - review & editing.

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