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## Total flavonoid content, total antioxidant activities and phytochemical constituents of selected medicinal plant extracts used for oxidative stress related chronic diseases in Sri Lanka

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### Abstract

The present study was conducted to evaluate the total flavonoid content, total antioxidant activities and to determine the phytochemical constituents of selected aqueous medicinal plant extracts. The total flavonoid content was determined by aluminium chloride method. The antioxidant activities were determined by three methods namely phosphomolybdenum assay, ferric thiocyanate assay and thiobarbituric acid. L-ascorbic acid was used as the reference compounds in expressing the antioxidant activities by the three assays. The total flavonoid content of plant extracts varied from  $1.0 \pm 0.1$  -  $7.1 \pm 0.1$   $\mu\text{gQE/g}$  of dry weight. The antioxidant activities ranged from ascorbic acid equivalents of  $102.6 \pm 9.2$  -  $542.3 \pm 7.2$  mg AAE/g of the extract for phosphomolybdenum assay, percentage of inhibition of  $56.3$  -  $\pm 2.4$  -  $81.1 \pm 0.2\%$  for ferric thiocyanate assay and  $\text{IC}_{50}$  of  $112.4 \pm 2.4$  -  $390.8 \pm 5.2$   $\mu\text{g/mL}$  for thiobarbituric acid assay method. The study demonstrates that the selected medicinal plant extracts possess antioxidant activities with relatively high content of flavonoids.

**Keywords:** Ferric thiocyanate assay, flavonoid content, medicinal plant extracts, phosphomolybdenum assay, thiobarbituric acid method

### Introduction

Flavonoids are heterocyclic molecules that have been associated with beneficial effects on human health such as reducing the risk of cancer, diabetes mellitus, cardiovascular and brain diseases. Recent interest in flavonoids has been stimulated by the potential health benefits arising from their antioxidant and free radical scavenging potentials<sup>[1, 2, 3]</sup>.

Free radicals are reactive oxygen molecules that are generated by oxidation-reduction reactions in the cell<sup>[4]</sup>. Insufficient levels of antioxidant molecules or antioxidant enzymes, cause increased production of free radicals lead to cellular oxidative stress from which may damage all components of the cell, including proteins, lipids and nucleic acid. The oxidative stress is thought to be involved in the development of many of the chronic diseases. Antioxidants are reducing agents such as thiols, ascorbic acid, or polyphenol molecules that inhibit or delay the oxidation process<sup>[4]</sup>. Therefore, antioxidants play a vital role in maintenance of human health and prevention of diseases caused by free radicals. The use of antioxidant compounds to halt the proliferation of free radical chain reactions has gained impetus recently, owing to their beneficial properties. Accordingly, there is a significant interest towards discovery of novel antioxidants from medicinal plant extracts which can protect the cells from oxidative damage<sup>[5, 6]</sup>.

Since ancient times, herbal medications have been used for relief of symptoms of disease. Despite the great advances observed in modern medicine, medicinal plants still make an important contribution to the health care in most of the developing countries including Sri Lanka. Much interest, in medicinal plants however, emanates from their long use in folk medicines as well as their prophylactic properties<sup>[7]</sup>. A large number of medicinal plants have been used in Sri Lankan traditional medicine for the treatment of wide range of oxidative stress related chronic diseases such as diabetes mellitus, rheumatism, chronic kidney disease, cardiovascular diseases and liver disease<sup>[8, 9]</sup>. Medicinal plants/parts selected for the investigation are listed in Table 1.

The selected medicinal plants were grown in the Southern region of Sri Lanka and widely used by Ayurveda physicians for the management of chronic diseases. The objectives of the study were to determine the total flavonoid content, total antioxidant activities of the aqueous plant extracts and to determine the phytochemicals present in selected species.

## Materials and methods

### Plant material

A total of 10 medicinal plant species from 10 families were collected during May-July 2015 from the Southern region of Sri Lanka (Table 1). The botanical identities of all plants were determined by the descriptions given by Jayaweera (9).

### Chemicals and reagents

All chemicals and solvents were of analytical grade and used without any purification.

### Preparation of aqueous plant extracts

Selected plant parts were cut into small pieces, dried at 40 °C until a constant weight was reached. The powdered plant material (2.50 g) was dissolved in 60.0 mL of distilled water and refluxed for two hours. The mixture was filtered and the final volume was adjusted to 50.0 mL. The concentration of the final extract was 0.05 g/mL.

### Determination of total flavonoid content

The flavonoid content of the plant extracts was determined using aluminum chloride colorimetric method as described previously [10, 11]. The plant extract (0.50 mL) was mixed with ethanol 95% (1.5 mL) followed by aluminium chloride 10% (0.10 mL), 1M potassium acetate (0.10 mL) and distilled water (2.8 mL). The resultant mixture was incubated at 27 °C for 30 minutes. The absorbance of the reaction mixture was measured spectrophotometrically at 415 nm. The results are expressed as micrograms of quercetin equivalent (QE)/g of the dry weight of plant material.

### Determination of total antioxidant activity

The total antioxidant activity was determined using phosphomolybdenum assay, ferric thiocyanate assay and thiobarbituric acid assay methods [12].

### Phosphomolybdenum assay

The method described by Prieto *et al.* [13] was followed. A volume of 0.1 mL of extract was combined with 1.0 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tube was capped and incubated in a boiling water bath at 95 °C for 90 minutes. L-Ascorbic acid was used as the reference compound. After cooling the sample to room temperature, the absorbance of the sample was measured using spectrophotometrically. The antioxidant capacity is expressed as equivalents of ascorbic acid (mg AAE/g of extract).

### Ferric thiocyanate assay

The assay was done according to the method described by Kikuzaki *et al.* [14]. A volume of 1.0 mL of the plant extract (50 µg/mL), 4.1 mL of 2.51% linoleic acid in ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0) and 3.9 mL of distilled water were mixed. Then the tubes were placed in a water bath at 40 °C. A volume of 0.1 mL of the reaction mixture is transferred to a test tube and 9.7 mL of 75% (v/v) aqueous ethanol, followed by 0.1 mL of 30% aqueous ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride in 3.5%

hydrochloric acid were added. Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance of the resulting mixture was measured at 500 nm using spectrophotometrically. L-Ascorbic acid (50 µg/mL) was used as the reference compound.

### Thiobarbituric acid method

The method described by Ottolenghi [15] was followed. A volume of 2.0 mL of 20% trichloroacetic acid and 2.0 mL of 0.67% of thiobarbituric acid were added to 1.0 mL of the sample solution (plant extract). The mixture was placed in a boiling water bath for 10 minutes and the absorbance of the resultant solution was measured at 552 nm using spectrophotometrically. L-Ascorbic acid was used as the standard compound. The antioxidant activity is expressed in terms of IC<sub>50</sub> (micromolar concentration required to inhibit peroxy radical formation by 50%).

### Statistical analysis

Results were expressed as mean ± standard deviation of the three analytical triplicates.

### Results and discussion

In the present study, we determined the total flavonoid content of selected medicinal plant extracts grown in Sri Lanka by classical aluminum chloride method and the total antioxidant activities using phosphomolybdenum assay, ferric thiocyanate assay and thiobarbituric acid assay methods. Quercetin and L-Ascorbic acid were used as reference compounds for the determination of total flavonoid content and antioxidant activity respectively.

Flavonoids comprise the most common group of plant polyphenols. Epidemiological studies have been highlighted the health benefits of flavonoids in recent decades. Flavonoids/flavonoid derivatives exert a wide range of antidiabetic, anti-inflammatory, antibacterial, antiviral, anticancer and antiallergic activities [16, 17, 18]. Although the bioactivity of flavonoids appears to be mediated through a variety of mechanisms, particular attention has been focused on their direct and indirect antioxidant potentials. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures and act as free radical scavengers, reducing agents and metal chelators [19]. As shown in Table 1, total flavonoid content of the selected aqueous medicinal plant extracts were in a range of 1.0 ± 0.1 -7.1±0.1 µgQE/g of dry weight. The highest and the lowest flavonoid contents were shown in the fruit extract of *Benincasa hispida* and the bark extract of *Coscinium fenestratum* respectively. The flavonoid content of the selected medicinal plant extracts was comparable to the reported medicinal plant extracts of Sri Lankan origin [20].

Three standard chemical assays were selected in the present study, based on the ability of plant antioxidants to act as reducing agents. However, any single universal method could not be applied in the determination of antioxidant activity of natural extracts due to the presence of wide variation of antioxidant molecules. Therefore, the assays are selected to assess the overall antioxidant activity of plant extracts, encompassing different antioxidant mechanisms, compound polarity, rate of reaction, and so forth. The antioxidant activity is expressed with respect to the L-ascorbic acid. L-ascorbic acid has been widely used as a reference compound in the determination of *in vitro* antioxidant potentials of aqueous herbal extracts in a number of previous studies [21, 22].

Phosphomolybdenum assay is based on the reduction of Mo

(VI) to Mo (V) by the sample (plant extract) and subsequent formation of a green phosphate Mo (V) complex at an acidic Ph<sup>23</sup>. The highest antioxidant activity was shown in the bark extract of *Terminalia arjuna* with a value of 542.3±7.2 mg AE/g extract. This activity may be due to the presence of relatively high flavonoid content in the extract. In contrast, the lowest antioxidant activity was found in the fruit extract of *Benincasa hispida* (Table 1).

The antioxidant activity of the selected medicinal plant extracts was further determined by peroxidation of linoleic acid using the ferric thiocyanate method in the study. During linoleic acid peroxidation, peroxides are formed and these convert the ferrous into ferric. The ferric ion formed a complex with thiocyanate, which had a maximum absorbance at 500 nm<sup>24</sup>. The percentage of inhibition of linoleic acid emulsion by the plant extracts at the concentration of 50µg/mL was in the range of 56.3±2.4- 81.1±0.2%. The results indicate that the selected aqueous extracts can moderately inhibit the peroxidation of linoleic acid and reduce the formation of hydroperoxide.

Thiobarbituric acid assay method was used to evaluate the inhibition of production of carbonyl compounds degraded from the peroxides at a later stage<sup>25</sup>. As shown in Table 1, results showed that all ten extracts caused a decrease in the production of carbonyl compounds. The bark extracts of *Nauclea orientalis*, *Terminalia arjuna*, seed extract of

*Coriandrum sativum* and bark extract of *Syzygium cumini* showed relative high antioxidant activity in thiobarbituric acid assay method. Similar results were described for other plant extracts in the literature<sup>26, 27</sup>. The high antioxidant activity shown in the above medicinal plant extracts may be due to the presence of high amount of flavonoids. It is reported that the efficacy of flavonoids depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups<sup>28, 29</sup>.

The results of phytochemical analysis of the plant extracts are shown in Table 2. Polyphenol compounds and flavonoids are present in all plant extracts. These phytochemicals may be prominently contributed to the antioxidant activity of the selected plant extracts. The total polyphenol content and total antioxidant activity of the selected plant extracts of Sri Lankan origin using DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging assay, FRAP (ferric reducing power) assay, and NO (nitric oxide) inhibition assay were reported previously by our group<sup>30</sup>. However, in comparison with the previous results, the antioxidant activities of the selected plant extracts shown in the present study corroborates the antioxidant and radical scavenging potentials determined by DPPH, FRAP and NO inhibition assays. In contrast, high polyphenol content was not always accompanied by high flavonoid content in the selected plant extracts. This may be due to the complex structure of polyphenol compounds.

**Table 1:** Total flavonoid content and total antioxidant activities of selected medicinal plant extracts

Plant name	Family	Plant part tested	Total flavonoid content (µgQE/g of dry weight)	Total antioxidant activity	Ferric thiocyanate assay (Percentage of inhibition: %)	Thiobarbituric acid method IC <sub>50</sub> (µg/mL)
				Phosphomolybdenum assay (mg AAE/g of the extract)		
<i>Terminalia arjuna</i>	Combretaceae	bark	6.8±0.3	542.3±7.2	80.5±1.9	120.2±4.7
<i>Benincasa hispida</i>	Cucurbitaceae	fruit	1.0±0.1	102.6±9.2	77.6±2.2	390.8±5.2
<i>Vetiveria zizanioides</i>	Gramineae	root	2.6±0.2	442.1±3.6	83.2±2.5	260.34±2.4
<i>Osbeckia aspera</i>	Melastomataceae	leaves	3.4±0	302.9±5.6	81.1±0.2	198.5±3.8
<i>Azadirachta indica</i>	Meliaceae	leaves	2.8±0.1	202.8±3.2	56.3±2.4	229.23±2.2
<i>Coscinum fenestratum</i>	Menispermaceae	stem	7.1±0.1	367.9±2.9	63.2±2.3	300.45±6.2
<i>Syzygium cumini</i>	Myrtaceae	bark	1.9±0	126.3±1.3	67.4±3.8	178.4±3.2
<i>Nauclea orientalis</i>	Rubiaceae	bark	5.7±0.1	467.3±9.7	78.3±3.3	112.4±2.4
<i>Withania somnifera</i>	Solanaceae	root	1.8±0.1	142.7±2.3	59.1±4.2	312.45±1.9
<i>Coriandrum sativum</i>	Umbelliferae	seeds	3.3±0.2	242.3±4.8	67.2±1.1	153.5±2.2
Ascorbic acid			N/A	N/A	98.2±3.9	43.34±0.2

All values are the mean of three measurements and expressed as mean ± SD.

**Table 2:** Phytochemical constituents of the plant extracts

Plant name	Saponins	Cardenolide glycosides	Polyphenol compounds	Alkaloids	Flavonoids	Tannins	Reducing substances	Proteins
<i>Terminalia arjuna</i>	+	-	+	+	+	+	-	-
<i>Benincasa hispida</i>	+	-	+	+	+	+	-	+
<i>Vetiveria zizanioides</i>	+	-	+	-	+	+	-	-
<i>Osbeckia aspera</i>	+	-	+	+	+	+	+	+
<i>Azadirachta indica</i>	+	-	+	-	+	+	-	+
<i>Coscinum fenestratum</i>	+	+	+	+	+	+	+	-
<i>Syzygium cumini</i>	+	-	+	+	+	+	-	-
<i>Nauclea orientalis</i>	+	-	+	+	+	+	-	+
<i>Withania somnifera</i>	+	+	+	-	+	-	+	-
<i>Coriandrum sativum</i>	-	+	+	+	+	+	+	-

Cyanogenic glycosides were not detected. - sign indicates the absence, + sign indicates the presence

## Conclusions

The study demonstrates that the selected medicinal plant extracts possess antioxidant activities with relatively high content of flavonoids. This could be due to the difference in stoichiometry of reactions between the antioxidant compounds

in the crude extracts and various radicals or oxidized molecules in different assays. The extracts of *Terminalia arjuna*, *Coscinum fenestratum* and *Nauclea orientalis* are with excellent antioxidative potentials. The relatively high total flavonoid content might be responsible for the antioxidant

activities of the plant extracts. The scientific data obtained from the investigation scrutinize the use of them in the management of oxidative stress related diseases.

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