Comparison of antioxidant activity, Phenolic and Flavonoid contents of selected medicinal plants in Sri Lanka

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Abstract: Oxidative stress related diseases are as a result of accumulation of free radicals in the cellular organs. Plant based antioxidants play a defensive role by preventing the generation of free radicals and therefore the main focus of this study was to screen twenty five Sri Lankan medicinal plants for comparison of antioxidant capacity. Aqueous extracts of twenty five plants were screened for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Six plants that gave high antioxidant activity Terminalia chebula Retz., Punica granatum L., Flueggea leucopyrus Willd., Cassia fistula L., Piper betle L. and Phyllanthus amarus were selected for further analysis. Methanol extracts of the selected plants were subjected to IC50 by DPPH assay. Total phenolic and flavonoid contents were analyzed by Folin Ceocalteu method and Aluminium Colorimetric method respectively. Statistical analysis was done by Minitab 17 package using regression analysis techniques to identify the effect of IC50 by phenolics and flavonoids. Seeds of T. chebula Retz. showed high antioxidant activity with 102 mg/ml of IC50 value. Antioxidant activity of rest of the plants in the ascending order was F. leucopyrus Willd. (twigs) > C. fistula L. (bark) > P. granatum L. (leaves) > P. betle L. (leaves) > P. amarus (twigs). The relationship between IC50 by phenolic and flavonoid content was statistically significant. Further IC50 has a strong negative relationship between phenolics and flavonoids. Data from present study revealed that Terminalia chebula Retz., Punica granatum L., Flueggea leucopyrus Willd., Cassia fistula L., Piper betle L. and Phyllanthus amarus possess high antioxidant capacity compared to other medicinal plants.

Keywords : Terminalia chebula, Flueggea leucopyrus, Cassia fistula, DPPH, Phenolics, Flavonoids.

INTRODUCTION

Antioxidants are compounds that prevent oxidation through one or more mechanisms. They scavenge free radicals that can harm the human body. There are many reviews on the relationships between oxidative damages and various diseases including cancer, liver disease, aging, arthritis, diabetes, atherosclerosis, acquired immune deficiency syndrome (AIDS) etc. (Ullah et al., 2016; Tribble et al., 1994; Wilcox et al., 2004). As a result many diseases have been treated with antioxidants to prevent oxidative damage.

The natural antioxidants, vitamin E, vitamin C, and polyphenols have been investigated for their possible use in preventing above diseases. Phenolic compounds present in plants have been reported as the main contributors to the antioxidant activity of fruits, vegetables, and medicinal plants (Stankovic, 2011). It was reported in France that drinking red wine has been linked to the low incidence of coronary heart disease (CHD), and later it was confirmed that polyphenols, flavonoids and anthocyanins in red wine play an important role in the prevention of CHD (Kanner et al., 1994).

Sri Lanka has different varieties of medicinal plants whose effectiveness has been proven across many generations as herbal treatments for control of diseases. Some of the diseases with complicated etiologies such as cancer, diabetes, arthritis have been recognized to be controlled or cured using these herbal medicines. Antioxidants present in plants may play a major role in controlling these diseases (Ayurvedic Medicinal plants of Sri Lanka, 2017). There were many reports on antioxidant activity of medicinal plants but antioxidant activities have not been compared among each other. Therefore aim of this study was to compare antioxidant capacity of medicinal plants that are extensively used in traditional medicines. In the present investigation, twenty five medicinal plants were screened by 2,2-diphenyl-1-picrylhydrazyl(DPPH) assay. Plants that showed high antioxidant activity Terminalia chebula Retz., Punica granatum L., Flueggea leucopyrus Willd., Cassia fistula L., Piper betle L. and Phyllanthus amarus were selected to determine IC50 by DPPH assay, total polyphenol content and flavonoid content.

MATERIALS AND METHODS

Chemicals

Aluminum Chloride, Sodium Nitrite, Sodium Carbonate were purchased from Dea Jung South Korea. DPPH and Folin Ciocalteu were obtained from Sigma Chemicals. Gallic acid and Quercetin were purchased from Sisco Research Laboratories. All other solvents and chemicals were of analytical grade.

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Selection and collection of the plant materials

Twenty five medicinal plants were selected by following discussions with several local Ayurveda practitioners and based on literature survey. The scientific names, vernacular (Sinhala) names and the plant parts selected are detailed in Table 1. The plant part used are the same as those generally used in medicinal preparations in the traditional Sri Lankan medicine such as leaves, stems/ bark, fruits, seeds, rhizomes or whole plant. For the initial screening of medicinal plant extracts for antioxidant capacity, all the plant materials were bought from reputed indigenous medicinal shops in Kandy and were verified by a local physician. Plants that showed high antioxidant activity by DPPH assay were collected freshly with the permission of garden owners from different localities of Sri Lanka. Bark of C. fistula and the seeds of T. chebula were collected from Moneragala (Uva Province). Twigs of F. leucopyrus were collected from Rantambe (Central Province). P. amarus was collected from Hemmathagama (Sabaragamuwa Province) and leaves of P. granatum and P. betle were collected from Udaperadeniya (Central Province). Plants were authenticated by comparison with the herbarium specimens at the National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka. A voucher specimen of each plant is deposited at the National Herbarium (Deposition Number: 6/01/H/03).

Preparation of water extract of plants for antioxidant activity

Plant material was washed with distilled water to clean off dirt and dust. They were cut into small pieces and dried in an oven at 30°C until constant weight was attained. Dried samples were pulverized using a domestic blender. Each powder sample was transferred separately into an air-tight bag and was stored in a refrigerator (-4°C) until extraction. Dried powdered plant material (0.5g) was transferred into a screw-capped test tube, together with 10.0 ml of distilled water and incubated at 95°C in a water bath for 1 hour. The resulting water extract was centrifuged at 3900 rpm for 5 minutes and the supernatant was stored in a freezer at-20°C for further analysis.

Screening of water extracts for free radical scavenging activity by DPPH assay

This assay is based on the determination of the concentration of DPPH solution, after adding the antioxidants. An aliquot (40 µl) of plant extract was added to 3 ml of 8 x10^-5 M DPPH and samples were incubated at room temperature for 20 minutes in the dark. Absorbance was measured at 517 nm.

Table 1: Scientific name, vernacular names and plant parts.

| Scientific Name          | Vernacular names | Plant part |
|--------------------------|------------------|------------|
| Asparagus racemosus Willd. | Hathawariya     | leaves     |
| Azadirachta indica A. Juss. | Kohomba         | Bark       |
| Boerhavia diffusa L.     | Saarana          | roots      |
| Cardiospermum halicacabum L. | WelPenela      | whole plant|
| Cassia fistula L.         | Ehela            | Bark       |
| Coriandrum sativum        | Kohthamalli      | Seeds      |
| Coscinium fenestratum     | Weniwelgeta      | Stem       |
| Cyperus rotundus          | Kalanduru        | rhizomes   |
| Fluggea leucopyrus Willd. | Katupila         | twigs      |
| Kalanchoe pinnata         | Akkapan          | leaves     |
| Mollugo cervina           | Pathpadagam      | whole plant|
| Munronia pinnata L.       | BimKohomba       | whole plant|
| Pavetta indica L.         | Pavatta          | Stem       |
| Phyllanthus amarus        | Pitawakka        | whole plant|
| Piper betle               | Bulath           | leaves     |
| Piper longum L.           | Thippili         | Fruit      |
| Pterocarpus santalinus L. | RathHandun       | Stem       |
| Punica granatum           | Delum            | leaves     |
| Solanum trilobatum L.     | WelThibbotu      | whole plant|
| Solanum virginianum L.    | KatuwelBatu      | whole plant|
| Sphaeranthus indicus L.   | Mudumahana       | whole plant|
| Terminalia chebula Retz.  | Aralu            | seeds      |
| Tinospora cardifolia      | Rasakinda        | stem       |
| Vetiveria zizanioides     | Savandara        | roots      |
| Zingiber officinale Roscoe.| Inguru          | rhizomes   |

*Commonly used Sri Lankan (Sinhala) names
Determination of flavonoid content by Aluminium Chloride Colorimetric assay

Total flavonoid content was determined by Aluminium colorimetric method (Nunes et al., 2012; Thangaraj et al., 2016). Quercetin (QE) calibration standards (500µl) were pipetted into 5ml volumetric flasks with 2ml of distilled water and 0.15ml of 5% NaNO₂, vortex thoroughly, and incubated at room temperature for 5 minutes. After incubation, 0.15ml of 10% AlCl₃ was added to each mixture, except for the blank to which same volume of distilled water was added, and incubated again at room temperature for 6 minutes. 1ml of 4% NaOH was added to the mixture, vortexed and made the volume up to 5ml with distilled water. Absorbance was measured at 430 nm after 15 minutes. All dilutions were analyzed in triplicate and average absorbance was used to plot the calibration graph. The standard graph was drawn with absorbance against the concentration of QE dilutions. Data are expressed as mean±SD of three replicates. Same procedure was repeated with the plant extract and the content of the flavonoids was expressed in terms of QE equivalents per gram of plant extract.

Statistical analysis

All graphs were drawn using Microsoft Excel. Data were expressed as means, and standard deviation. Statistical analysis was done by Minitab 17 statistical package using Regression analysis techniques to identify the effect of IC₅₀ by phenolics and flavonoids.

RESULTS AND DISCUSSION

Antioxidant activity of plant extracts by DPPH assay

There are various methods to evaluate antioxidant capacity. DPPH analysis is one of the accurate and frequently used methods for evaluating antioxidant capacity (Pérez-Jiménez et al., 2008). DPPH is a stable free radical due to delocalization of the extra electron on the whole molecule and therefore DPPH does not dimerize as it happens with other free radicals. This property gives rise to a deep violet colour for DPPH that can absorb UV light at about 517 nm (Sharma and Bhat, 2009). Antioxidants react with DPPH radical, forming reduced form of DPPH that the intensity of the resulting colour is proportional to the remaining concentration of DPPH after reaction with the antioxidant (Molyneux, 2004).

The results of antioxidant activity of water extracts by DPPH assay are given in Figure 1. Antioxidant activity was expressed as ascorbic acid equivalents per gram of plant material on a dry basis (Wong et al., 2006). Out of twenty five samples analyzed, six had high antioxidant capacity (> 0.5 mmol/g dry weight of sample). T. chebula, Retz. gave the highest antioxidant activity of 5.41 mmol/g of dry weight. The order of radical scavenging capacity as T. chebula > P. granatum > F. leucopyrus Willd. > C. fistula > P. betle > P. amarus. The results of antioxidant activity of methanol extracts at different concentrations, estimated by DPPH assay are given in Figure 2. The concentration of
sample required to give 50% scavenging activity of DPPH free radical is considered as the total antioxidant activity. IC\textsubscript{50} value was determined from the plotted graphs and is shown in the Table 2. According to the results, methanol extract of T.chebula Retz. showed high total antioxidant activity with IC\textsubscript{50} value of 102 mg/l (r\textsuperscript{2} = 0.98) compared to the other plants. The IC\textsubscript{50} of F. leucopyrus Willd. (r\textsuperscript{2} = 0.97), C. fistula L. (r\textsuperscript{2} = 0.98), P. granatum (r\textsuperscript{2} = 0.99), P. betle (r\textsuperscript{2} = 0.98) and P. amarus (r\textsuperscript{2} = 0.99) were 353 mg/l, 1,489 mg/l, 508 mg/l, 623 mg/l and 1037 mg/l respectively. Ascorbic acid was used as the reference standard and IC\textsubscript{50} value was 3.18 mg/L (r\textsuperscript{2} = 0.98).

![Figure 1](image1.png)

**Figure 1:** DPPH Radical scavenging activity of water extract of 25 medicinal plants (n=3 each, error bars represent standard deviation) expressed as ascorbic acid equivalents per gram of plant material on a dry basis.

![Figure 2](image2.png)

**Figure 2:** DPPH free radical scavenging activity at different concentrations of methanol extracts of Cassia fistula, Flueggea leucopyrus, Phyllanthus amarus, Piper betle, Punica granatum and Terminalia chebula.
Total phenol content by Folin- Ciocalteu reagent

Phenolic compounds are a group of antioxidants that act as free radical terminators. The amount of total phenol was determined using Folin Ciocalteu reagent (Brand-Williams et al., 1995). This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (Folin-Ciocalteu) and yielding a blue colour with a broad maximum absorption at 765 nm (Singleton et al., 1999; Agbor and Vinson, 2014). Gallic acid was used as a standard compound and total phenols were expressed as mg/g of gallic acid equivalents. Figure 3 shows the standard graph of Gallic acid. Total phenols were expressed as mg/g of gallic acid equivalents using the standard graph equation: \[ y = 0.8243x + 0.0135, \quad R^2 = 0.99, \]

where \( y \) is absorbance at 765 nm and \( x \) is total phenolic content. Table 3 shows the total phenol content that was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent. T. chebula showed high total phenol content of 121.72 mg/g. Total phenol content of plant extracts reduces in the order of T. chebula > C. fistula > F. leucopyrus > P. granatum > P. betle > P. amarus. The relationship between IC_{50} (mg/l) by phenolic contents was statistically significant (p < 0.05). Further, IC_{50} has a strong negative relationship between phenolics, the correlation value (r) of -0.82 with antioxidant activity (Figure 4).

Table 2: Radical scavenging capacity of crude methanolic extract of C. fistula, F. leucopyrus, P. amarus, P. betle, P. granatum and T. chebula.

| Name of the plant            | DPPH (IC_{50}±SD) mg/l |
|------------------------------|-------------------------|
| Cassia fistula Linn.         | 489 ± 10                |
| Flueggea leucopyrus Willd.   | 353 ± 15                |
| Phyllanthus amarus           | 1037 ± 11               |
| Piper betle                  | 623± 40                 |
| Punica granatum L.           | 508 ± 9                 |
| Terminalia chebula Retz      | 102 ± 4                 |

Each value in the table is represented as mean ± SD (n = 3).

Table 3: Total phenolic content and flavonoid content.

| Name of the plant            | Total phenolic content mg/GAE/g extract | Total flavonoid content mg/QE/g extract |
|------------------------------|----------------------------------------|----------------------------------------|
| Cassia fistula Linn.         | 43.57 ±1.16                            | 4.99 ± 0.10                            |
| Flueggea leucopyrus Willd.   | 39.17±0.74                             | 4.58 ± 0.09                            |
| Phyllanthus amarus           | 11.30± 0.38                            | 3.64 ± 0.08                            |
| Piper betle                  | 14.83±0.06                             | 4.43 ± 0.03                            |
| Punica granatum L.           | 29.97±0.57                             | 5.48 ± 0.04                            |
| Terminalia chebula Retz      | 121.72±6.04                            | 8.31 ±0.43                             |

Each value in the table is represented as mean ± SD (n = 3).

Figure 3: Standard calibration graph of gallic acid.
Total flavonoid content by Aluminium chloride colorimetric method

Total flavonoid content was estimated by Aluminium chloride (AlCl₃) colorimetric method. AlCl₃ forms acid stable complexes with C-4 keto groups and either C-3 or C-5 hydroxyl group of flavones and flavonols (Thangaraj, 2016, Bhaigyabati et al., 2014). The content of flavonoids was expressed as mg/g of quercetin equivalents. Figure 5 shows the standard graph and the equation is, \( y = 1.112x - 0.0164, R^2 = 0.9977 \), where \( y \) is absorbance at 430 nm and \( x \) is total flavonoid content. Table 3 shows the mean ±SD of flavonoid content in terms of quercetin equivalent. The high content of flavonoids was observed in \( T. chebula \) as 8.31 ±0.43 mg/quercetin/g. The relationship between IC₅₀ (mg/L) by flavonoid contents was statistically significant (p < 0.05). IC₅₀ has a strong negative relationship with flavonoids, which gave a correlation value(r) of -0.82 with antioxidant activity (Figure 6).

Antioxidant activity of fruits of \( T. chebula \) has been tested in many different assays. Harza et al., 2010 reported that IC₅₀ of seeds by DPPH as 1.73 μg/ml, comparatively higher antioxidant capacity than our results. High phenolic and flavonoid contents were observed as 127.6 mg Gallic acid equivalent per 100 mg plant extract and 219.30 mg quercetin equivalent per 100 mg plant extract respectively. In \( F. leucopyrus \), Soysa et al 2014 reported that the phenolic content was 22.15 μg/g of Gallic acid equivalents and DPPH radicals by 50% was 11.16 ± 0.45 μg/mL. There were many reports on the antioxidant activity of different parts of \( C. fistula \). According to Ilavarasan et al., 2005, IC₅₀ of the methanol extract of bark of \( C. fistula \) was 203 μg/mL by DPPH assay. Though there were many reports on antioxidant activity by different assays, a remarkable differences in the values and cannot be compared each other.

Medicinal value of plants

\( T. chebula \) is a perennial medicinal plant used widely in indigenous and ayurvedic medicinal systems (Ayurveda Department, 2002), mainly for the treatment of asthma, sore throat, vomiting, hiccup, diarrhea, bleeding piles, gout, heart and bladder diseases (Cheng et al., 2003) and cancer (Saleem et al., 2002). Juice of \( P. granatum \) has been used to treat sore throats, cough, urinary infections, skin disorders and arthritis (Bhowmik et al., 2013). However modern research suggests that pomegranates might be useful in treating cancer, osteoarthritis and diabetes (Ephraim and Robert, 2007). Limited work has been reported from the leaves of pomegranate, but leaves use in eye infections, dysentery, worm diseases, asthma in ayurvedic medicine in SriLanka (Ayurvedic Medicinal plants of Sri Lanka, 2017) \( F. leucopyrus \) is extensively used in the treatment of cancer and tumors in traditional Ayurvedic medicinal practice in Sri Lanka (Al-Sabagh et al., 2014) \( C. fistula \) is an important medicinal plant belonging to family Caesalpiniaceae. Stem-bark is useful in the treatment of many diseases such as skin diseases, inflammatory diseases, rheumatism, and jaundice (Saleem et al., 2002). \( P. amarus \) has been widely used in the treatment of jaundice, constipation, diarrhea, kidney ailments, ringworm, ulcers, malaria etc. (Lim and Murtijaya, 2007).
CONCLUSIONS
Natural products have been proven relatively safe for human consumption and the plants have been used for thousands of years in the treatment of different diseases. The wide use of these plants in the Sri Lankan indigenous systems may be in part due to their antioxidant potency. As shown in terms of other antioxidant assays, plants used in this medicinal system convey a very high antioxidant potential. The mechanism of action of these plants require further study and isolation of the secondary metabolites responsible for the antioxidant activity has to be taken up which may result in the discovery of modern drugs from these plants.

LIST OF ABBREVIATIONS
DPPH- 2,2-diphenyl-1-picrylhydrazyl
AIDS - acquired immune deficiency syndrome
CHD- coronary heart disease

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