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IN VITRO ANTIOXIDANT ACTIVITY OF LEAF EXTRACTS IN THREE MEDICINAL PLANTS; *Costus specious* (Koen.) Smith, *Coccinia grandis* (L.) J. Voigt AND *Wattakaka Volubilis*

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Abstract: The present study was aimed at investigating the antioxidant activity of leaf extracts of three important medicinal plants; *Costus specious* (Thebu), *Coccinia grandis* (Kowakka) and *Wattakaka volubilis* (Kiri Anguna). The antioxidant activity of methanolic extracts of leaf samples were evaluated by using *in vitro* assays; DPPH assay (IC_{50} value) and Folin-Ciacalteu method calculated as Gallic acid equivalents (GAE). Values of DPPH assay were compared to the standard antioxidant butylated hydroxyltoluene (BHT). All samples showed effective scavenging activity and none of the samples exerted an obvious pro-oxidant activity. Highest antioxidant activity was shown in *Coccinia grandis*; 0.501 ± 0.003 mg mL^{-1} and highest total phenolic content was shown in *Wattakaka volubilis*; 975.82 ± 11.28 (mg/GAE/ 100 g) although there is no significant difference between *Wattakaka volubilis* and *Coccinia grandis.* The antioxidant activity was increased with the increasing amount of doses. The free radical scavenging activity may be attributed to the presence of phenolic compounds. Present study reported a positive relationship (r = 0.647) between phenolic content and the antioxidant activity. Results obtained from the present study suggested that the leaves of *Costus specious*, *Coccinia grandis* and *Wattakaka volubilis* as potential sources of natural antioxidants

Keywords: Anti-oxidant activity, Medicinal plants, BHT (Butylated hydroxytoluene)

1. Introduction

Free radicals which can be identified as species having very short half-life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids have been postulated as two kind of; namely Reactive Oxygen Species (ROS) which are oxygen derived free radicals and Reactive Nitrogen Species (RNS) which are nitrogen based free radicals. Oxygen based free radicals (ROS) are O_2^- (superoxide), $HO^\cdot$ (hydroxyl), HO_2 (hydroperoxyl), $ROO^\cdot$ (peroxyl), $RO^\cdot$ (alkoxyl) as free radicals and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly $NO^{\cdot -}$ (nitric oxide), $ONOO^{\cdot -}$ (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide).Three sources of ROS generation are depicted; namely (1) Endogenous sources - detoxification reaction, electron transport chain (2) Exogenous sources - cigarette smoking, industrial waste products, ozone, asbestoses fibers, viral and bacterial infection [1] & (3) Pathological sources - infections cancer, immune cell activation, metabolism of environmental pollutions & certain drugs.

Wide variety of toxic oxidative reactions as peroxidation of membrane lipids, direct inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross

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linking of molecules like DNA, enzymes and proteins are some of the adverse effects of free radicals which ultimately cause to cell death [2].

However with in the human body, a balance is maintaining between pro-oxidants (free radicals) and antioxidants and when in absence or shifting towards pro-oxidants of this so called balance will create the condition called as "oxidative stress" [3].

Antioxidants may be defined as any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate [4] Anti-oxidants can be divided into two major classes as enzymatic and non-enzymatic. Superoxide dismutase, catalase, and glutathione peroxidase which are produced endogenously are enzymatic kind of antioxidants. The class non-enzymatic antioxidants involve tocopherols, carotenoids, ascorbic acid,

flavonoids and tannins which are obtained from natural plant sources [5].

Although there are many synthetic antioxidants used in processed foods such as BHT (butylated hydroxytoluene), (BHA) butylated hydroxyanisole and tertiary butyl hydroquinone, it has been suggested those affect liver damage and mutagenesis [6]. Therefore there is a great tendency towards natural antioxidant sources such as plant derivatives.

Flavonoids and other phenolic compounds of plant origin or in natural sources which are aromatic hydroxylated compounds have been testified as inhibitors of free radicals [7].

Costus specious (Thebu) which is also known as crepe ginger or spiral flag in English is a perennial rhizomatous herb with upright or spreading stem with important medicinal value [8]. This plant being in order Ziniberales and family Zingiberaceae is native to Malay peninsula of the south-east Asia. Many Asian counties including Sri Lanka have broadly discussed its medicinal value. A recent study has shown that methanol and water extracts of leaves have effectively diminished the insulin resistance in vivo [9].

Coccinia grandis (Kowakka) belonging to the family of Cucurbitaceae has been reported as an anti-diabetic plant in Sri Lanka [10]. In Indian folk medicine the leaves of this species are widely used for remedy for diabetes mellitus [11]. Hypouricaemic activities and xanthine oxidase inhibitory activities have been shown in studies conducted on crude hydromethanolic extract of the leaves [12].

Wattakaka volubilis (Anguna) belonging to family Asclapadesiae has shown to have constituents as flavonoids, sterols/terpenoids, phenolic acids [13]

The objective of this study is to analyze the *in vitro* antioxidant potential of ; *Costus specious*, *Coccinia grandis* and *Wattakaka volubilis*. Total phenolic contents were also determined in order to evaluate a relationship between the antioxidant activity and the phytochemical constituents.

2. Material and methods

2.1 Sample Collection and Preparation

The leaf samples were collected from Kandy in Central province, Sri Lanka and specimens were kept in national herbarium, for identification. The samples were washed well and dried at room temperature for 24 h. The samples were ground in to a fine powder in a laboratory mechanical blender. The homogenized leaf samples in 80% methanol were kept in a mechanical shaker (250 rpm) for 4 h in room temperature. The extracts were filtered using Whatman no.1 filter paper. Filtrates were taken to assess the total phenolic content and antioxidant potential.

2.2 DPPH radical scavenging assay

2,2-diphenil-1-picrylhydrazil (DPPH)) scavenging activity was evaluated using a spectrophotometric method described by Brand / William *et al.*,(1995) with some modifications. Freshly prepared DPPH solution (100µM in absolute Methanol) was used for each experiment. For the reaction mixture 2.5 ml of 100 µM DPPH solution and 0.5 ml of sample dissolved in methanol were used and for control sample 2.5 ml of DPPH solution and 0.5 ml of methanol were used instead. Five concentrations from each sample were prepared and each sample was analyzed in triplicates. All samples were incubated at room temperature for 30 minutes in dark. Absorbance was measured at 517 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). Using the equation mentioned below, the percentage of DPPH radical scavenging activity was determined in all five concentrations. BHT (Butylated hydroxytoluene) was used as the reference standard.

% scavenging activity = $((A_0 - A_s)/A_0) \times 100$

Where,

A_0 – Absorbance of the DPPH solution of the control sample

A_s- Absorbance of the DPPH solution in the presence of plant extract

The sample concentration which had given 50% scavenging activity according to the equation was estimated as IC_{50} value from regression analysis using Minitab 16.

2.3 Determination of total phenolic content

For evaluation of phenolic content the method described by Slinkard and Singleton (1977) was used. Powdered sample of each plant material was used and extracted using 80% methanol. The reaction mixture was prepared using 0.5 ml of extracted sample with 2.5 ml of Folin-Ciocalteu reagent which was diluted 10 times. After 3 minutes 5.0 ml of 7.5% sodium carbonate (w/v) solution was added to the above mixture. It was kept at 45 ºC for 10 minutes in a water bath. Then absorbance of each plant extract was measured at 765 nm using the UV-Vis spectrophotometer (SHIMADZU UV mini 1240). Methanol (80%) was used as the blank sample.

Total phenolic content was expressed as mg Gallic acid equivalent/100g using the equation obtained from the calibration curve for Gallic acid (R^2 = 0.9999). Data are expressed as mean±SD of four replicates.

2.4 Statistical Analysis

All experiments were performed in triplicate (n=3) and results were expressed as mean ± SD. Statistical analysis

was carried out with (Minitab version 16) using ANOVA followed by Turkey's test (P<0.05).

3. Results and Discussion

DPPH assay

DPPH is a stable free radical in which nitrogen centered is widely used to test the antioxidant activity of compounds or extracts in plant materials as hydrogen donor capacity [14]. Decrease in absorbance in the above mentioned range represents the ability of natural antioxidants in plant material to reduce DPPH free radical. As the positive control butylated hydroxyltoluene was used.

Sample concentration needed to scavenge 50% percent of free radical (IC_{50} value) which is inversely proportional to antioxidant activity was compared in this study.

Table 1: Radical Scavenging Activities of plant leaf extracts[a]

Sample	*Coccinia grandis*	*Wattakaka volubilis*	*Costus specious*	BHT
IC_{50} value (mg mL^{-1})[b] DPPH	0.501±0.003[c]	0.88900± 0.002[b]	1.143±0.002[a]	0.0115±0.000

[a]The value of IC_{50} were determined by triplicates in individual experiments. Values are mean± SD of three determinations

[b]The concentration of sample required to scavenge 50% of the radical scavenging activity.

Values are expressed as mean± SEM of three parallel measurements. Values within a column followed by different letters are significantly different (P<0.05).

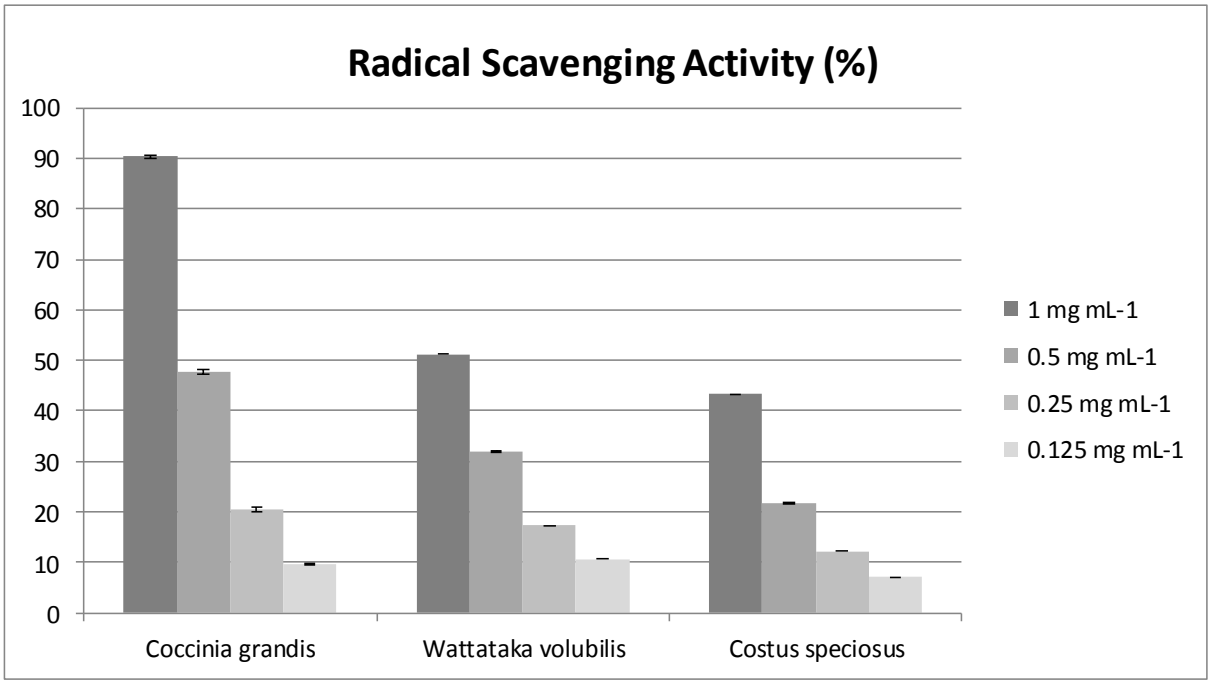

Figure 1: Radical Scavenging Activity (%)

A previous study done on *Coccinia gardins* leaf extracts by Tamilselvan *et al*.,2011 have reported that it contains phytochemicals as Alkaloids, Steroids, Tannins, Saponins ,Ellagic acid, Phenols , Glycosides , Lignans and Triterpenoids, which is an evidence to its profound antioxidant activity. (Table 1)

The results of the previous study of Arumugasamy *et al*.,2012 [15] showed the presence of phytochemical constituents such as alkaloids, glycosides, carbohydrates, cardiac glycosides, phenolic compounds, flavonoids,

steroids, tannins, phlobatannins, anthraquinones, phenols, sugars, saponins, and resins in *W. volubilis* and the result of extractive value has shown higher percentage in the methanol followed by chloroform, petroleum ether, and aqueous solvent. Sujatha (2012) [16] has reported that *Wattakaka volubilis* can be used as a substituted for "Jeewanthi" in Indian Ayurveda medicine.

However for *Costuss peciosus,* Kalpa *et al*.,2014 [17] reported much higher DPPH radical activity value than the present study (0.320 ± 0.01 mg/mL) in ESR spectrophotometer using freeze dried leaves although sun dried leaves have been used in the present study.

However BHT which was used as the commercial antioxidant in this study reported an IC_{50} value of 0.0115 mg mL^{-1}. Synthetic antioxidants have shown to possess higher performance levels than the natural ones, since the natural antioxidants show a greater hesitancy when donating hydrogen atoms in preventing oxidation. [18]

When considering about this assay, DPPH is a stable nitrogen radical and therefore it doesn't contain any similar groups or similarity to highly reactive and short-lived peroxyl radicals. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility and it has been reported the preference is given to small compounds [19]. Interpretation becomes more complicated when the test compounds have spectra that are

likely to overlap DPPH at 515 nm. Compounds as carotenoids may interfere [20]

Some authors have reported that the initial fast reactions may be ignored by long time incubations which may also give undue weight to slow reactions, so should be abandoned .On the other hand, multiple solvents should be utilized for DPPH assay, at a minimum MeOH (EtOH is not preferred because of generating reactive radicals which may obstruct with the assay) [19]

Glutathione, aromatic amines (such as *p*-phenylenediamine and *p*-aminophenol), and α-tocopherol (Vitamin E - 2:1 stoichiometry) and polyhydroxy aromatic compounds (such as hydroquinone and pyrogallol) have been reported as compounds active in this DPPH reaction. On the other hand, compounds such as monohydric phenols (such as tyrosine), simple sugars (such as glucose), purines and pyrimidines, are not responsive, while proteins tent to be precipitated [21]

Total Phenolic Content

Wattakaka volubills and *Coccinia gardins* have shown similar phenolic content and values are not significantly different from each other

Several studies have reported on the relationships between phenolic content and antioxidant activity. Some authors found a correlation between the phenolic content and the antioxidant activity, while others reported no such relationship. A study conducted in selected fruits, vegetables and grain products by Velioglu *et al*., (1998) [22] reported a strong relationship between total phenolic content and antioxidant activity. No correlation between antioxidant activity and phenolic content was found in the study on some plant extracts containing phenolic compounds done by Kähkönen *et al*.,1999 [23]

Table 2 : Total Phenolic Content

Sample	*Coccinia grandis*	*Wattakaka volubilis*	*Costus specious*
Total phenolic content (mg/ GAE /100 g)	966.80 ± 12.11^{a}	975.82 ± 11.28^{a}	476.78 ± 11.23^{b}

Values are expressed as mean± SEM of four parallel measurements. Values within a row followed by different letters are significantly different ($P<0.05$)

Present study also reported a positive relationship (r = 0.6476) (Figure 2) between phenolic content and the antioxidant activity. Although a significant difference was not apparent in the phenolic content between *Wattakaka volubilis* and *Coccinia gardins*, significantly higher pyrocatechol equivalent/mg) in chloroform extract of leaves. Methanolic extract of *C. gardins* have been reported to contain phenols and flavonoids [11].

*Wattakaka volubilis*also have been reported to contain phenols and flavonoids [15].

Additionally, Seabra *et al*.,2006 [24] reported the synergistic effects of phenolics with other antioxidants, namely ascorbic acid, β carotene and α tocopherol, and regulation of antioxidant activity was reported in *Coccinia gardins.* This may have been due to the presence of higher Secondary metabolites other than phenolics and flavonoids in *Coccinia gardins.* Umamaheswari and Chatterjee have reported the phenolic content of *Coccinia gardins*as 47.66±0.88 µg intracellular glutathione levels. This also should be taken into consideration.

However this study implies the need of further studies on antioxidant capacity by different anti-oxidant analyzing methods. Proceeding further experiments using higher number of samples from different areas may increase the accuracy of results. Further studies to evaluate the *in vivo* potential of these plants in various animal models should be carried out.

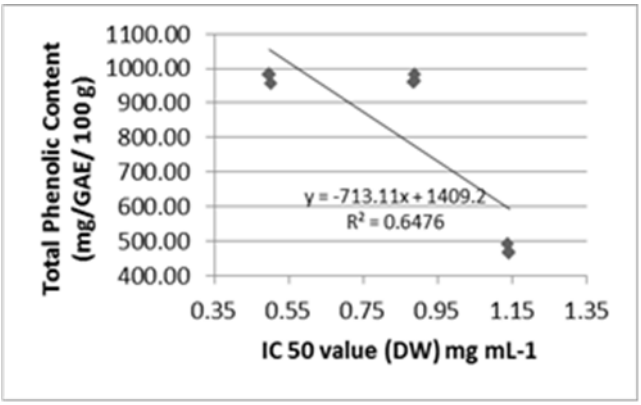

Figure 2: Correlation between total phenolic content and antioxidant activity

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