

Free radical Scavenging Activity and Phenolic content of decoctions of some medicinal plants

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Received on : 03-04-2013

Accepted on: 08-08-2013

Abstract

Free radicals are normally generated in substantial amounts in the body; excessive amounts cause oxidative damage through chain reactions forming disorders like diabetes mellitus, cardiovascular and pulmonary diseases. In diabetic conditions, a non enzymatic reaction occurs with proteins and reducing sugars forming glycated protein named Amadori products. Further rearrangement, oxidation and reduction of Amadori products leads to form Advanced Glycated End products which causing spontaneous damage to proteins in physiological system leading various complications like Nephropathy, Neuropathy, Retinopathy and this process accompanying the formation of free radicals. In this process oxidation plays an important role to form Advanced Glycated End Products. Therefore antioxidants are highly important in prevention or slowing the glycation reaction. Humans have evolved a complex antioxidant system, but this may not be sufficient to maintain optimal cellular functions in diabetic conditions. Medicinal plants usually contain different phenolic compounds having antioxidant properties. Therefore, a study was carried out to examine the *in vitro* free radical scavenging activity and total phenolic content of the decoctions of plants, *Cassia auriculata* (Ranawara, flower) *Phyllanthus emblica*.(Nelli, fruit) and *Scoparia dulcis* (Walkottamalli, whole plant) which are used in the treatment for diabetics. Three fresh samples from each plant collected from different areas where they are grown and commercial dried sample from the traditional market was selected to prepare the decoctions and compared phenolic contents and antioxidant activity. The total phenolic content of each extract was determined using Folin-Ciocalteu reagent and evaluation of free radical scavenging activity was assessed using DPPH assay and ABTS assay. Decoctions of the commercial samples of *P.emblica* showed the highest total

phenolic content as 625 mgGAE/g and *C. auriculata* and *S. dulcis* showed 459 and 131 mgGAE/g respectively. Samples dried under laboratory conditions of *C. auriculata* had total phenolic content from 226 – 287 mgGAE/g, *P. emblica* from 479 -517 mgGAE/g and *S. dulcis* from 167 – 186 mgGAE/g. The highest DPPH antioxidant activity showed the commercial sample of *P. emblica* as 27 µg/ml and other samples dried under laboratory conditions were in the range of 41 – 49 µg/ml. No significant difference between the DPPH activity of *C. auriculata* commercial sample and other samples dried in the dehydrator and were in the range of 248 -309 µg/ml while *S. dulcis* all the samples showed DPPH antioxidant activity from 437 – 540 µg/ml. The reference standard ,Butylated Hydroxy Toluene showed 20 µg/ml. ABTS antioxidant activity was high in all commercial samples of *C. auriculata*, *P. emblica* and *S. dulcis* and were as 648,625 and 615 mmol/g while other samples dried using the dehydrator showed 313 – 536 mmol/g,479 -517 mmol/g and 549-550 mmol/g respectively.

Key words: Antioxidant properties, Glycation, Diabetes, DPPH assay, ABTS assay

Introduction

Free radicals are normally generated in substantial amounts as a byproduct of various internal metabolic processes in aerobic organisms such as phagocytosis, neutrophils defense, carboxylation and hydroxylation reactions (Ajaikumar *et al.*, 2005). In diabetic conditions, a non enzymatic reaction occurs with proteins and reducing sugars forming glycated protein named Amadori products. Further rearrangement, oxidation and reduction of Amadori products leads to form Advanced Glycated End products which causing spontaneous damage to proteins in physiological system leading various complications like Nephropathy, Neuropathy, Retinopathy and this process also accompanying the formation of free radicals. In this process oxidation plays an important role to form Advanced Glycated End Products. Therefore antioxidants are highly important in prevention or slowing the glycation reaction. Although the presence of free radicals to a certain extent is beneficial for the immune system, excessive amounts cause oxidative damage to lipid, protein and nucleic acids. Therefore, they have been implicated in the pathogenesis of many human sufferings like cardiovascular and pulmonary diseases, some types of cancers cataracts immune and autoimmune diseases and brain dysfunction like Parkinson's diseases.(Bayani *et al.*, 2009).In conditions of a diabetes mellitus the formation of free radicals enhances with the non enzymic reaction of reducing sugars with free amino groups of proteins, lipids and nucleic acid forming Advanced Glycated End Products (AGEPs).Humans have evolved highly complex antioxidant system, enzymic and nonenzymic which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. Endogenous antioxidants may not be required to maintain optimal cellular functions.(Khalid R.,2007). Medicinal plants usually contain different phenolic compounds having antioxidant properties. Therefore a study was carried out to examine the *in vitro* free radical scavenging activity and total phenolic content of plants, *Cassia auriculata*(Ranawara,flower) *Phyllanthus emblica* (Nelli, fruit) and *Scoparia dulcis* (Walkottamalli, whole plant) which are used in the treatment of diabetes mellitus.

Materials and Methods

Plant materials

Three plants were chosen which are being used for the treatment of diabetes mellitus using the information gathering by discussing with Ayurvedha practioners and referring the traditional medical books. From each plant, one commercial sample was collected from the traditional market at Gabos lane, Colombo and three other fresh samples from three different sites; areas where the species are grown.

Chemicals and Reagents

DPPH ((2,2 diphenyl-2-picryl hydrazyl hydrate), BHT (ButylatedHydroxyToluene), Trolox(6 – Hydroxy -2,5,7,8 – tetramethylchroman -2-carboxylic acid)ABTS (2,2' – azinobis (3 – ethyl benzothiazoline -6-sulphonic acid) diammonium salt (Sigma Chemicals Co. (USA), Gallic acid(3,4,5 – tri hydroxyl benzoic acid (FlukaChem ,Swetzerland),Potassium per sulphate(),Methanol (Sigma, Aldrich), Sodium carbonate (Sigma, Aldrich), Folin Ciocaltue phenol reagent(Fluka Chem.,Switzerland)

Extraction Procedure

Samples collected were cleaned washed and dried in a dehydrator at 55 °C for 24 hours, ground to obtain fine powder using a domestic grinder. Water extracts were prepared according to the traditional method used to prepare 'kwatha'in Ayurvedha medicine . As the sample was finely powdered one 60 g (12 kalan) was simmerly boiled with 960 ml of water (4 patha) to get a concentrate of 240 ml.(one patha)The water extract was filtered using a fine silk cloth and freeze dried to obtain a fine powder. Samples were kept at 4°C in a cold room in an air tight containers.

Evaluation of antioxidant activity

DPPH assay:

The experiment was carried out according to the method of (Brand-William *et al.*, 1995) with slight modifications. Radical scavenging activity of samples were carried out against stable DPPH (2,2 diphenyl-2-picryl hydrazyl hydrate (sigma) using a spectrophotometric method. Freshly prepared DPPH solution was used for each experiment. Reaction mixture was prepared using 2.5 ml of 6.5×10^{-5} M DPPH solution and 0.5 ml of sample dissolved in methanol and control sample with 2.5 ml of 6.5×10^{-5} M DPPH and 0.5 ml methanol. Samples were tested in five concentrations and each sample was done in triplicate .All samples were incubated at room temperature for 30 minutes in dark and then absorbance was measured at 540 nm using Uv-Vis spectrophotometer (SHIMADZU UV mini 1240). The percentage of DPPH radical scavenging activity was determined in five concentrations using the equation mentioned below.

$$\% \text{ scavenging activity} = \frac{A_b - A_s}{A_b} \times 100$$

A_b – 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 gives 50 % scavenging activity was estimated as IC_{50} value from regression analysis using Minitab 14.

ABTS assay:

2,2'-azinobis (3 - ethyl benzothiazoline -6-sulphonic acid) diammonium salt (sigma) radical cation decolorization assay was used to measure the antioxidant activity (Miller et al., 1996) with slight modifications. The stock solutions of 7mM ABTS solution and 2.4 mM Potassium per sulphate solution were prepared and the working solution was prepared mixing equal parts from each and allowing the mixture to stand in the dark at room temperature for 12 - 16 hrs before use. The solution was then diluted mixing 1.0 ml ABTS⁺ solution with 16.0 ml phosphate buffer to obtain initial absorbance as 0.700 ± 0.01 . ABTS⁺ solution was freshly prepared for each assay. Freeze dried water extract of each sample was prepared in 3 concentrations dissolving in phosphate buffer solution and the reaction mixture was prepared mixing 100 μ l of plant extract with 3.0 ml ABTS⁺ solution. ABTS⁺ radical scavenging activity was determined after measuring the fall of absorbance exactly after 15 min. Trolox (6 - Hydroxy -2,5,7,8 - tetramethylchroman - 2-carboxylic acid) was used as the standard and the blank sample was prepared adding 100 μ l phosphate buffer to 3.0 ml ABTS⁺ solution. Data are reported as mean \pm SD of the three replicates as Trolox equivalents using the equation obtained from the calibration curve of Trolox.

Determination of Total Phenolic content.

The total phenolic content of each extract was determined using Folin-Ciocaltue reagent (Kohkonen *et al.*, 1999) Freeze dried sample (0.01 g) from each plant material was used for the extraction and extracted using 70 % methanol. The reaction mixture was prepared using 0.5 ml of extracted sample with 2.5 ml of Folin-Ciocaltue reagent which diluted 10 times using Methanol. 2.0 ml of 7.5% Sodium carbonate (w/v) solution was added after 3 minutes to the above mixture and kept at 45 °C for 10 minutes in an incubator (Microsil). Then absorbance of each plant extract and prepared blank adding 0.5 ml of methanol instead of the plant extract was measured at 765nm using the UV-Vis spectrophotometer (SHIMADZU UV mini 1240). Total phenolic content was expressed as mg Gallic acid equivalent/g using the equation obtained from the calibration curve for Gallic acid. Data are expressed as mean \pm SD of three replicates.

Results

DPPH radical scavenging activity

The antioxidant potential is inversely proportional to IC_{50} values which were calculated using regression analysis of percentage of scavenging activity versus extract concentrations using Minitab 14. Among the three plants selected for the study *Phyllanthus emblica* commercial sample and other three samples collected from Bibile, Anuradhapura and Balangoda showed the highest antioxidant activity ranging from 27 - 46 μ g/ml in DPPH assay. Commercial sample of *Cassia auriculata* had the IC_{50} value 274 μ g/ml comparable to the other samples from Anuradhapura, Bibile and Balangoda which were dried under laboratory conditions (248 - 309 μ g/ml), but not showed a significant difference among them.. *Scoparia dulcis* showed the lowest antioxidant activity (437 - 540 μ g/ml) in DPPH assay in this study but no significant difference from the commercial sample and other samples. Butylated Hydroxy Toluene (BHT) used as the standard and it showed IC_{50} value as 20 μ g/ml.

Table I: Results of total phenolic content and free radical scavenging activity by DPPH assay and ABTS assay

Plant	Plant part	Location	Total phenolic content mg GAE /g	DPPH IC50 µg/ml	Trolox Equivalents mmol/g sample
<i>Cassia auriculata</i>	flower	Commercial sample	459 ±8.7	237±7.2	648±5.3
<i>Cassia auriculata</i>	flower	Anuradhapura	215 ±11.6	248±6.5	480±2.2
<i>Cassia auriculata</i>	flower	Katharagama	226 ±5.43	309±4.9	536±6.1
<i>Cassia auriculata</i>	flower	Mathugama	287 + 5.2	263±6.0	313±4.4
<i>Phyllanthus emblica</i>	fruit	Commercial sample	625±3.2	27.1±3.2	625±3.2
<i>Phyllanthus emblica</i>	fruit	Bibile	479±2.4	40.9±3.5	479±2.4
<i>Phyllanthus emblica</i>	fruit	Anuradhapura	517±3.0	45.4±5.2	517±3.0
<i>Phyllanthus emblica</i>	fruit	Balangoda	491±4.4	49.5±6.0	491±4.4
<i>Scoparia dulcis</i>	whole plant	Commercial sample	131±1.3	490±2.1	615±4.2
<i>Scoparia dulcis</i>	whole plant	Bandarawela	167 ±6.9	437±3.1	549±5.8
<i>Scoparia dulcis</i>	whole plant	Wijerama	186±3.4	540±4.2	553±4.9
<i>Scoparia dulcis</i>	whole plant	Rukmale	170±4.04	450±2.2	550±7.2
BHT				20.21±2.6	

ABTS radical Scavenging activity

The ABTS scavenging activity was given as Trolox equivalents with the water soluble α tocopherol analogue using the equation $y=0.2435x + 0.0591$ of the calibration curve of Trolox concentration $\mu\text{mol/L}$ versus fall of absorbance at 734 nm. All the 12 samples showed a high antioxidant activity in *invitro* ABTS assay. Decoction of *Cassia auriculata* flowers showed high antioxidant activity (648 Trolox Equivalents mm/g sample) comparing to the other three samples collected as fresh from Anuradhapura, Katharagama and Mathugama and dried under laboratory conditions.

(313 – 480 Trolox Equivalents mm/g sample) Commercial sample of *Phyllanthus emblica* decoction had high antioxidant activity in ABTS assay also , and was 625 Trolox Equivalents mmol/g sample, and other three samples Bibile, Anuradhapura and Balangoda showed 491 – 517 Trolox Equivalents mm/g sample. *Scoparia dulcis* commercial sample and other samples from Bandarawela, Wijerama and Rukmale also showed high free radical scavenging activity (615 – 549 Trolox Equivalents mmol/g sample) although they showed a low activity in DPPH assay.

Total phenolic content

Total phenolic content was calculated as Gallic acid equivalents using the equation $y=10.169x +0.0302$ obtained from the calibration curve of concentration of Gallic acid versus absorbance at 765 nm. Among the three plants the decoction of *Phyllanthus emblica* had the highest phenolic content ranging from 479 – 625 mgGAE/g. *C auriculata* samples dried under the laboratory conditions had 215 – 287 mg GAE/g while commercial sample contained 459 mg GAE/g. *S dulcis* Commercial sample and other samples freshly collected and dried under laboratory conditions had the lowest phenolic content ranging from 162 – 170 mg GAE/g.

Discussion

In this study three different plant materials which are being used for the treatment of diabetes mellitus were investigated for their free radical scavenging activities using *in vitro* DPPH assay and ABTS assay and the total phenolic content. Antioxidants in plants may be water soluble, fat soluble, insoluble or bound to cell walls. As the water extracts which are being mostly used in Ayurvedha medicine were used in this experiment, the water soluble antioxidants that is available in decoctions of the above plants taken into account for the antioxidant activity. As the decoctions were freeze dried the organoleptic properties of them subjected to minimal changes. In general, water soluble antioxidants react with oxidants in the cell cytosol and the blood plasma while lipid soluble antioxidants protect cell membrane from lipid peroxidation (Hass *et al.*, 2006)

According to the results of both assays there is a high antioxidant activity in all *Phyllanthus emblica* samples which contained significantly high phenolic content. Among the three samples the commercial samples showed high ABTS antioxidant activity than the samples which collected as fresh and dried under laboratory conditions. More than 4000 phenolic compounds (flavonoids, monophenols and polyphenols) are found in vascular plants. Phenolic compounds such as quercetin, rutin, catechine. Caffeic acid and Gallic acid are very important plant constituents. (Safaa *et al.*, 2010) The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers.(Miller *et al.*, 2000) The molecular antioxidant response of phenolic compounds depends on their chemical structure.

Conclusion

Decoctions prepared using 60 g of powdered samples (12' kalan') in 960 ml of water (4 'patha') simmerly boiling to get the concentrate of 240 ml (one patha,) of all the selected plants, *Cassia auriculata* flowers (Ranawara), *Phyllanthus emblica* (fruit) (Nelli) and *Scoparia dulcis* (whole plant) (Walkottamalli) showed positive antioxidant activity in both *invitro* assays. Commercial samples of *Cassia auriculata* flowers and *Phyllanthus emblica* (fruit) showed high antioxidant activity in both assays and the high phenolic content comparing to the other samples collected as fresh samples and dried under laboratory conditions. Commercial sample of *Scoparia dulcis* (whole plant) showed high activity in ABTS assay but other samples dried in laboratory also showed high ABTS activity than DPPH assay. Among the three plant species *Phyllanthus emblica* (fruit) (Nelli) contained high phenolic content than other two plants. The decoction of *Phyllanthus emblica* (fruit) (Nelli) is good therapeutic potential for oxidative stress develop with the diabetic condition.

Acknowledgements

Financial support from the University grant(ASP/08/RE/2008/09) of University of Sri Jayewardenepura, Gangodawila, Nugegoda is gratefully acknowledged.

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