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Full Length Research Paper

Colebrookia oppositifolia: A valuable source for natural antioxidants

Tauheeda Riaz[1], Muhammad Athar Abbasi[1]*, Tayyaba Shahzadi[1], Aziz-ur-Rehman[1], Sabahat Zahra Siddiqui[1] and Muhammad Ajaib[2]

[1]Department of Chemistry, Government College University, Lahore-54000, Pakistan.
[2]Department of Botany, Government College University, Lahore-54000, Pakistan.

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Methanolic extract of Colebrookia oppositifolia Smith. was dissolved in distilled water and partitioned with n-hexane, chloroform, ethyl acetate and n-butanol sequentially. Phytochemical screening showed the presence of phenolics, flavonoides and cardiac glycosides in chloroform, ethyl acetate and n-butanol fraction. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated by four methods: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, total antioxidant activity, Ferric Reducing Antioxidant Power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolics. The polar fractions showed noteworthy antioxidant potential. The results revealed that ethyl acetate soluble fraction showed the highest value of percentage inhibition of DPPH (83.43 ± 1.27) at concentration of 60 µg/ml. The IC_{50} of this fraction was 27.2 ± 0.23 µg/ml, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/mL. It also showed highest FRAP value (172.23 ± 1.66 µg of trolox equivalents), as well as highest value of inhibition of lipid peroxidation (57.61 ± 1.1%) as compared to the studied fractions. The chloroform fraction showed highest total antioxidant activity (0.711 ± 0.031), as well as highest total phenolic contents (72.5 ± 0.34).

Key words: Colebrookia oppositifolia Smith, DPPH assay, total antioxidant activity, FRAP value, total phenolics, Inhibition of lipid peroxidation (%).

INTRODUCTION

The consumption of fruits and vegetables is strongly and inversely associated with the incidence and/or mortality rate of several forms of cancer, cardiovascular, cerebro-vascular and neuro-degenerative diseases (Bazzano et al., 2003; Liu, 2003; Riboli and Norat, 2003). Since the occurrence of oxidative stress can either lead to or accompany the progression of the above disorders, the high presence of antioxidants in fruits and vegetables generally has been regarded as an important basis for the health-protecting effects associated with their consumption (Gate et al., 1999; Terry et al., 2001). In addition to fruits and vegetables, herbs of no particular nutritional value can also constitute an important source of antioxidants (Warren, 1999; Ng et al., 2000). The term herb refers not only to herbaceous plants, but also to the leaves, bark, roots, seeds, fruits and flowers of shrubs and trees. More recently, the study of other herbs as a potential source of antioxidants has also stemmed from the need to screen endangered floras, and has been prompted by the increasing assumption that, given their natural origin, plant-derived antioxidants would comprise a group of relatively innocuous compounds. Number of synthetic antioxidants, such as BHA, BHT and TBHQ, have been added to foodstuffs. Although these synthetic antioxidants are efficient and relatively cheap, there are some disadvantages, becausethey are suspected of having some toxic properties. Therefore search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones (Khan et al., 2010).

Epidemiological and in vitro studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in

*Correspondence author. E-mail: atrabbasi@yahoo.com. Tel: (+92)-42-111000010 Ext.264.

biological systems (Souri et al., 2008). *Colebrookia oppositifolia* Smith locally known in Pakistan as “Bhinda”, “Pathan” or “Kala Behakar,” is widespread in Pakistan and India and belongs to the family Lamiaceae/Labiateae, whose flowering period is July–September. *C. oppositifolia* Smith. is also distributed in semi-hilly and hilly areas of Pakistan and India up to an altitude of 49,000 m. Ethno-medicinal uses of *C. oppositifolia* Smith. include the use of their leaves as applied to wounds and ulcers as an antiseptic. Roots’ extract, which is used in epilepsy treatment, contains flavones (Khan et al., 2003). Ansari et al. (1982) performed chemical investigation and screening of active compounds from roots of *C. oppositifolia* Smith. Stem bark of *F. bengalensis*, roots of *Asparagus racemosus*, fruits of *Annona squamata*, and shoots of *C. oppositifolia* Smith. along with stem and leaves are crushed and eaten on an empty stomach as a cure for urinary problems (Paudyal, 2000). *C. oppositifolia* Smith. reportedly contains a number of flavonoids and glycoflavonoids (Fan et al., 1996). Pure acteoside of high hepatoprotection was isolated from aerial parts of *C. oppositifolia* Smith. (Qazi et al., 2006). To the best of our knowledge, no detailed antioxidant studies have been carried out on *C. oppositifolia* Smith., therefore, in the present investigation, we described the comparative in vitro antioxidant potential of aqueous and organic fractions of this species by four methods: 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, total antioxidant activity by phosphomolybdenum complex (PC) method, Ferric Reducing Antioxidant Power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolic contents relative to conventionally used standards.

MATERIALS AND METHODS

Plant Material

The plant *C. oppositifolia* Smith. was collected from Kotli, Azad Kashmir in June 2009, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A Voucher specimen (GC. Herb. Bot. 622) has been deposited in the Herbarium of the Botany Department of the same university.

Extraction and fractionation of antioxidants

The shade-dried ground whole plant (17 kg) was exhaustively extracted with methanol (20L × 5) at room temperature. The extract was evaporated to yield the residue (1026 g), which was dissolved in distilled water (2 L) and partitioned with n-hexane (1L × 4), chloroform (1L × 4), ethyl acetate (1L × 4) and n-butanol (1L × 4) respectively. These organic fractions and remaining water fraction was concentrated separately on rotary evaporator (n-hexane at 35°C, chloroform at 37°C, ethyl acetate at 45°C, n-butanol at 50°C and water at 60°C). The yields of n-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, n-butanol soluble fraction and remaining aqueous fraction were 201, 148 124, 285 and 268 g, respectively. All these fractions were studied to evaluate their in vitro antioxidant potential.

Chemicals and standards

DPPH$^{\cdot}$ (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin Ciocalteu’s phenol reagent and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (n-hexane, chloroform, ethyl acetate, n-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride, ferrous chloride, ceric sulphate, hydrocloric acid, copper sulphate, aluminium chloride, lead acetate, acetic acid and ammonia from Merck (Pvt.) Ltd. (Germany).

Phytochemical screening

The Phytochemical screening was performed using the standard methods (Sofowara, 1993; Trease and Evans, 1989; Ayoola et.al., 2008) as described futher.

Test for alkaloides

For the test of alkaloids, the TLC card having spots of the studied samples was sprayed with Draggendorff’s reagent. Appearance of orange colour indicates the presence of alkaloids.

Test for terpenoides

Two methods were used to test THE presence of terpenoides. First, Ceric sulphate solution was sprayed on TLC card having spots of samples. TLC card was heated on TLC heater. Appearance of brown colour indicates the presence of terpenoides. Second, to 0.5 g of each of the extract was added 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoides.

Test for saponins

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for tannins

2 ml of sample was taken in test tube and 5 ml of n-butanol-HCl solution was added. Mixture was warmed for 1 h at 95°C in a water bath. Appearance of red colour indicated the presence of tannins.

Test for sugars

Sample solutions (0.5 g in 5 ml water) were added to boiling Fehling’s solution (A and B) in a test tube. Formation of red precipitates indicated the presence of sugars.

Test for phenolics

Neutral ferric chloride was added to each fraction. Appearance of

bluish green colour indicated the presence of phenolics.

Test for flavonoides

Four methods were used to test for flavonoides. First, dilute ammonia (5 ml) was added to a portion of sample solution in water. Concentrated sulphuric acid (1 ml) was added. A yellow colouration that disappears on standing indicated the presence of flavonoides. Second, a few drops of 1% aluminium chloride solution were added to sample solution. A yellow colouration indicated the presence of flavonoides. Third, the TLC card having spots of samples was sprayed with Benedict's reagent. Green fluorescence in UV light indicated the presence of flavonoides. Fourth, the TLC card having spots of samples was sprayed with lead acetate solution. Green fluorescence in UV light indicated the presence of flavonoides.

Test for cardiac glycosides (Keller-Killiyani test)

To 0.5 g of each sample diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Antioxidant assays

The following antioxidant assays were performed on all the studied fractions.

DPPH radical scavenging activity

The DPPH radical scavenging activities of various fractions of plant were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the reported method (Lee and Shibamoto, 2001). Briefly, various concentrations of the samples (1000, 500, 250, 125, 60, 30 and 15 µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for one hour. Then absorbance was measured at 517 nm against methanol as a blank in the UV-visible spectrophotometer (CECIL Instruments CE 7200 Cambridge England). Lower absorbance of spectrophotometer indicated higher free radical scavenging activity. The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = A_{control} - A_{sample} / A_{control} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total antioxidant activity by phosphomolybdenum complex method

The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method (Prieto et al., 1999). Briefly, 500 µg/mL of each sample was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95°C for 90 min. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of BHT. All determinations were assayed in triplicate and mean values were calculated.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to Benzie and Strain (1996) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa.3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2,4,6-Tripyridyl-s-triazine) solution in 40 mM Hydrochloric Acid and 20 mM Ferric chloride hexahydrate solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. The solutions of plant samples and that of trolox were formed in methanol (250□µg/mL). 10 µL of each of the sample solution and BHT solution were taken in separate test tubes and 2990 µL of FRAP solution was added in each to make the total volume up to 3 mL. The plant samples were allowed to react with FRAP solution in the dark for 30 min. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm by UV-visible spectrophotometer. The FRAP values were determined as micromoles of trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

Total phenolic contents

Total phenolics of various fractions of plant were determined using the method of Makkar et al. (1993). The 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10% Sodium carbonate and 0.1 mL of 2N Folin-Ciocalteu's phenol reagent. After 40 min, absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolic contents were expressed as micrograms of gallic acid equivalents (GAE) per gram of sample using the standard calibration curve constructed for different concentrations of gallic acid. The curve was linear between 50 to 500 µg/mL of gallic acid. Results were expressed in GAE µg/mL.

Ferric thiocyanate (FTC) assay

The antioxidant activities of various fractions of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method (Valentao et al., 2002). The 0.1 mL of each of sample solution (0.5 mg/ mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40°C. The mixture without extract was used as control. The mixture (0.1 mL) was taken and mixed with 5.0 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely, 3 min after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP%) [IP% = {1-(abs. of sample) /(abs. of control)} × 100]. The antioxidant activity of BHT was assayed for comparison as reference standard.

Statistical analysis

All the measurements were done in triplicate and statistical analysis was performed by Statistical software. All the data were expressed

Table 1. Phytochemical constituents of various fractions of *Colebrookia oppositifolia* smith.

Test	*n*-hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate soluble fraction	*n*-Butanol soluble fraction	Remaining aqueous fraction
Alkaloides	–	+	+	+++	–
Terpenoides	+++	+++	++	+++	+
Saponins	–	–	++	++	++
Tannins	–	+	++	+	++
Sugars	–	+	++	+++	++++
Phenolics	–	+++	+++	++	+
Flavonoides	–	+++	+++	++	+
Cardiac glycosides	+	++	++	++	+

'+' represents presence and '–' represents absence.

as ± S.E.M. Statistical analysis were determined using one way analysis of variance (ANNOVA) followed by post-hoc Tukey's test.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening was done on all the studied fractions and results is shown in Table 1. It was observed from the results that chloroform fraction, ethyl acetate fraction and n-butanol fraction contain phenolics and flavonoides, as well as alkaloids, while n- hexane fraction and aqueous fraction showed absence of all these compounds. Cardiac glycosides were present in all fractions but in more amount in chloroform fraction, ethyl acetate fraction and n-butanol fraction as compared to n-hexane fraction and aqueous fraction. Terpenes were detected in all the fractions. Tannins and sugars were also present in all fractions except n-hexane fraction. Saponins were present in ethyl acetate fraction, n-butanol fraction and aqueous fraction, while absent in n-hexane and chloroform fraction.

DPPH radical scavenging activity

The effects of phenolic compounds on DPPH radical scavenging are thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Siddaraju and Dharmesh, 2007). It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow (Meir et al., 1995). DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples. The method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule. DPPH radical decolourizes in the presence of antioxidants. It contains an odd electron, which is responsible for visible deep purple colour (Lee and Shibamoto, 2001). Reduction of DPPH radical was observed by the decrease in absorbance at 517 nm, whereas colour changes from purple to yellow. The various fractions of *C. oppositifolia* reduced DPPH radicals significantly. The values of percent scavenging of DPPH radical have been shown in Table 2. It was observed that activity was increased by increasing the concentration of the samples. The various concentrations of ethyl acetate soluble fraction exhibited the highest percent inhibition of DPPH radical as compared to other fractions. It showed 83.43 ± 1.27 inhibition of DPPH radical at a concentration of 60 µg/ml. The various concentrations of the fractions, which showed percent inhibition greater than 60% were found to be significant ($p < 0.05$). IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds (Gayatri Nahak and R.K. Sahu). IC_{50} values of all the fractions were also calculated and results are shown in Table 3. The lower the IC_{50} value, the higher the scavenging power. Ethyl acetate soluble fraction exhibited lowest IC_{50} value, that is, 27.2 ± 0.23 as compared to other fractions. The IC_{50} values n-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, n-butanol soluble fraction and aqueous fraction were found to be 557.32 ±6.55, 50.43 ± 0.79, 38.53 ± 0.86 and 98.12 ± 1.96 relative to butylated hydroxytoluene (BHT), a reference standard, having IC_{50} of 12.1 ± 0.92 µg/ml. The IC_{50} values of chloroform soluble fraction, ethyl acetate soluble fraction, n-butanol soluble fraction and aqueous fraction were found to be significant ($p < 0.05$), while that of n-hexane soluble fraction was found to be non significant ($p > 0.05$) when compared with BHT, a reference standard.

Table 2. Free radical scavenging activity of various fractions of *Colebrookia oppositifolia* using 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH).

S/N	Sample	Concentration in assay (µg/mL)	percentage scavenging of DPPH radical ± S.E.M[a)]
1	*n*-Hexane soluble fraction	1000	70.13 ± 0.43*
		500	48.58 ± 0.38
		250	35.6 ± 0.79
2	Chloroform soluble fraction	250	83.08 ± 1.05*
		125	62.31 ± 1.17*
		60	37.03 ± 0.76
3	Ethyl acetate soluble fraction	60	83.43 ± 1.27*
		30	56.05 ± 0.76
		15	35.11 ± 0.30
4	*n*-Butanol soluble fraction	125	91.46± 1.18*
		60	66.57 ± 1.30*
		30	41.49 ± 0.17
		15	21.24 ± 0.19
5	Aqueous fraction	500	90.63 ± 0.07*
		250	74.72 ± 1.85*
		125	55.11 ± 0.19
		60	27.88 ± 0.54
6	BHT[b)]	60	91.35 ± 0.14*
		30	75.46 ± 0.08*
		15	42.57 ±0.05
		8	23.47 ±0.34

[a)] Standard mean error of three assays; [b)] Standard antioxidant. *$p < 0.05$ when compared with negative control, that is, blank/solvent ($p<0.05$ is taken as significant).

Total antioxidant activity by phosphomolybdenum complex method

Total antioxidant activity of the studied fractions was determined by phosphomolybdenum method. This method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidants and the subsequent formation of a green phosphate Mo (V) complex at acidic pH. Electron transfer occurs in this assay, which depends upon the structure of the antioxidant (Prieto et al., 1999). The phosphomolybdenum method usually detects antioxidants, such as ascorbic acid, some phenolics, tocopherols and carotenoids. The total antioxidant activities of these fractions were compared with the standard antioxidant BHT and the results are shown in Table 3. It was revealed from the results that chloroform fraction showed highest total antioxidant activity, that is, 0.711 ± 0.031 as compared to other fractions. The total antioxidant activity of ethyl acetate was found to be 0.615 ± 0.009, while that of n-butanol fraction and aqueous fraction was 0.417 ± 0.02 and 0.59 ± 0.04. The n-hexane fraction showed lowest antioxidant activity (0.217 ± 0.01). The results were compared with BHT, a reference standard having total antioxidant activity of 0.818 ± 0.09. The total antioxidant activity shown by chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction were found to be significant ($p < 0.05$), while that of n-hexane and aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Ferric reducing antioxidant power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on

Table 3. IC_{50}, total antioxidant activity, FRAP values, total phenolics and lipid peroxidation inhibition values of different fractions of *Colebrookia oppositifolia*.

S/N	Sample	DPPH-radical scavenging activity (IC_{50}; µg/mL) ± S.E.M [a)]	Total antioxidant activity ± S.E.M [a)]	FRAP value TE (µM/ml) ± S.E.M [a)]	Total phenolics (GAE µg/g of sample) ± S.E.M [a)]	Inhibition of lipid peroxidation (%) ± S.E.M [a)]
1	*n*-Hexane soluble fraction	557.32 ±6.55	0.217 ± 0.01	10.43 ± 1.31	10.38 ± 2	9.63 ± 0.72
2	Chloroform soluble fraction	50.43 ± 0.79**	0.711 ± 0.031**	75.31 ± 0.69*	72.5 ± 0.34*	52.21± 0.57**
3	Ethyl acetate soluble fraction	27.2 ± 0.23**	0.615 ± 0.009**	172.23 ± 1.66*	54.17 ± 0.58*	57.61 ± 1.1**
4	*n*-Butanol soluble fraction	38.53 ± 0.86**	0.417 ± 0.02**	76.49 ± 1.44*	30.05 ± 1.18*	45.46 ± 0.74**
5	Aqueous fraction	98.12 ± 1.96**	0.59 ± 0.04**	20.57 ± 0.28	12.89 ± 0.23	11.3 ± 0.85
6	BHT [b)]	12.1 ± 0.91	0.818 ± 0.09	-	-	62.91 ± 0.60

[a)] Standard mean error of three assays; [b)] Standard antioxidant. *p< 0.05 when compared with negative control i.e. blank/solvent (p<0.05 is taken as significant); **p < 0.05 when compared with reference standard (BHT).

the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine [TPTZ] forming an intense blue Fe^{2+}–TPTZ complex with an absorbance maximum at 593 nm (Benzie and Strain, 1996). Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied fractions were calculated and results are shown in Table 3.

Among all the fractions, the ethyl acetate fraction showed highest FRAP value (172.23 ± 1.66 TE µM/mL). Chloroform fraction and n-butanol fraction showed almost equal FRAP values, that is, 75.31 ± 0.69 and 76.49 ± 1.44 TE µM/mL respectively, while that of aqueous fraction and n-hexane fraction were found to be poor sources. High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents.

The FRAP values of chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction were found to be significant ($p < 0.05$), while that of n-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank.

Total phenolic contents

Phenolic compounds and flavonoids are very important plant secondary metabolites. These compounds have numerous defense functions in plants, and thus several environmental factors, such as light, temperature, humidity, and internal factors, including genetic differences, nutrients, hormones, etc., contribute to their synthesis (Strack, 1997). Similarly, other factors, such as germination, degree of ripening, variety, processing and storage, also influence the content of plant phenolics (Bravo, 1998). It was reported that the phenolics are responsible for the variation in the antioxidant activity of the plant (Cai et al., 2004).

They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorney, 2001; Pitchaon et al., 2007) or chelate metal ions and protect against pathogens and predators (Balasundram et al., 2006). Most commonly encountered flavonoides are flavonols, quercetin, flavanols and anthocyanins. Table 3 shows the phenolic concentration in the different fractions, expressed as microgram of gallic acid equivalents (GAEs) per gram of fraction. Among these five fractions, the chloroform soluble fraction showed the highest amount of total phenolic compounds (72.5 ± 0.34 GAE µg/g). The total phenolic contents of n-hexane soluble fraction, ethyl acetate soluble fraction, n-butanol soluble fraction and aqueous fraction were found to be 10.38 ± 2, 54.17 ± 0.58, 30.05 ± 1.18 and 12.89 ± 0.23 GAE µg/g, respectively. The results for total phenolic contents of chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction were found to be significant ($p < 0.05$), while that of n-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank.

Ferric thiocyanate (FTC) assay

Peroxidation of lipids occurs both in vivo and in vitro and gives rise to cytotoxic and reactive products. These products disturb the normal functioning of the cell and can give rise to damaged or modified DNA. Oxygen reacts with

unsaturated double bond on the lipid, which results in generation of free radicals and lipid hydroperoxides. Hydrogen donating antioxidants can react with lipid peroxyl radicals and break the cycle of generation of new radicals. The FTC method is used to measure the amount of peroxide at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate, a reddish pigment (Aqil et al., 2006). The various fractions of plant were tested by this assay and results are shown in Table 3. Significantly, lower absorbances as compared to control were observed for chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction, which indicated that these fractions have greater antioxidant activities. The fractions, which showed greater values of percent inhibition of lipid peroxidation might contain primary antioxidant compounds, which are able to react aggressively with free radicals, particularly hydroxyl radicals, thereby terminating the radical-chained reaction and retarding the formation of hydroperoxides (Ismail et al., 2010). The highest percentage of inhibition of lipid peroxidation was exhibited by ethyl acetate fraction (57.61 ± 1.1%), while n-hexane soluble fraction had the lowest percentage of inhibition of lipid peroxidation (9.63 ± 0.72%). Chloroform, n-butanol and aqeuous fractions exhibited percent inhibition of lipid peroxidation: 52.21± 0.57, 45.46 ± 0.74 and 11.3 ± 0.85%, respectively. The inhibition of lipid peroxidation by BHT (standard) was 62.91% ± 0.60. The results for percent inhibition of lipid peroxidation of chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction were found to be significant ($p < 0.05$), while that of n-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Conclusion

The results showed that chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction contain large number of phenolics and flavonoides, while n- hexane fraction and aqueous fraction showed absence of all these compounds. Cardiac glycosides were present in large amounts in chloroform fraction, ethyl acetate fraction and n-butanol fraction as compared to n- hexane fraction and aqueous fraction. Due to presence of such compounds, chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction showed good antioxidant activity, while n- hexane fraction and aqueous fraction showed no activity due to absence of all these compounds. Ethyl acetate soluble fraction showed highest value of percentage inhibition of DPPH (83.43 ± 1.27) at concentration of 60 µg/ml. The IC_{50} of this fraction was 27.2 ± 0.23 µg/ml, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/mL. It also showed highest FRAP value (172.23 ± 1.66 µg of trolox equivalents), as well as highest value of inhibition of lipid peroxidation (57.61 ± 1.1%) as compared to the studied fractions. The chloroform fraction showed highest total antioxidant activity (0.711 ± 0.031), as well as highest total phenolic contents (72.5 ± 0.34). The overall antioxidant activity results for the chloroform soluble fraction, ethyl acetate soluble fraction and n-butanol soluble fraction were found to be significant ($p < 0.05$), while that of n-hexane soluble fraction and aqueous fraction were mostly found to be non significant ($p > 0.05$) when compared with BHT/blank. So, it was concluded that chloroform fraction, ethyl acetate fraction and n-butanol fraction are rich in strong antioxidants. These fractions are potentially valuable sources of natural antioxidants and bioactive materials, which would be expected to increase shelf life of foods and fortify against peroxidative damage in living systems in relation to aging and carcinogenesis.

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