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Original Article

IN VITRO ANTIOXIDANT AND RADICAL SCAVENGING ABILITIES OF AQUEOUS METHANOLIC EXTRACTS OF *CASSIA OBTUSA* L. PLANT PARTS (CAESALPINIACEAE)

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ABSTRACT

Objective: The present study was carried out to examine the *in vitro* antioxidant activity of the aqueous methanolic extracts of *Cassia obtusa* plant parts.

Methods: The activity was assessed using various *in vitro* assay models *viz.,* ferric reducing antioxidant power, DPPH•, OH•, $O_2^{\bullet-}$, ABTS•+ scavenging activity and lipid peroxidation inhibiting activities. The results were substantiated with well known natural as well as synthetic antioxidants.

Results: Among the samples analyzed, *C. obtusa* leaf extracts determined ($P<0.05$) effective antioxidant power in terms of all the assays tested.

Conclusion: Based on these findings, *C. obtusa* leaf can be considered as a potent source of nutraceutical contributor which could offer protection and ameliorate oxidative stress-induced physiological malfunctions.

Keywords: *Cassia obtusa,* Antioxidant, Aqueous methanolic extract, Free radical scavenging activity.

INTRODUCTION

Oxidative damage is caused by free radicals and reactive oxygen species, mostly generated endogenously. Excessive generation of free radicals aggravates the progression of many clinical diseases, including cancer, ageing, inflammation, cardiovascular diseases, liver injury, atherosclerosis and neuro generative diseases [1]. Over the past decades, much research has aimed to counterbalance the adverse effects of free radicals by complementing the endogenous antioxidant defence system with medicinal plants. Natural antioxidants offer a wide range of biochemical entities, including inhibition of reactive oxygen species (ROS) generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential [2]. Their broad range of effects on biological and medicinal systems has been studied extensively in many experimental investigations [3, 4]. Consequently, there has been overwhelming evidence indicating the potential health promoting properties of phytochemicals. Literature data abounds in examples where phenolic compounds are the bioactive components responsible for the prophylactic benefits of traditionally used plant sources [5]. Therefore identification and development of safer and natural antioxidants from plant sources are more beneficial to human health.

Cassia obtusa L. is a diffuse, perennial herb belonging to the family Caesalpiniaceae. A majority of the plants in this family are used as medicine or as vegetable. The seeds and leaves of *C. obtusa* are purgative and anthelmintic. The dried, pulverized leaves and root are soaked in hot water and are being served as tea by common people. The leaves and root are used to cure ulcers, burns and pityriasis. It is also used in traditional medicines to treat fever, sores, diabetes, diarrhoea, skin diseases and ophthalmia [6]. The hot water extract of root material is being used as tonic for liver diseases. The extract of leaves is reported to have antibacterial properties [7]. *C. obtusa* leaves contain anthraquinones, sennosides, flavonoids, sterols, polysaccharides, alkaloids, emodins and aloe-emodins. Nonetheless, two new anthroquinones viz., 1,3-dihydroxy-6-methoxy-7-methylanthroquinone and 1-hydroxy-3,7-diformylanthroquinone have been isolated and reported from their root [6].

Despite several medicinal claims, the present study was carried out to investigate the *in vitro* antioxidant potential of the aqueous methanolic extracts of *C. obtusa* plant parts. The antioxidant activities were measured using ferric reducing power, free radical scavenging activities (DPPH•, OH•, $O_2^{\bullet-}$, ABTS•+) and lipid peroxidation inhibiting activities.

MATERIALS AND METHODS

Procurement and preparation of plant materials

Leaf, stem, root, flower and pods of C. obtusa were harvested from Perundurai, Erode, Tamil Nadu, India. The authenticity of the selected plant material was duly identified and confirmed by comparison with reference specimen preserved in the herbarium at Botanical Survey of India, Southern Circle, Coimbatore. The voucher specimens (vide no: BSI/SC/5/23/10-11/Tech-535) were lodged in the departmental herbarium for further reference. The plant materials were cleaned, washed with copious amount of distilled water, shade dried, chopped into bits and coarsely powdered in a Willy mill (Nippon Electricals, Chennai, India) to 60-mesh size for extraction.

Preparation of crude plant extracts

50g of coarsely powdered plant samples were exhaustively extracted with methanol/water (70/30, v/v) using a round bottom flask with an attached reflux condenser for 3 h at a controlled temperature. The extracts were filtered and concentrated to dryness under reduced pressure using rotary vacuum evaporator (RE300; Yamato, Japan), lyophilized (4KBTXL-75; Vir Tis Benchtop K, New York, USA) to remove traces of water molecules and the lyophilized powders were stored at - 20°C until used directly for the assessment of various *in vitro* antioxidant activities.

DPPH• radical scavenging activity

The antiradical efficiency was assessed using DPPH• method as described by Blios [8]. In this method commercially available methanol soluble stable free radical DPPH was used. In its radical form, DPPH has an absorption band of 515 nm, which disappears upon reduction by an antioxidant compound or a radical species. For the photometric assay, different volumes of extracts (100 - 500µg) were taken in different test tubes. The volume was adjusted to 100 µL with methanol. 5.0 mL of 0.1 mM methanolic solution of DPPH• was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27°C. The control was prepared as above but without the test extract and methanol was used for the baseline correction. Changes in the absorbance of the samples were monitored at 517 nm. Results were compared with the activity of

rutin, quercetin, BHA and BHT. The per cent DPPH· discolouration of the samples were calculated using the following formula:

DPPH radical scavenging activity (%) = [(A_{517} of control - A_{517} of sample) /A_{517} of control] × 100.

Antioxidant activities of the extracts were expressed as IC_{50}, the microgram of extract to scavenge 50% of the DPPH radicals and were obtained by interpolation from the linear regression analysis. A lower IC_{50} value corresponds to greater antioxidant power.

Hydroxyl radical scavenging activity

The scavenging activity for the sample extracts on hydroxyl radical was measured according to the method of Klein *et al*. [9]. Different concentrations of the extract (100 - 500µg) was added with 1.0 mL of iron – EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. The reaction was terminated by the addition of 1.0 mL of ice – cold TCA (17.5% w/v). Then, 3.0 mL of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2.0 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at laboratory temperature for 15 min. The reaction mixture without the sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against the reagent blank. Results were compared with the activity of standard antioxidants *viz*., rutin, quercetin, BHA and BHT. The analysis was performed in triplicate. The hydroxyl radical scavenging activity (HRSA) % was calculated using the following formula:

Hydroxyl radical scavenging activity (%) = (control OD-sample OD / control OD) × 100.

The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Superoxide radical scavenging activity

Superoxide radicals were generated by the modified method of Beauchamp and Fridovich [10]. The assay was based on the capacity of the sample to inhibit formation by scavenging superoxide radicals generated by riboflavin-light-NBT in the system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200 - 1000µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

Superoxide radical scavenging activity (%) = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Lipid peroxidation inhibiting activity

The antioxidant capacity of the extract was evaluated using β – carotene- linoleate model system [11]. 1.0 mg of β – carotene was dissolved in 10 mL of chloroform and mixed with 20 mg of linoleic acid and 200 mg of Tween – 40 emulsifier mixture. Chloroform was completely removed at 45°C under vacuum using a rotary vacuum evaporator. 50 mL of oxygenated distilled water was added slowly to the semi-solid residue with vigorous agitation, to form an emulsion. A 5.0 mL aliquot of the emulsion was dispensed into tubes containing 100 µg/ mL of the sample extract. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using spectrophotometer. Subsequent absorbance readings were recorded at 15 min intervals by keeping the sample tubes in a water bath at 50°C until the visual colour of β – carotene in the control sample disappeared (about 120 min). A blank, devoid of β – carotene, was prepared for background subtraction. Rutin, quercetin, BHA and BHT were used as standards. All determinations were performed in triplicate and averaged.

The antioxidant activity (AA) was measured in terms of reduction in β – carotene bleaching activity using the following formula:

AA (%) = [1- (A_s^0 – A_s^{120}) / (A_c^0 – A_c^{120})] × 100

Where, $A_s^{\ 0}$ is the absorbance of the sample at 0 min, $A_s^{\ 120}$ is the absorbance of the sample at 120 min, A_c^0 is the absorbance of control sample at 0 min, and A_c^{120} is the absorbance of control sample at 120 min.

Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity was performed using an improved $ABTS^{\cdot+}$ method proposed by Siddhuraju and Manian [12]. The ABTS radical cation ($ABTS^+$) was generated by a reaction of 7 mmol/ L ABTS and 2.45 mmol/ L potassium persulfate after incubation for 16 h at laboratory temperature in dark. Blue – green ABTS+ was formed at the end of this period. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to obtain an absorbance of 0.700 ± 0.02 at 734 nm, the wavelength of maximum absorbance in the visible region. The stock solution of the sample extracts in ethanol was diluted such that, after introduction of a 10 µL aliquot of each dilution into the assay, they produced between 20 - 80% inhibition of the blank absorbance. After the addition of 1.0 mL of diluted ABTS+solution to 10µLof sample extracts or Trolox standards (final concentration 0-15 µM) in ethanol, absorbance was recorded at 30°C, exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicates were maintained for the experiments and the per cent inhibition of the blank absorbance at 734 nm was plotted as a function of Trolox concentration [13]. The unit of total antioxidant activity (TAA) was defined as the concentration of Trolox having the equivalent antioxidant activity expressed as µmol/ g sample extracts on the dry weight basis.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacities of the sample extracts were estimated according to the procedure described by Pulido *et al*. [14]. FRAP reagent (900 µL), prepared freshly and incubated at 37°C, was mixed with 90 µL of distilled water and 10 µL of test sample or methanol (for the reagent blank) was added. The test samples and reagent blank were incubated at 37°C for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ and 25 mL of 0.3 mol/L acetate buffer (pH 3.6) as described by Siddhuraju and Manian [12]. At the end of incubation, the absorbance readings were taken immediately at 593 nm, using spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranging from 100 to 2,000 µmol/L, ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. The parameter equivalent concentration (EC1) is defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L $FeSO_4.7H_2O$. The EC1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/L concentration of Fe (II) solution, determined using the corresponding regression equation.

Statistical analysis

For *in vitro* antioxidant activity of the extracts, the results were recorded as mean ± standard deviation (SD) (n = 3) and subjected to one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using SPSS (version 9, SPSS Inc., Chicago, USA). $P < 0.05$ was chosen as the criterion for statistical significance.

RESULTS AND DISCUSSION

Free radical scavenging ability using DPPH assay

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The antioxidant

reacts with the stable free radical DPPH (deep violet colour) and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. The decrease in absorption was taken as a measure of the extent of radical scavenging [15]. In the present study, all the assessed samples were able to interact intensively with DPPH and reduce the stable violet DPPH radical to the yellow DPPH-H, reaching their 50% reductive plateau ranging between 178.7 and 253.8 µg/mL. Among the plant parts examined, the crude aqueous methanolic leaf extract of *C. obtusa* was able to quench DPPH radicals more effectively than their corresponding plant parts analyzed (Table 1). Apparently, these values were significantly higher than the standard antioxidants tested (Table 1). However, substantial DPPH radical scavenging capacity of the leaf extract reported in the study could be due to the presence of high levels of DPPH radical quenching biomolecules with pronounced antioxidant activity and the aqueous methanol was able to extract those compounds very efficiently. In similar lines, Siddhuraju *et al*. [16] also reported that high concentration of tannins (proanthocyanidins) extracted from the stem bark of *C. fistula* also possess elevated DPPH radical quenching capacity.

Hydroxyl radical scavenging activity

Hydroxyl radical is the most dangerous radical in the body. It can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as copper or iron. Hydroxyl radicals can react with lipid, polypeptides, proteins and DNA, especially thiamine and guanosine. The resulting radical can undergo further reactions, such as reacting with oxygen to give peroxyl radicals, or decomposing to phenoxyl-type radicals by water elimination [17]. In the present study, the $OH^{\bullet}$ scavenging data indicates that all the plant part extracts of *C. obtusa* generally registered good hydroxyl radical scavenging ability in a concentration dependent manner. Among them, the crude aqueous methanolic extract of leaf registered the highest $OH^{\bullet}$ scavenging potential (122.7 µg/mL) and these values were higher than the standard antioxidants tested (Table 1). The prowess of the leaf extracts to quench hydroxyl radicals seems to insinuate the prevention of propagation of the process of lipid peroxidation, and hence good scavengers of active oxygen species thus reducing the rate of chain reaction [18]. Scavenging of hydroxyl free radical was also reported in several anthraquinone, anthrone and flavonoids [19, 20]. Therefore it is apparent that the presence of phenolic, flavonoid and anthraquinone constituents [6, 21] reported in this species might have implicated in antioxidant metabolism [22].

Superoxide radical scavenging activity

Superoxide anion is an oxygen-centered radical with selective reactivity. It is biologically quite toxic. These precursor signals active free radicals to react with biological macromolecules and thereby inducing tissue damage [23]. Superoxide radical is normally formed first and it produces other kinds of free radicals and oxidizing agents. They are derived from dissolved oxygen by riboflavin/methionine/illuminate system and reduces NBT in the system. In this method, superoxide anions reduces the yellow dye (NBT^{2+}) to produce blue colour formazan which is measured spectrophotometrically at 590 nm. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance (590 nm) with an antioxidant indicates the consumption of superoxide anion in the reaction mixture. In the present investigation, all the assessed plant samples were able to quench superoxide radicals effectively in the reaction mixture. Among the plant parts analyzed, the leaf extracts (375.6µg/mL) displayed remarkably highest radical quenching ability than their corresponding plant parts examined (Table 1). However, these values were higher than the standard antioxidants tested.

Lipid peroxidation inhibiting activity

Lipid peroxidation contains a series of free radical- mediated chain reaction process and is also associated with several types of biological damage. The role of free radicals and ROS is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, ageing and atherosclerosis [24]. At the given concentration of 100 µg/mL, the leaf (28.6%) and pod (26.3%) extracts exerted effective antioxidant activity percentage. Interestingly, these values were comparably higher than those of the natural antioxidants tested viz., Rutin (12.4%) and Quercetin (24.9%) (Table 1). Accordingly, it can be suggested that the lipid peroxidation inhibiting activity of the various plant parts of *C. obtusa* may be attributed to the presence of phenolics, flavonoid and anthraquinone constituents [6]. It is believed that lipid peroxidation is one of the causes of the occurrence of cardiovascular diseases [25] and cancer [26, 27]. Furthermore, the high inhibition rate of the extracts obtained in the present study might therefore contribute a big way to their therapeutic potential.

Table 1: Radical scavenging abilities and lipid peroxidation inhibiting activity of the aqueous methanolic extracts of *C. obtusa* plant parts

Sample	IC_{50} values (µg/mL)#			Lipid peroxidation inhibiting activity (%)
Plant parts	$DPPH^{\bullet}$ scavenging activity	$OH^{\bullet}$ scavenging activity	$O_2^{\bullet-}$ scavenging activity	
Leaf	178.7±4.79^{b}	122.7±1.39^{b}	375.6±0.57^{b}	28.6±0.17^{ab}
Stem	194.6±3.49^{bc}	169.7±1.34^{bc}	407.8±0.67^{c}	24.3±0.17^{b}
Root	253.8±2.24^{cd}	146.5±0.99^{b}	556.8±1.26^{d}	19.7±0.08^{c}
Flower	195.5±6.74^{bc}	198.5±3.15^{bc}	522.8±1.09^{d}	19.9±0.11^{c}
Pod	202.6±6.16^{c}	132.9±1.41^{b}	445.8±0.80^{c}	26.3±0.19^{ab}
Standards				
Rutin	15.8±0.01^{a}	15.7±0.8^{a}	18.8±0.01^{a}	12.4±0.8^{d}
Quercetin	20.7±0.05^{a}	34.9±3.5^{a}	23.0±0.07^{a}	24.9±1.6^{b}
BHA	21.4±0.1^{a}	35.5±1.9^{a}	26.4±0.51^{a}	38.0±0.6^{a}
BHT	34.7±0.3^{a}	45.6±0.8^{a}	39.5±0.63^{a}	28.7±0.7^{ab}

*Values are mean ± standard deviation (SD) of three independent experiments. Mean values followed by different superscript in a column are significantly different (P<0.05). #Values are the amount of sample necessary to decrease initial concentration/activity of free radicals/enzymes by 50% (IC_{50}) under the experimental condition.

$ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ is a protonated radical, which has a characteristic maximum absorbance band at 734 nm, that decreases with the scavenging of proton radical [28]. The decolorization of $ABTS^{\bullet+}$ cation radical is an unambiguous way to measure the Trolox equivalent antioxidant capacity of the test drugs. Since, TEAC is a measurement of the effective antioxidant activity of the extract, a higher TEAC value would imply greater antioxidant activity of the sample. This assay was calibrated with the water soluble α-tocopherol analogue, Trolox. In the evaluation of total antioxidant capacity by $ABTS^{\bullet+}$ method, all the sample extract of *C. obtusa* plant parts were able to quench $ABTS^{\bullet+}$ radicals more efficiently with their TEAC values ranging between 4073 and 1056 µmol Trolox equivalent/g extract (Fig. 1a). Among the plant parts analyzed, both the flower (4073 µmol Trolox equivalent/g extract) and stem extracts (3565 µmol Trolox equivalent/g extract) of *C. Obtuse was* able to quench $ABTS^{\bullet+}$ radicals more efficiently than the other plant parts examined. The high antioxidant power of the extract reported in the study may be contributed by the hydrogen-donating compounds likely to be

present in the polar solvents [29]. Hagerman *et al.* [30] have reported that the high molecular weight phenolics have more ability to quench free radicals ($ABTS^{\bullet+}$) and that effectively depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group substitution, rather than their specific functional groups.

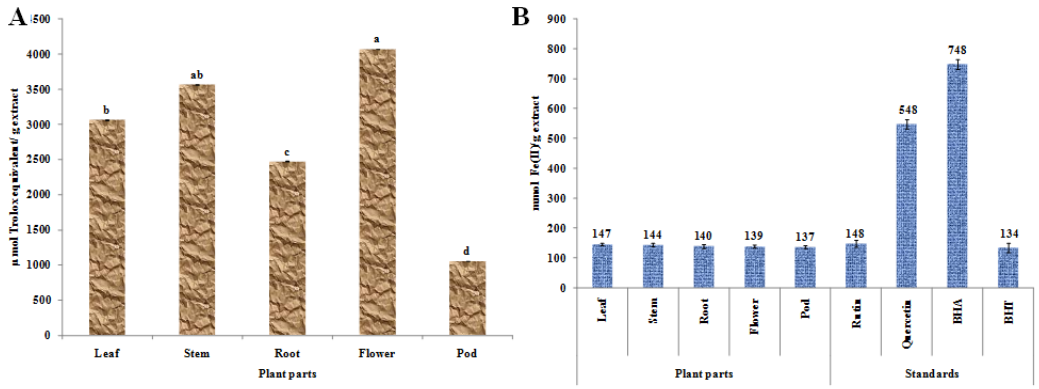

Fig. 1: Total antioxidant capacity (a) and ferric-reducing/antioxidant power assay (FRAP) (b) of the aqueous methanolic extracts of *C. obtusa* plant parts*.

*Values are mean of three replicate determinations ± standard deviation. Vertical bars labelled with different letters are significantly different (P<0.05)

Ferric reducing antioxidant power (FRAP) assay

FRAP assay directly measures antioxidants or reductants in a sample that reacts with ferric tripyridyltriazine (Fe^{3+} TPTZ) complex and produces a coloured ferrous tripyridyltriazine (Fe^{2+} TPTZ). The antioxidant capacities of the aqueous methanolic extracts of *C. obtusa* plant parts varied significantly (P<*0.05*) (fig. 1b). However, the leaf (147 mmol Fe (II) /g extract) and stem (144 mmol Fe (II)/g extract) showed the higher FRAP antioxidant activity and these values were comparably higher than the well known standard antioxidant, BHT (134 mmol Fe (II)/g extracts). It is well established that the reducing ability of polyphenols and anthroquinones in these extracts may be an important factor dictating their free radical-scavenging capacity as observed by earlier workers [31]. Therefore, it is speculated that these sample extracts can act as electron donors and react with free radicals and convert them to stable products, thus terminating the radical chain reactions [18].

CONCLUSION

It is concluded that the aqueous methanolic extracts of *C. obtusa* leaf exhibited a wide range of antioxidant capacities in terms of the assays used for the determination of antioxidant potential, thus making them a valuable source of natural antioxidants. However, the data pertaining to the bioactive compounds responsible for such activities will delineate the future use of this species in the field of pharmaceutical/ nutraceuticals.

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CONFLICT OF INTEREST

We have no conflict of interest.

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