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Antioxidant Properties of the Methanol Extracts of the Leaves, Seeds and Stem of *Cassia occidentalis*

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ABSTRACT

In the present study, the antioxidant potency of methanolic extracts of leaves, stems and seeds of *C. occidentalis* plant was investigated. Employing various established *in vitro* systems such as nitric oxide scavenging activity, β -carotene-linoleic acid model system, hydroxyl radical scavenging activity, reducing power, metal chelating activity and superoxide radical scavenging activity. The methanolic extract of seeds were found to have highest hydroxyl radical, superoxide radical and β -carotene-linoleic acid scavenging potential as compared to corresponding leaves and stem extracts. However, methanolic extract of leaves and stem were found to possess highest metal chelating and nitric oxide radical scavenging potential respectively as compared to seed extract. Overall, the data obtained in the *in vitro* models clearly establish the antioxidant potency of *C. occidentalis* plant.

Key words: *Cassia occidentalis*, free radicals, phenolics, flavonoids, reducing power

INTRODUCTION

Life on earth survives only due to presence of oxygen. Oxygen gives us energy by oxidation of food which is essential for living. During this process highly reactive and harmful oxygen species are also generated which can damage living organisms. Antioxidants either prevent these reactive oxygen species being formed or remove them before they can damage the cellular components. Ayurveda, Unani Chinese and other traditional medical systems, provide substantial lead to find active and therapeutically useful antioxidant compounds from plants. Presently, there has been an amplified interest worldwide to identify antioxidant compounds which are pharmacology effective and have low or no side effects for use in preventive medicine and the food industry (Sati *et al.*, 2010).

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are highly reactive and potentially damaging transient chemical species are continuously produced in the human body, as a consequence of exposure to a plethora of exogenous chemicals in our ambient environment and a number of exogenous metabolic processes involving redox enzymes and bioenergetics electron transfer (Ali *et al.*, 2008). Antioxidants are the compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Martino *et al.*, 2009; Jain *et al.*, 2011). The antioxidative effects are mainly due to phenolic components, such as flavonoids (Ozsoy *et al.*, 2008; Annegowda *et al.*, 2010), phenolic acids and phenolic diterpenes (Ohnishi *et al.*, 1994). The antioxidative activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decreasing peroxides (Osman *et al.*, 2009; Butkhup and Samappito, 2011; Couliadiati *et al.*, 2011).

Cassia occidentalis (Caesalpinaceae) is called as Kasmard in Sanskrit, Kasondi in Hindi and Coffee Senna in English. *C. occidentalis* plant have ethnomedicinal importance like paste of leaves is externally applied on healing wounds, sores, itch, cutaneous diseases, on bone fracture, fever, ringworm, skin diseases and throat infection. This plant was known to possess antibacterial (Saganuwan and Gulumbe, 2006), antimalarial (Tona *et al.*, 1999), hepatoprotective (Jafri *et al.*, 1999), antimutagenic (Sharma *et al.*, 2000), antiplasmodial (Tona *et al.*, 2004) and anticarcinogenic activity (Yadav *et al.*, 2010). The leaves (aqueous extract) of this plant are chiefly utilized in food and drinks in West Africa (Kouakou-Siransy *et al.*, 2010) and seeds are used as coffee substitute in some parts of India (Yadav *et al.*, 2010). Recently, some researchers have investigated the hydrogen peroxide and hypochlorous acid scavenging activity of leaves of *C. occidentalis* from West Africa region (Kouakou-Siransy *et al.*, 2010). However, no work has been reported on the stem of this plant. So, in search of some more promising antioxidant activities and phenolics, an attempt was made to investigate the free radical scavenging activity of methanolic extract of *C. occidentalis* plant from Haryana region by *in vitro* techniques.

MATERIALS AND METHODS

Plant collection: The leaves, seeds and stems of *C. occidentalis* were collected from the local areas of Rohtak district of Haryana on October, 2008. The plant was identified and authenticated by comparing the herbarium specimen (MDU 2504) available in the Department of Genetics, M.D. University, Rohtak, India.

Preparation of the extract: The plant parts (leaves, stems, seeds) were cleaned with deionized water, oven dried at 50°C for 48 h and powdered in a grinder. The plant material (200 g) was extracted with methanol (2000 mL) by using Soxhlet apparatus for 24 h at a temperature not exceeding the boiling point of the solvent. The obtained extracts were filtered by using Whatman filter paper No. 1 and then concentrated under vacuum at 40°C by using a rotary evaporator. The individual extracts were then lyophilized (Allied Frost lyophilizer, New Delhi, India) to powdered form at -55°C under vacuum conditions.

Preliminary phytochemicals and total phenolic content: Preliminary analysis of saponins, steroids, flavanoids and phenolic compounds were carried out by using the different methods (Harbone, 1988). The total phenolic content of the extracts was determined by using method of Mcdonald *et al.* (2001), with some modifications. Calibration curve was prepared by mixing ethanolic solution of gallic acid (1 mL; 0.025-0.400 mg mL⁻¹) with 5 mL of Folin-Ciocalteu reagent (diluted ten fold) and sodium carbonate (4 mL; 0.7 M). The absorbance of the solution was noted at 765 nm and drew the calibration curve. One milliliter of plant extract in different concentrations (0.2-1.0 mg mL⁻¹) was also mixed with the reagents above and after two hours the absorbance was measured to determine total plant phenolic contents. All determinations were carried out in triplicate. The total content of phenolic compounds of the extract was calculated in gallic acid equivalents (% dw GAE).

Nitric oxide radical scavenging activity: Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction (Hyoung, 1992). In this investigation, Griess Illosvoy reagent was generally modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of the use of 1-naphthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside

(10 mM, 2 mL), phosphate buffer saline (0.5 mL) and the extract (0.2-1.0 mg mL⁻¹) were incubated at 25°C for 150 minutes. After incubation, 0.5 mL of the reaction mixture mixed with 1 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completion of the reaction process of diazotization. Further, 1 mL of the naphthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30 min at 25°C. The absorbance was taken at 546 nm. Curcumin was used as a standard. The Percent Inhibition (PI) was calculated using the formula:

$$PI = \frac{A_{(Control)} - A_{(Sample\ or\ Standard)}}{A_{(Control)}} \times 100$$

Where:

A_(Control) = Absorbance of control reaction
A_(Sample or Standard) = Absorbance of sample extract or standard

Hydroxyl radical scavenging activity: The hydroxyl radical scavenging activity was measured by the deoxyribose method (Halliwell *et al.*, 1987; Mathew and Abraham, 2006). The reaction mixture which contained extract in different concentrations (0.2 mg mL⁻¹ to 1.0 mg mL⁻¹), deoxyribose (3.75 mM), H₂O₂ (1 mM), Potassium phosphate buffer (20 mM, pH 7.4), FeCl₃ (0.1 mM), EDTA (0.1 mM) and Ascorbic acid (0.1 mM), was incubated in a water bath at 37±0.5°C for 1 h. The extent of deoxyribose degradation was measured by the TBA (Thio-barbituric acid) method (Ohkawa *et al.*, 1979). One milliliter of TBA (0.1% w/v) and 1 mL of TCA (2.8% w/v) were added to the mixture and heated in a water bath at 100°C for 20 min. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm. All the analysis was done in triplicates and the average values were taken. Percent Inhibition (PI) of deoxyribose degradation was calculated according to the equation:

$$PI = \frac{A_{(Control)} - A_{(Sample\ or\ Standard)}}{A_{(Control)}} \times 100$$

Where:

A_(Control) = Absorbance of control reaction
A_(Sample or Standard) = Absorbance of sample extract or standard

Reducing power assay: The reductive potential of the extract was determined according to the method of Oyaizu (1986). Different concentrations of extracts and standard (0.2, 0.4, 0.6, 0.8, 1.0 mg mL⁻¹) in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v) was added to the mixture, which was then centrifuged for 10 min at 1000 g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. Higher the absorbance of the reaction mixture indicated greater reductive potential.

Metal chelating activity: The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis *et al.* (1994). Extracts were added in different concentrations

(0.2 to 1 mg mL⁻¹) to a solution of 1mM FeCl₂ (0.05 mL). The reaction was initiated by the addition of 1 mM Ferrozine (0.1 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. All test and analyses were done in triplicate and averaged. The percentage of inhibition of ferrozine-Fe²⁺ complex formation was calculated using the formula given below.

$$PI = \frac{A_{(Control)} - A_{(Sample\ or\ Standard)}}{A_{(Control)}} \times 100$$

Where:

A_(Control) = Absorbance of control reaction

A_(Sample or Standard) = Absorbance of sample extract or standard

β-Carotene-linoleic acid (linoleate) assay: The antioxidative activity of crude methanolic extracts of leaves, seeds and stems of *C. occidentalis* was evaluated using a β-carotene-linoleic acid model system (Miller, 1971). Briefly, 1 mL of β-carotene (0.2 mg mL⁻¹) dissolved in the chloroform was pipetted in to a small round-bottom flask. After removing the chloroform by using a rotary evaporator, 20 mg of linoleic acid, 200 mg of Tween 40 and 50 mL of aerated distilled water were added to the flask with vigorous stirring. Five milliliter aliquot of the prepared emulsion were transferred to a series of tubes containing (0.2-1.0 mg mL⁻¹) of plant extract. Ascorbic acid was used as a positive control. The test systems were placed in the water bath at 50°C for 2 h. The reaction was performed in triplicates and absorbance was measured using the spectrophotometer at 470 nm, immediately after sample preparation (t = 0 min) and then at the end of experiment (t = 120 min). The antioxidant activity of the plant extracts under investigation was expressed as:

$$\%AA = 100 \left[\frac{1 - (A_{1(t=0)} - A_{1(t=120)})}{(A_{0(t=0)} - A_{0(t=120)})} \right]$$

Where:

% AA = Antioxidant activity of the plant extract

A_{1(t=0)} = Absorbance of the test sample/standard at zero time.

A_{1(t=120)} = Absorbance of the test sample/standard after 120 min.

A_{0(t=0)} = Absorbance of the aqueous control sample at zero time.

A_{0(t=120)} = Absorbance of the aqueous control sample after 120 min.

Superoxide anion scavenging activity: Measurement of superoxide anion scavenging activity of extracts was based on the method described by Liu *et al.* (1997) with slight modification of Oktay *et al.* (2003). Superoxide radicals are generated non-enzymatically in PMS-NADH systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL Tris-HCl buffer (16 mM, pH 8.0) containing NBT (Nitroblue tetrazolium) (50 μM) solution and NADH (78 μM) solution with varying concentrations of the extract (0.2-1 mg mL⁻¹). The reaction was started by adding PMS (Phenazine Methasulfate) Solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the

absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The Percentage Inhibition (PI) of superoxide anion generation was calculated using the following formula:

$$PI = \frac{A_{(Control)} - A_{(Sample\ or\ Standard)}}{A_{(Control)}} \times 100$$

Where:

$A_{(Control)}$ = Absorbance of control reaction

$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard

Statistical analysis: The experimental results are expressed as means±Standard Deviation (SD) of triplicate measurements. The results were processed using Microsoft Excel 2000 and the data were subjected to one way analysis of variance and the significance of differences between samples means were calculated by Graph pad prism 5.0 (San Diego, USA) software using Duncan's multiple range test. The p-values = 0.05 were regarded as significant. Correlation analysis between antioxidant tests and total phenolic content was also carried out by using the same software.

RESULTS AND DISCUSSION

Total phenolic content: Phenolics or polyphenols are the plant secondary metabolites and are very important by virtue of their antioxidant activity by chelating redox active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals. The Folin-Ciocalteu procedure has been proposed to rapidly estimate the level of total phenolics in food and supplements (Prior *et al.*, 2005). The total phenolic content of the of *C. occidentalis* extracts varied from seeds (18.63±1.09% dw GAE)>leaves (16.57±0.40% dw GAE) >stem (10.95±0.68% dw GAE).

Nitric oxide radical scavenging activity: In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al.*, 1991). Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for two hours resulted in a linear time-dependent nitrite production, which is reduced by the tested extracts of *C. occidentalis*. This may be due to the antioxidative principles (chiefly phenolics) in the extracts which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The nitric oxide radical scavenging activity of methanolic extract of *C. occidentalis* was in the following order: Stem (56.15±0.26%)>seeds (40.55±0.41%)>leaves (34.23±0.22%) (Fig. 1).

Hydroxyl radical scavenging activity: Generally molecules that inhibit deoxyribose degradation are those that can chelate the iron ions and thereby prevent them from complexing with deoxyribose and render them inactive in a Fenton reaction (Smith *et al.*, 1992). The antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation has been correlated with iron chelating properties (Ohnishi *et al.*, 1994). The hydroxyl radical scavenging activity of seeds of *C. occidentalis* was found to be maximum followed by stem and leaf extracts respectively as depicted in Fig. 2.

Reducing power assay: In this assay, the yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of each extract. The presence of reductants (antioxidants) in the plant extract causes the reduction of Fe^{3+} /Ferricyanide complex to ferrous form. Therefore, Fe^{2+} can be monitored by measuring formation of Perle's Prussian blue at 700 nm (Chung *et al.*, 2002).

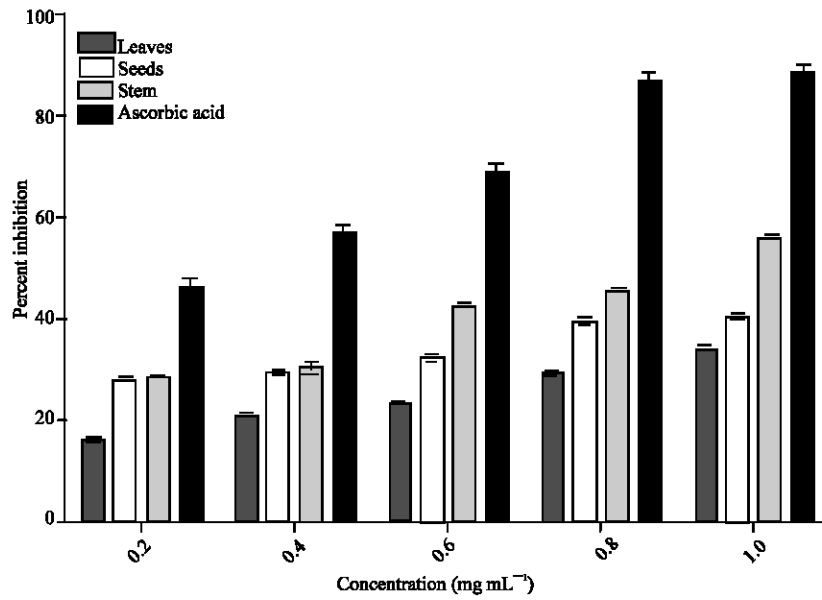


Fig. 1: Nitric oxide radical scavenging activity of methanol extracts of leaves, stem and seeds of *C. occidentalis*

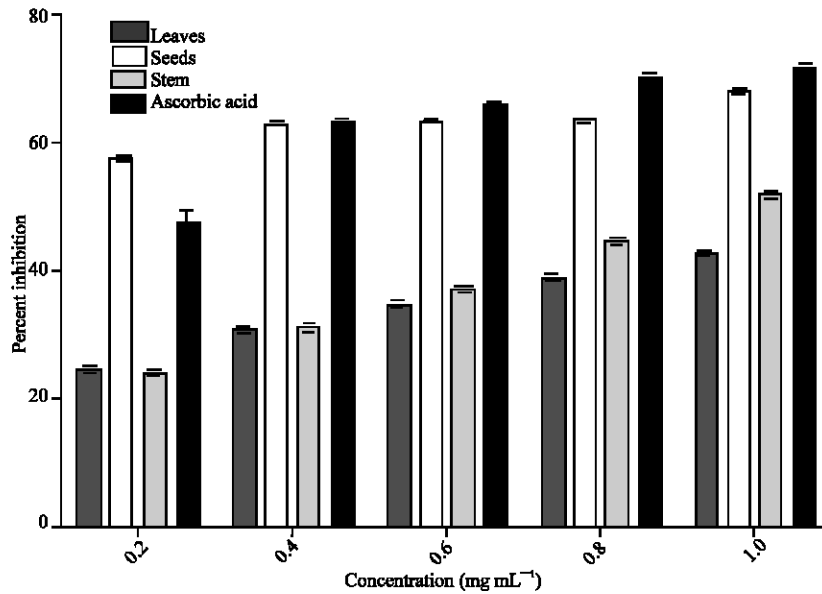


Fig. 2: Hydroxyl radical scavenging activity of methanol extracts of leaves, stem and seeds of *C. occidentalis*

Metal chelating activity: Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. The formation of Ferrozine- Fe^{2+} complex is not complete in the presence of plant extract, indicating their ability to chelate the iron. The absorbance of Ferrozine- Fe^{2+} complex decreased linearly in a dose-dependent manner (0.2 to 1 mg mL⁻¹). The standard compound Ascorbic acid did not exhibit any metal chelating activity at all the tested concentrations. Reaction of ascorbic acid with $FeCl_2$ might enhance the degradation of ascorbic acid and increase the ascorbyl acid radical concentration (Sato and Sakagami, 1997). Maximum chelation of metal ions was observed from the leaf methanolic extract followed by seeds and stem extracts respectively (Table 1).

β -carotene-linoleic acid assay: In β -carotene-linoleic acid model, β -carotene undergoes rapid discoloration in the absence of an antioxidant. During oxidation, an atom of hydrogen is abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds (Frankel, 1998). The pentadienyl free radical so formed then attacks highly unsaturated β -carotene molecule in an effort to reacquire a H-atom. As the β -carotene molecule loses their conjugation, the carotenoids lose their characteristic orange color. The presence of a phenolic antioxidant can hinder the extent of β -carotene destruction by neutralizing the linoleate free radical and any other free radicals formed within the system. In this work, the greatest antioxidative efficacy found was from the natural antioxidant ascorbic acid, which significantly inhibited the β -carotene consumption throughout the incubation period. The seed extract of *C. occidentalis* was found to possess maximum inhibition (50.81±0.22%) activity as compared to the leaves and stem methanolic extracts as shown in Table 1.

Superoxide radical scavenging activity: In PMS/NADH-NBT system, superoxide anions derived from dissolved oxygen by PMS/NADH coupling reaction reduce NBT. The decrease in the absorbance at 560 nm with the antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. In the present investigation, the reference compound, ascorbic acid, showed a pro-oxidant effect. Ascorbic acid is a potent reducing agent and acts as a free radical scavenger.

Table 1: Antioxidant activity of different parts of *C. occidentalis* plant (in percent inhibition)

Concentration (mg mL ⁻¹)	Metal chelating activity			B-carotene linoleic acid assay			Superoxide radical scavenging activity		
	Seed	Leaf	Stem	Seed	Leaf	Stem	Seed	Leaf	Stem
0.2	45.35 ±0.17	55.54 ±0.44	24.32 ±0.59	20.66 ±0.40	20.43 ±0.48	24.29 ±0.61	17057 ±0.38	17.29 ±0.13	23.36 ±0.55
0.4	62.67 ±0.40	56.43 ±0.32	27.32 ±0.59	33.56 ±0.38	33.31 ±0.19	28.69 ±0.60	35.52 ±0.32	35.54 ±0.44	26.06 ±0.95
0.6	68.19 ±0.11	65.36 ±0.54	32.95 ±0.84	37.54 ±0.40	37.54 ±0.40	34.99 ±0.95	41.47 ±0.50	41.69 ±0.28	32.95 ±0.84
0.8	69.28 ±0.15	66.34 ±0.28	43.66 ±1.16	44.69 ±0.31	44.29 ±0.35	39.92 ±0.97	55.67 ±0.29	55.42 ±0.22	39.95 ±0.96
1.0	75.40 ±0.52	77.12 ±0.10	52.62 ±0.47	50.81 ±0.22	50.42 ±0.35	44.39 ±1.13	58.82 ±0.22	58.63 ±0.21	42.63 ±0.54

However, it may act as a pro-oxidant in the presence of metals (Bendich *et al.*, 1986). In Case of *C. occidentalis*, methanolic extract seeds were found to possess maximum superoxide radical scavenging activity as compared to leaves and stem extracts.

CONCLUSION

These observations were clearly evidence that the extracts of leaves, stem and seeds of *C. occidentalis* have significant antioxidant capacities in different extracts. A further detailed study to characterize the active principles and to elucidate the exact mechanism of action of this extract is the subject of ongoing investigation in our group.

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