COMPARATIVE ASSESSMENT OF RELATIVE ANTIOXIDANT ACTIVITY OF SEQUENTIAL LEAF EXTRACTS OF *CASSIA OCCIDENTALIS* L. AND *C. TORA* L.

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Summary

The present study was designed to compare the antioxidant potential of petroleum ether, benzene, chloroform, methanol and water extract of leaves of *C. occidentalis* and *C. tora* by nitric oxide scavenging activity, β-carotene-linoleic acid tests, reducing power and metal chelating activity. The extracts of different solvents revealed significant inhibition of free radicals in a dose-dependent manner. The aqueous leaf extract of *C. occidentalis* showed maximum inhibition of 64.65±0.27%, 50.81±0.22% and 79.44±0.18% at 1mg/ml concentration as compared to the corresponding aqueous and methanolic extracts of *C. tora* for nitric oxide, β-carotene-linoleic acid test and metal chelating activity respectively. The results were compared with the positive control standards (curcumin and ascorbic acid) and significance was observed. The maximum total phenolic content was observed in the aqueous extract of the leaves of *C. occidentalis* (21.37±0.33% dw Gallic Acid Equivalent (GAE)) as compared to the methanolic extract of *C. tora* leaves (13.15±0.78% dw GAE). The data obtained in vitro models clearly establish the high antioxidative potential of leaf extracts of *C. occidentalis* as compared to *C. tora*.

Keywords: *Cassia occidentalis, Cassia tora,* antioxidant activities, total phenolics.

Introduction

Tissue damage resulting from an imbalance between reactive oxygen species (ROS) generating and scavenging systems ('oxidative stress') has been implicated in the pathogenesis of a variety of disorders, including degenerative disorders of CNS such as Alzheimer's disease (1, 2). Antioxidants possess the ability to protect the cellular organelles from damage caused by the free radicals induced oxidative stress. The beneficial effects of antioxidants on promoting health is believed to be achieved through several possible mechanisms, direct reaction with and quenching of free radicals, chelation of transition metals, reduction of peroxides and stimulation of the antioxidative enzyme defense system (3).

Many synthetic antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) are very effective but they possess certain health risks and toxic properties to human health. Research results indicate that plant polyphenolics and their metabolites may scavenge oxygen and nitrogen free radicals, which controls oxidative stress and oxidative damage to blood lipids and proteins (4). Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidant in reducing such free radicals induced tissue injury (5, 6).

Cassia occidentalis L. and *C. tora* L. are the Ayurvedic medicinal plants and have ethnomedicinal importance. *C. occidentalis* is called as Kasmard in Sanskrit, Kasondi in Hindi, Coffee senna in English and *C. tora* is called as Chakramard in Sanskrit, Puvad in Hindi and Foetid senna in English. Both plants belong to family Caesalpiniaceae. *C. occidentalis* and *C. tora* both are found in wild, along the road sides in Haryana from April-May to August-September. The former plant is more abundant in Haryana as compared to the latter one. Leaves of *C. occidentalis* plant have been used for healing wounds, sores, itch, cutaneous diseases, on bone fracture, fever, ringworm, skin diseases and throat infection. *C. tora* is chiefly used for skin diseases, fever, malaria, stomach disorders, pain and cardiac disorders. *C. occidentalis* (7) and *C. tora* (8) both plants were known to possess antimicrobial, hepatoprotective, antihelminthic, antioxidant, anticancerous and antimutagenic properties.

Although the chemical constituents of these plants have been reported, so far little information was available concerning the antioxidant properties of their leaves (7). In

Haryana, leaves of both *C. occidentalis* and *C. tora* have been consumed as food. Chiefly, leaves of *C. tora* (local name – puvad) are used as a food ingredient in traditional recepies by local people of Haryana. A perusal of literature reveals that there was no scientific documentation available on the comparative assessment of antioxidant activity of leaf extracts of these plants. Recently, some researchers (9, 10) observed the antioxidant potential of leaf extracts of both *C. occidentalis* and *C. tora*. But still there is need of detailed in vitro antioxidant assays to evaluate the antioxidative potential of leaves extracts of these plants. Moreover, our preliminary experiments for phytochemicals of leaf extracts of *C. occidentalis* exhibited considerable bioactivity on microbes (11). The present study was aimed to provide information on antioxidant properties *C. occidentalis* and *C. tora* plant extracts prepared in different solvents.

Methods

Plant material and extract preparation

The leaves of *C. occidentalis* and *C. tora* were collected from the local areas of Rohtak district of Haryana in October, 2008. The plant were identified and authenticated by comparing the herbarium specimen MDU 2504 (*C. occidentalis*), MDU 2505 (*C. tora*) available in the Department of Genetics, M. D. University, Rohtak. The powdered leaf material (200 gm) was sequentially extracted in solvents according to their increasing polarity (petroleum ether, benzene, chloroform, methanol and water in 2000 ml separately by using Soxhlet apparatus for 24 hours at a temperature not exceeding the boiling point of the respective solvent. The extracts were then concentrated under vacuum at 40^0C by using a rotary evaporator and lyophilized to powdered form at -55^0C under vacuum conditions.

Determination of Total phenolic content

The total phenolic content of the leaf extract was determined by using Folin-Ciocalteu reagent method (12). The results were expressed in percent dry weight of gallic acid equivalents (% dw GAE).

Nitric oxide radical scavenging activity

Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction (13). In brief, 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) 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 dihydrochoride 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.

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

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 (14). Different concentrations of extracts and standard (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) 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 minutes at 1000×g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. The results were expressed as increased absorbance along the concentration.

Metal chelating activity

The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis and co workers (15).

β-Carotene-linoleic acid (linoleate) Assay

The antioxidative activity of crude methanolic and aqueous extracts of leaves of *C. occidentalis* was evaluated using a β-carotene-linoleic acid model system (16).

Statistical analysis

The experimental results were expressed as means ± Standard Deviation (S.D.) of triplicate values. The results were analyzed using Microsoft Excel 2000 and the data were subjected to one way analysis of variance (ANOVA) 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. P values ≤ 0.05 were regarded as significant. Correlation analysis between different antioxidant activities was also carried out by using the same software.

Results and Discussion

Total phenolic content

It is reported that the antioxidant activity of plant origin components can be ascribed mainly to the presence of phenolic compounds (17). 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. The Folin-Ciocalteu phenol reagent assay is used widely for a crude estimation of the amount of phenolic compounds present in an extract. This method is based on the reducing power of the phenolic hydroxyl groups, which react with the Folin-Ciocalteu phenol reagent to form chromogens that can be detected spectrophotometrically at 760 nm (18). According to our results, the maximum total phenolic content was observed in the aqueous extract of the leaves of *C. occidentalis* (21.37±0.33% dw GAE) as compared to the aqueous extract of leaves of *C. tora* (11.22±0.12% dw GAE). Similarly, methanolic extract of *C. occidentalis* showed high phenolic content (16.57±0.40% dw GAE) than corresponding methanolic extract of leaves of *C. tora* (13.15±0.78% dw GAE). However, in contrast, chloroform (3.10±0.22% dw GAE), benzene (0.95±0.69% dw GAE) and petroleum ether (0.71±0.18% dw GAE) extracts of leaf of *C. occidentalis* showed less total phenolic content than the corresponding extracts of *C. tora*. The total phenolic content of leaf extracts of *C. occidentalis* and *C. tora* have previously been studied (9, 10) which is in agreement with the present study.

Nitric oxide radical scavenging activity

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (19). Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25^0C for two hours resulted in a linear time-dependent nitrite production, which is reduced by the tested leaf extracts of *C. occidentalis* and *C. tora*. The leaf extracts of *C. occidentalis* showed significant nitric oxide radical scavenging activity as shown in Table 1. The highest nitric oxide radical scavenging activity was found in the aqueous extract (64.65±0.27% at 1 mg/ml) of leaves of *C. occidentalis* as compared to the aqueous extract of *C. tora*. But, on the other hand, methanolic extract of *C. tora* showed maximum activity (40.44±0.49% at 1 mg/ml) as compared to the methanolic extract of *C. occidentalis* (34.23±0.22% at 1 mg/ml). The percentage inhibition was increased with increasing concentration of extract in a dose-dependent manner. By this study it seems that the antioxidant activity of leaf extracts of both *C. occidentalis* and *C. tora* are due to antioxidative principles (chiefly phenolics) in the extracts which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite which can be shown by a highly significant and positive correlation coefficient between the nitric oxide radical scavenging activity and total phenolic content of *C. occidentalis* and *C. tora* (Table 4). The present study showed strong correlation with previously reported studies on the leaf extracts of *C. occidentalis* and *C. tora*. In case of *C. tora*, we observed that the methanolic extract showed 40.44±0.49% inhibition at 1 mg/ml of the extract while in previously reported (in Kerala) study it showed 68% inhibition against nitric oxide radicals at 400 µg/ml concentration (9). This type of variation in these studies may be due to some seasonal effect on phytochemicals. As *C. tora* is a perennial plant in Kerala and annual plant in Haryana.

Table 1: Nitric oxide radical scavenging activity of leaf extracts of *Cassia occidentalis* and *C. tora*

	Leaf extracts										
Concentration (mg/ml)	**PE**		**BE**		**CE**		**ME**		**AE**		**CUR[b]**
	CO[a]	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**	
0.2	0.55 ± 0.10	1.1.20 ± 0.11	2.73 ± 0.12	2.56 ± 0.29	1.56 ± 0.24	3.49 ± 0.43	16.46 ± 0.28	13.62 ± 0.23	22.74 ± 0.33	7.38 ± 0.43	65.37 ± 0.54
0.4	4.19 ± 0.036	2.43 ± 0.30	5.05 ± 0.12	4.69 ± 0.43	5.58 ± 0.34	5.52 ± 0.43	21.24 ± 0.19	17.38 ± 0.45	41.46 ± 0.32	10.44 ± 0.34	76.33 ± 0.32
0.6	12.84 ± 0.15	5.91 ± 0.081	7.55 ± 0.10	6.71 ± 0.40	8.25 ± 0.19	8.56 ± 0.41	23.67 ± 0.32	23.57 ± 0.45	53.78 ± 0.52	16.62 ± 0.47	81.11 ± 0.11
0.8	29.94 ± 0.11	8.55 ± 0.44	10.38 ± 0.15	9.43 ± 0.30	9.59 ± 0.22	10.59 ± 0.44	29.25 ± 0.41	32.68 ± 0.54	61.25 ± 0.25	18.64 ± 0.37	85.78 ± 0.28
1.0	36.14 ± 0.17	10.42 ± 0.32	11.45 ± 0.31	11.81 ± 0.23	11.59 ± 0.31	13.53 ± 0.38	34.23 ± 0.22	40.44 ± 0.49	64.65 ± 0.27	23.51 ± 0.36	89.38 ± 0.06

[a]Values are expressed as mean ± S.D., ***p<0.0001 vs curcumin by Duncan's multiple range test in percent inhibition

[b]Reference compound Curcumin (mean ± S.D.)

PE Petroleum ether, *BE* Benzene extract, *CE* Chloroform extract, *ME* Methanol extract, *AE* Aqueous extract, *CUR* Curcumin, *CO Cassia occidentalis*, *CT Cassia tora*

Table 2: β-carotene-linoleic acid test of leaf extracts of *Cassia occidentalis and C. tora*

	Leaf extracts										
Concentration (mg/ml)	PE		BE		CE		ME		AE		AS[b]
	CO[a]	CT[a]	CO[a]	CT[a]	CO[a]	CT[a]	CO[a]	CT[a]	CO[a]	CT[a]	
0.2	17.35 ± 0.54	0.63 ± 0.32	6.50 ± 0.41	3.54 ± 0.11	15.44 ± 0.49	5.66 ± 0.32	20.43 ± 0.48	15.80 ± 0.32	22.36 ± 0.46	12.75 ± 0.34	43.59 ± 0.45
0.4	21.41 ± 0.36	1.58 ± 0.15	11.51 ± 0.39	4.8 ± 0.25	21.53 ± 0.39	6.58 ± 0.22	33.31 ± 0.19	23.70 ± 0.35	26.36 ± 0.36	16.75 ± 0.34	57.39 ± 0.39
0.6	26.46 ± 0.26	3.55 ± 0.41	14.61 ± 0.33	5.85 ± 0.16	28.37 ± 0.24	8.59 ± 0.38	37.45 ± 0.40	33.02 ± 0.05	28.41 ± 0.43	21.85 ± 0.17	62.44 ± 0.26
0.8	29.52 ± 0.13	5.58 ± 0.38	20.80 ± 0.23	8.82 ± 0.16	35.51 ± 0.44	11.65 ± 0.29	44.29 ± 0.35	43.36 ± 0.38	37.18 ± 0.21	27.72 ± 0.46	68.27 ± 0.16
1.0	34.23 ± 0.24	6.65 ± 0.08	23.32 ± 0.22	11.41 ± 0.23	38.44 ± 0.49	16.84 ± 0.35	50.42 ± 0.35	51.59 ± 0.36	50.81 ± 0.22	30.93 ± 0.10	73.15 ± 0.10

[a]Values are expressed as mean ± S.D., ***p<0.0001 vs ascorbic acid by Duncan's multiple range test in percent inhibition

[b]Reference compound Ascorbic acid (mean ± S.D.)

PE Petroleum ether, *BE* Benzene extract, *CE* Chloroform extract, *ME* Methanol extract, *AE* Aqueous extract, *AS* Ascorbic acid, *CO Cassia occidentalis*, *CT Cassia tora*

Table 3: Metal chelating activity of leaf extracts of *Cassia occidentalis* and *C. tora*

	Leaf extracts									
Concentration (mg/ml)	**PE**		**BE**		**CE**		**ME**		**AE**	
	CO[a]	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**	**CO[a]**	**CT[a]**
0.2	4.32 ± 0.37	1.67 ± 0.47	3.29 ± 0.67	4.59 ± 0.44	7.21 ± 0.71	4.92 ± 0.06	55.55 ± 0.18	17.81 ± 0.22	44.44 ± 0.13	13.60 ± 0.38
0.4	9.18 ± 0.12	3.63 ± 0.54	6.18 ± 0.48	6.55 ± 0.50	16.39 ± 0.18	6.81 ± 0.31	56.11 ± 0.71	21.96 ± 0.06	62.23 ± 0.58	18.60 ± 0.35
0.6	14.4 ± 0.18	4.74 ± 0.27	7.14 ± 0.42	7.58 ± 0.51	26.50 ± 0.78	9.33 ± 0.79	65.10 ± 0.11	31.92 ± 0.97	68.34 ± 0.87	19.63 ± 0.33
0.8	22.3 ± 0.98	5.96 ± 0.95	15.69 ± 0.37	9.37 ± 0.34	35.1 ± 0.61	10.19 ± 0.73	66.66 ± 0.78	33.63 ± 0.45	69.83 ± 0.98	21.53 ± 0.48
1.0	25.4 ± 0.87	7.63 ± 0.54	21.39 ± 0.19	10.28± 0.60	46.84 ± 0.57	11.56 ± 0.50	77.11 ± 0.90	43.93 ± 0.12	79.44 ± 0.18	26.08 ± 0.65

[a]Values are expressed as mean ± S.D., ***$p<0.0001$ in percent inhibition

PE Petroleum ether, *BE* Benzene extract, *CE* Chloroform extract, *ME* Methanol extract, *AE* Aqueous extract, *AS* Ascorbic acid, *CO Cassia occidentalis*, *CT Cassia tora*

Table 4: Correlation analysis of different antioxidant activities of leaves of *C. occidentalis* and *C. tora* (at 1 mg/ml).

$r(R^2)^a$	Nitric Oxide Scavenging	Beta Carotene Linoloeic acid assay	Metal Chelating Activity
Nitric Oxide Scavenging	-	0.8884 (0.7892)*	0.6508 (0.4235)
Beta Carotene Linoloeic acid assay	0.9906 (0.9812)***	-	0.9598 (0.9211)**
Metal Chelating Activity	0.9965 (0.9930)***	0.9932 (0.9865)***	-

[a]The upper diagnol values for *C. occidentales* and lower for *C. tora*.

r = Correlation coefficient, R^2 = Coefficient of determination. The values in parentheses represent R^2 values.

***Significance level at p<0.0001 and **at p value < 0.05.

β-Carotene-linoleic acid (linoleate) Assay

In β-carotene-linoleic acid model, β-carotene undergoes rapid discolouration 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 (20). 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 (6). 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 comparative assessment of β-carotene-linoleic acid test of leaf extracts of *C. occidentalis* and *C. tora* was shown in Table 2. The antioxidant activity of aqueous extract (50.81±0.22% at 1 mg/ml) of leaf of *C. occidentalis* was found to be maximum followed by the methanol, chloroform, petroleum ether and benzene extract respectively. However, in case of *C. tora*, methanolic extract exhibited maximum inhibition (51.59±0.36% at 1 mg/ml) as compared to other tested extracts. The β-carotene-linoleic acid activity of leaf extracts of *C. occidentalis* and *C. tora* have been firstly reported in the current study.

Reducing power assay

Figure 1 depicts the comparative assessment of reducing powers of different extracts of leaf of *C. occidentalis* and *C. tora* at 1 mg/ml where aqueous extract showed maximum reducing potential. Among all the leaf extracts of *C. tora*, methanol showed maximum reduction potential than other tested extracts. 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 Perl's Prussian blue at 700 nm (21). In other words, the $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the semiquantitative determination of dilute concentrations of polyphenolics, which

participate in the redox reaction. Polyphenolics in the aqueous extracts appear to function as good electron and hydrogen donors and therefore should be able to terminate radical chain reaction by converting free radicals to more stable products. The results suggest that as electron donors, each extract could convert free radicals into more stable products, leading to termination of radical chain reactions.

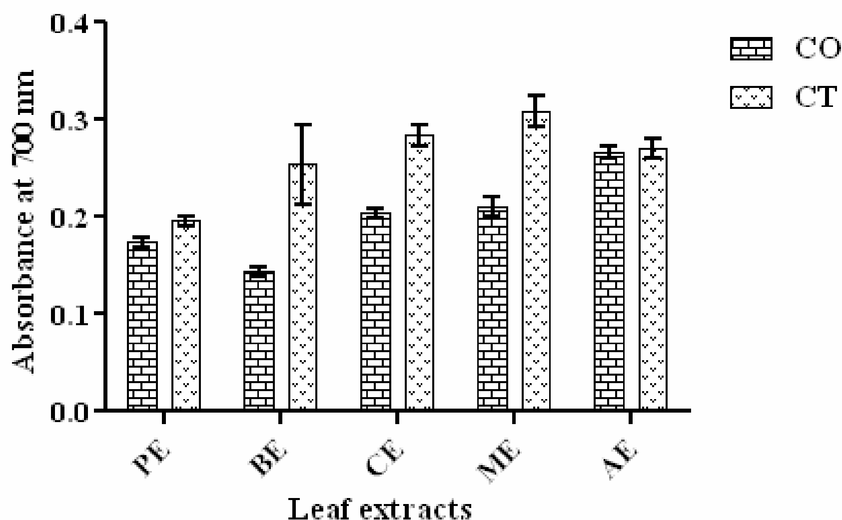

Figure 1: Reducing power of leaf extracts of *C. occidentalis* and *C. tora at* 1 mg/ml concentration.

[a]Values are expressed as mean ± S.D., ***p<0.0001

PE Petroleum ether, *BE* Benzene extract, *CE* Chloroform extract, *ME* Methanol extract, *AE* Aqueous extract, *CO Cassia occidentalis*, *CT Cassia tora*

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 was decreased. As shown in Table 3, the formation of Ferrozine-Fe^{2+} complex is not completed in the presence of plant extract, indicated their ability to chelate the iron. The absorbance of Ferrozine-Fe^{2+} complex decreased linearly in a dose-dependent manner (0.2 mg/ml to 1 mg/ml). 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 (22). The metal chelating activity of the leaf extracts of *C. occidentalis* and *C. tora* were shown in Table 3. The aqueous extract of *C. occidentalis* showed maximum metal chelating activity (79.44±0.18% at 1 mg/ml) as compared to the aqueous extract of leaves (26.08±0.65% at 1 mg/ml) of *C. tora*.

Correlation between different Antioxidant activity tests of leaf extracts of *C. occidentalis* and *C. tora*

To find the relationship between different antioxidant activities of *C. occidentalis* and *C. tora*, we performed a correlation analysis of the values of different antioxidant capacities at 1 mg/ml concentration. The correlation coefficient (r) and coefficients of determination (R^2) were determined. Most of the R^2 values were positive and significant at the $p<0.05$ significance level, suggesting that there were significant and positive correlations between different antioxidant activities. For example, In case of leaves extract of *C. occidentalis*, a highly significant and linear correlation was observed between nitric oxide scavenging activity and hydroxyl radical scavenging activity (r=0.9368). Similarly, a very significant and positive correlation was observed between hydroxyl radical scavenging activity and metal chelating activity (r=0.9133) suggesting the fact that antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation has been correlated with iron chelating properties (23). In case of *C. tora*, a highly significant and positive correlation between hydroxyl radical scavenging activity and metal chelating activity (r = 0.9980) was observed. From various antioxidant activity tests of leaf extracts of *C. occidentalis* and *C. tora*, aqueous extract of leaf of *C. occidentalis* and methanolic extract of leaf of *C. tora* was found to be most active against free radicals as compared to other tested extracts. We have performed a comparison of the most active extracts with different antioxidant activities tested to find out which types of free radicals inhibited most by these extracts. The aqueous extract of leaves of *C. occidentalis* showed maximum effect on metal chelating activity followed by the nitric oxide scavenging activity and β-carotene-linoleic acid test respectively. In case of methanolic extract of *C. tora*, following trend was monitored β-carotene-linoleic acid test> metal chelating activity> nitric oxide radical scavenging activity.

Conclusion

These observations were clearly evidence that the extracts of seeds of *C. occidentalis* are rich in phenolics and may be responsible for the observed antioxidant capacities of different extracts. The result obtained from this study has provided scientific credence to the ethno-therapeutic usage of this plant traditionally. Further detailed studies to characterize the active principles and to elucidate the exact mechanism of action of these extracts are the subject of ongoing investigation in our group.

Acknowledgement

Financial assistance from the Department of Science and Technology, India for the award of INSPIRE-DST JRF to Vedpriya is gratefully acknowledged. Financial support in the form of equipment grant received from the Haryana State Government is also acknowledged.

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