ANTIOXIDANT ACTIVITY AND TOTAL PHENOLICS IN LEAVES EXTRACTS OF *CASSIA TORA* L.

Vedpriya Arya, J. P. Yadav*

Department of Genetics,
Maharishi Dayanand University, Rohtak – 124001, Haryana, India

Author For correspondence:
Dr. J. P. Yadav
Professor and Head,
Department of Genetics, M. D. University,
Rohtak – 124001, Haryana, India
Tel.: 01262-393055
E-mail: yadav1964@ rediffmail.com

Summary

The leaves of *Cassia tora* (local name – puvad) are usually consumed as food ingredient in traditional recepies by local people of Haryana. In the present study, the antioxidant potency of sequential organic (petroleum ether, benzene, chloroform, methanol) and aqueous leaf extracts of *Cassia tora* plant was investigated by using 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 super oxide radical scavenging activity. The methanolic extract of the leaves of *C. tora* was found to be more effective against free radicals followed by aqueous and other organic extracts respectively. A preliminary study for the qualitative and quantitative estimation of phenolics was performed and the results were correlated with different antioxidant tests. A positive and significant correlation was observed (R^2 = 0.4626 to 0.9961) between various test systems and total phenolics showed that the phenolic compounds were responsible for the antioxidant activity of the extracts. The data obtained from the in vitro models clearly establish the antioxidant potency of leaf extracts of *C. tora*.

Keywords: Antioxidant activity, *Cassia tora,* Free radicals, Phenolics.

Introduction

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 (1). 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 (2). The antioxidative effects are mainly due to phenolic components, such as flavonoids (3), phenolic acids, and phenolic diterpenes (4). 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 (5).

Cassia tora L. (family: Caesalpiniaceae) is called as Chakramard in Sanskrit, Puvad in Hindi and Foetid senna in English. *Cassia tora* is a common edible leafy vegetable consumed by Indians. It is a well known ayurvedic medicinal plant used for skin diseases, fever, malaria, stomach disorders, pain and cardiac disorders (6, 7). The leaves of *Cassia tora* are used as food ingredient in traditional recepies by the local people in India. But so far little information was available concerning its detailed antioxidant features. The methanolic extract of leaves of this plant was found to possess antioxidant and antiproliferative activity (7). However, no research has been reported about the antioxidant activity of different organic and aqueous leaf extracts of *C. tora*. The objective of this study was to investigate the in vitro antioxidant activity and total phenolic contents of leaves of *C. tora*.

Methods

Preparation of plant extracts

The leaves of *C. tora* 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 2505) available in the Department of Genetics, M. D. University, Rohtak. The powdered leaf material (200 gm) was sequentially extracted with different solvents according to their increasing polarity: petroleum ether < benzene < chloroform < methanol < 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 concentrated under vacuum at 40^0C by using a rotary evaporator and lyophilized to powdered form at -55^0C under vacuum conditions.

Preliminary phytochemicals and Total phenolic content (TPC)

Preliminary analysis of saponins, steroids, flavanoids and phenolic compounds were carried out by using the different methods (8). The total phenolic content of the leaf extracts was determined by using method of McDonald et al. (9), with some modifications. The total phenolic content of the leaf extracts was calculated in Gallic acid equivalents (% of GAE).

Nitric oxide radical scavenging activity (NOS)

Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction (10).

Hydroxyl radical scavenging activity (HRS)

The hydroxyl radical scavenging activity was measured by the deoxyribose method (11-13).

Reducing power assay
The reductive potential of the extract was determined according to the method of Oyaizu (14) by the reduction of $FeCl_3$ in presence of extracts.

Metal chelating activity (MCA)
The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis et al. (15).

β-Carotene-linoleic acid (linoleate) Assay (BCL)
The antioxidative activity of different extracts of leaves of *C. tora* evaluated using a β-carotene-linoleic acid model system (16).

Super oxide anion scavenging activity (SRS)
Measurement of super oxide anion scavenging activity of extracts was based on the method described by Liu et al. (17) with slight modification of Oktay et al. (18).

Statistical analysis
The experimental results were expressed as means ± Standard Deviation (S.D.) of triplicate measurements (in percent inhibition). The results were processed using Microsoft Excel 2000 and the data were subjected to one way analysis of variance (ANOVA) and the significance of differences between samples means vs. standard were calculated by Graph pad prism 5.0 (San Diego, USA) software using Dunnett's multiple comparison test. *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. The data was subjected to linear regression analysis to calculate IC50 of the extract.

Results and Discussion

Total Phenolic Content (TPC)
Phenolic compounds react with Folin-Ciocalteu reagent (FC) only under basic conditions. Dissociation of a phenolic proton in basic medium leads to a phenolate anion, which is capable of reducing FCR in which the molybedate in the testing system is reduced forming blue coloured molybdenum oxide with maximum absorption near 700 nm. The phenolic compounds can donate hydrogen to radical and break the reaction of lipid oxidation at the initiation step (19). Investigation of the different organic (petroleum ether, benzene, chloroform, methanol) and aqueous extracts of leaf of *C. tora* revealed the presence of phenolics, tannins, steroids, flavonoids and saponins. The highest total phenolic content was found in the methanolic extract (13.15±0.78% dw GAE) followed by the aqueous (11.22±0.12% dw GAE), chloroform (9.66±0.57% dw GAE), petroleum ether (6.18±0.13% dw GAE) and benzene (6.17±0.31% dw GAE) extracts respectively. This is in accordance with the previously reported data (7). These results show that contents of total phenolics vary due to complex nature of these groups of compounds and the methods of extraction and analysis.

Nitric Oxide Radical Scavenging Activity (NOS)
Incubation of solution of sodium nitroprusside in phosphate buffer saline at 25^0C for two hours resulted in a linear time-dependent nitrite production (20), which is reduced by the tested leaf extracts of *C. tora*. 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.

Our experiments showed strong correlation with previously reported studies on the leaf extracts of *C. tora* (7). In case of *C. tora*, we observed that the methanolic extract showed 40.44±0.49% inhibition at 1 mg/ml of the extract (IC50 1.24 mg/ml) while in previously studied report from Kerala, India showed 68% inhibition (IC50 180 µg/ml) against nitric oxide radicals at 400 µg/ml concentrations (7). This type of variation in these studies may be due to some spatial and seasonal effect on phytochemicals. Moreover, the climatic conditions in these two distantly located states (Kerala and Haryana) of India varied drastically in terms of temperature, humidity, rainfall and soil.

β-carotene linoleic acid model System (BCL)

In β-carotene linoleic acid model, β-carotene undergoes rapid discoloration in the absence of an antioxidant. 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 (21) In this work, potent inhibitory activity of different extracts was observed with highest activity of methanolic extract (IC50 0.96 mg/ml) as shown in Table 1.

Reducing power assay

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 the formation of Perl's Prussian blue at 700 nm (22). At 1 mg/ml concentration, reducing power of the methanolic extract (0.30±0.01) is far superior to that of any other tested extract (Figure 1).

Metal Chelating Activity (MCA)

Ferrozine can quantitatively form complexes with Fe^{2+}. 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 (23). A significant and positive correlation was observed between the total phenolic content and metal chelating activity of the leaf extracts of *C. tora* at 1 mg/ml concentration as shown in Table 2. The methanolic extract showed maximum MCA as compared to other tested extracts with IC50 1.23 mg/ml.

Hydroxyl Radical Scavenging Activity (HRS)

The most reactive of the ROS that attacks almost every molecule in the body is the hydroxyl radical. It initiates the peroxidation of cell membrane lipids yielding malondialdehyde, which is mutagenic and carcinogenic. Generally molecules that inhibit deoxy-ribose degradation are those that can chelate iron ions and thereby prevent them from complexing with deoxyribose and render them inactive in a Fenton reaction (24). In the present work, methanolic extract of leaves of *C. tora* was found to inhibit maximally (IC50 0.69 mg/ml) as compared to other tested extracts of leaf of *C. tora*.

Super oxide Radical Scavenging Activity (SRS)

The formation of super oxide radical leads to a cascade formation of other ROS in the cell. Endogenously, super oxide could be produced in large amounts by various metabolic and physiological processes (25, 26). In the PMS/NADH-NBT systems, super oxide 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 super oxide anions in the reaction mixture.

In the presence study, ascorbic acid showed a pro-oxidant effect but BHT (Butylated hydroxyl toluene) showed remarkable antioxidant activity. The methanolic leaf extract of *C. tora* showed maximum inhibitory potential (IC50 0.69 mg/ml) as compared to other tested extracts in a dose dependent manner.

Correlation between antioxidant activity and TPC

To find the relationship between the antioxidant activity and phenolic contents, we have performed correlation analysis of the values of different antioxidant capacities with the total phenolic content at 1 mg/ml concentration. The correlation coefficient (r) and coefficient of determination (R^2) were listed in Table 2. Most of the r values are positive and significant at the $p<0.05$ significance level, suggesting that there were significant and positive correlation between antioxidant activities and TPC. For example, there was a highly significant and linear correlation between HRS and MCA (r = 0.9980) suggesting the fact that antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation has been correlated with iron chelating properties.

Table 1: Antioxidant activity of leaf extracts of *C. tora* in different solvents in comparison with standard antioxidants

Leaf extracts	Antioxidant activity (IC50[a] in mg/ml)				
	NOS	HRS	BCL	MCA	SRS
Petroleum ether	3.94	3.35	6.38	6.95	1.94
Benzene	4.17	3.01	4.96	6.56	4.26
Chloroform	3.86	6.10	3.52	5.58	2.54
Methanol	1.24	0.69	0.96	1.23	0.69
Aqueous	2.28	0.98	1.78	2.75	2.07
Standard[b]	0.26	0.23	0.29	-	0.30

[a]Extracts concentrations, which shows 50% antioxidant activity, were determined by interpolation through linear regression with 95% of confidence level.

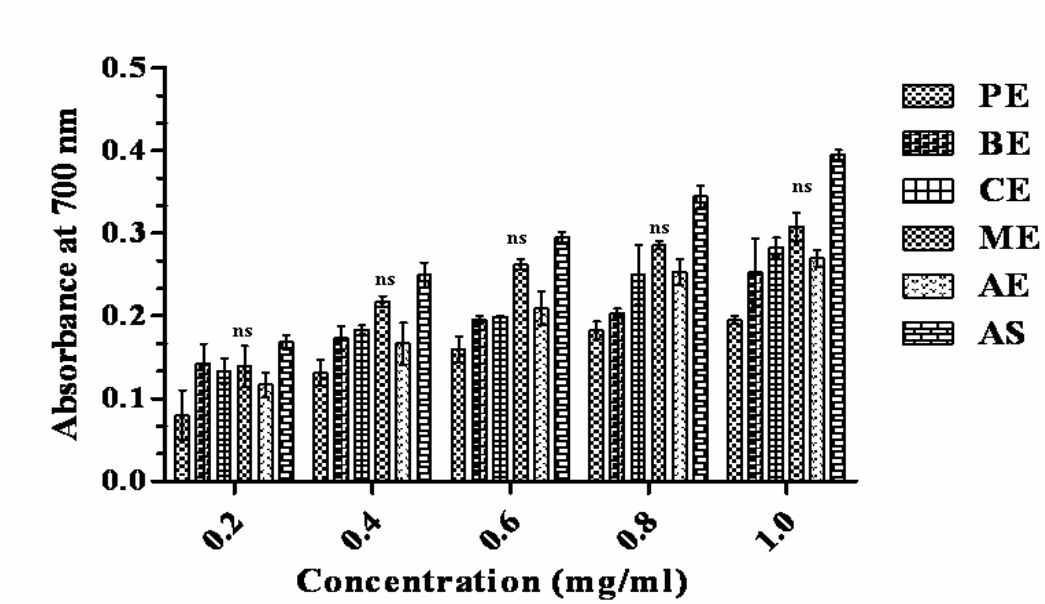
[b]Standard is Ascorbic acid in case of HRS and BCL, Curcumin in case of NOS and BHT in case of SRS

Table 2: Correlation analysis between different antioxidant tests and total phenolic content of leaf extracts of *C. tora* at 1 mg/ml concentration

$r(R^2)$[a]	NOS	HRS	BCL	SRS	MCA	TPC
NOS	-	0.9451	0.9906	0.9189	0.9965	0.8849
HRS	0.8932	-	0.9266	0.7657	0.9588	0.7763
BCL	0.9812	0.8585	-	0.8948	0.9932	0.9292
SRS	0.8443	0.5863	0.8007	-	0.8905	0.8275
MCA	0.9930	0.9194	0.9865	0.7931	-	0.8919
TPC	0.7831	0.6027	0.8635	0.6847	0.7955	-

[a]Values in upper diagonal represents correlation coefficient (r) and lower diagonal represents R^2 (coefficient of determination) with ***significance at $p<0.0001$

Figure 1: Reducing power assay of leaf extracts of *C. tora*



Values are mean ± SD of triplicates and *** significant at $P<0.001$ compared to Ascorbic acid standard and ns is not significant values by Dunnettt's multiple comparison test. PE petroleum ether, BE Benzene, CE chloroform, ME methanol, AE water and AS Ascorbic acid

Conclusion

These observations were clearly evidence that the extracts of the leaf of *C. tora* are rich in phenolics and may be responsible for the observed antioxidant capacities of 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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