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Identification of Flavonoids and Antioxidant Potential of *Cassia tora* L.

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ABSTRACT

Callus culture *Cassia tora* L. was done using nodal explant. Maximum callus induction was obtained on basal MS medium supplemented with 2, 4-D (2.5 mg L⁻¹), NAA (2.5 mg L⁻¹) and Kinetin (0.02 mg L⁻¹). Highest growth index was obtained at 6th week of subculture in both the plants. Analysis of plant parts revealed maximum content of flavonoids in leaves and minimum in stem. Callus culture also showed significant content of flavonoids. Plant parts and *in vitro* cultures were also studied for their antioxidant potential.

Key words: Callus culture, *Cassia tora*, flavonoids, antioxidant

INTRODUCTION

Medicinal plants possess myriads of metabolites having therapeutic potential (Sharma *et al.*, 2009; Vats *et al.*, 2012). This has lured scientists all over the world to explore their bioactivities (Bhatia *et al.*, 2008; Vats and Alam, 2013). *Cassia tora* L. (commonly called as chakod), is a member of the family Fabaceae. *C. tora* is an indigenous of paleotropical regions, from India and Ceylon eastward into Polynesia (Smith, 1985). It occurs abundantly in open pastures and pastures under coconuts and is very common on roadsides, riversides and wasteland of hot warm and low altitude regions (Swarbrick, 1997). In Ayurvedic system the leaves and seeds have been reported to be laxative, anthelmintic, liver tonic and expectorant (Ambasta, 1998). The seeds of *C. tora* L. have been used in Chinese traditional medicine for several years. It is used to enhance eye sight and to treat hypercholesterolemia and hypertension. The roasted seeds have been popularly used as a health drink tea (Yen *et al.*, 1998).

C. tora has varied bioactivities viz., hypolipidemic (Cho *et al.*, 2007), larvicidal (Jang *et al.*, 2002); antioxidant (Yen and Chuang, 2000); antiplasmodial (El-Tahir *et al.*, 1999); antihepatotoxic (Wong *et al.*, 1968). Yen and Chen (1995) evaluated the relationship between antioxidant activity and antimutagenicity. *C. tora* is rich in anthraquinones, emodin, rhein, sitosterol, stigmasterol and chrysophanol (Shibata *et al.*, 1969; Huang, 1993).

Flavonoids are polyphenolic compound almost universally present in plants. They have varied bioactivities like Antiatherosclerotic, anti-allergic, anti-inflammatory, Antithrombogenic etc. (Nijveldt *et al.*, 2001).

There is constant production of free radicals in our body due to metabolic processes, which is counter acted by enzymatic and non-enzymatic antioxidants. Excessive productions of free radicals cause various diseases such as atherosclerosis, cancer and Parkinson's disease (Setiadi *et al.*, 2003).

A large number of compounds from different plant sources have been shown to possess antioxidant properties. Some antioxidants of plant origin are vitamin E and C, selenium, flavonoids etc.

Excessive exploitation of plants for its activities has led to marked depletion of their biodiversity. Plant tissue culture technique has proved to be an alternative for the production of plant secondary metabolites. Various components of the tissue culture system can be manipulated for *in vitro* regulation of secondary metabolites.

The present work was undertaken to identify and quantify flavonoids *in vivo* and *in vitro* and to evaluate their antioxidant potential, which has been reported for the first time to the authors' best knowledge.

MATERIALS AND METHODS

Plant tissue culture: Nodal explants (2 cm) were washed in running tap water for 15 min and then treated with liquid soap containing 1% (v/v) Teepol for 4 min with intermittent agitation. The explants were then treated with HgCl₂ (0.1%)+Ciprofloxacin (0.25%) for 5 and 2 min, respectively. Sterilants were washed off thrice using sterile distilled water in a laminar airflow cabinet. Nodal segments were inoculated in the flasks containing MS medium supplemented with various concentrations of 2, 4-D, NAA, IBA, IAA and Kinetin (KN). Cultured flasks were incubated in the growth chamber maintained at 26±1°C. The light intensity (1200 lux) was provided from fluorescent tubes (40 W) and incandescent bulbs (40 W). A photoperiod of 16 h light was used. The cultures were observed and examined every week and final data were recorded.

PHYTOCHEMICAL ANALYSIS

Extraction: Different plant parts were harvested in the month of June, 2010 and kept at 100°C for 10 min to inactivate the enzymes and then dried at 60°C till constant weight was achieved. Powdered plant parts and *in vitro* grown callus was Soxhlet extracted for 24 h with 80% methanol (v/v). The methanol soluble fractions were filtered, concentrated *in vacuo* and the aqueous fraction was sequentially extracted with petroleum ether (60-80°C; Fr. I), diethyl ether (Fr. II-Free) and ethyl acetate (Fr. III-Bound) thrice. The residues were dissolved in small volume of EtOH for chromatographic analysis.

Identification: The extracts (Fr. II and Fr. III) were examined for flavonoids using silica gel G (E Merck) coated plates (20×20 cm; wet thickness 0.2-0.3 mm) using various solvent systems. The isolated flavonoids were identified by mp (performed in capillaries on Toshniwal Melting Point Apparatus), IR (Infra-red spectrophotometer: Perkin Elmer 337 Grating Infra-red spectrophotometer). RP-HPLC was done on a Nova-Pak C18 (Waters Associates, Milford, MA) column (3.9×150 mm, 4 µm) using methanol/water (50:50 v/v, pH 2.5 with trifluoroacetic acid) as mobile phase and UV detection (365 nm) at the flowrate of 1 mL min⁻¹ (Miean and Mohammed, 2001). Chromatograms were compared with the chromatograms of standards to get the results and expressed as Mean±S.E of 3 replicates.

DPPH (2, 2-DIPHENYL-1-PICRYLHYDRAZYL) ASSAY

Different plant parts and calli (1 g) were refluxed in methanol under water bath for 24 h and filtered. The filtrate was evaporated on a rotary evaporator and the different extracts were measured for radical scavenging activity using DPPH. Different extracts (1 mL) were mixed with 0.3 mM DPPH reagent (1 mL) and allowed to stand at room temperature for 30 min in dark. The absorbance was taken at 517 nm. Radical scavenging activity was calculated and expressed as IC₅₀ (Vats, 2012).

STANDARDS AND CHEMICALS

DPPH, ascorbic acid and standard flavonoids were purchased from Sigma Chemical Co. (St. Louis, USA), all other chemicals and reagents used were of analytical grade.

RESULTS AND DISCUSSION

The nodal explants responded differentially to various plant hormones supplemented in MS medium. Culture flask containing basal MS medium was not able to induce callus in the explants. Different concentrations of auxins tried alone and in combination with KN showed different degree of callusing. Initiation of callus started from the cut ends after six to eight weeks of incubation of culture. Lower concentrations of 2,4-D was not able to induce callus. On the other hand maximum callusing was observed in MS medium supplemented with 2,4-D (2.5 mg L⁻¹) + NAA (mg L⁻¹) and KN (mg L⁻¹) with percentage callus induction as 82±1.16. This was followed by 2,4-D (5 mg L⁻¹) and KN 0.02 (mg L⁻¹). Callusing on MS medium supplemented with only 2,4-D (5 mg L⁻¹) induced comparatively less callusing (Table 1). This suggests that kinetin induce in rapid proliferation of cells (Vats *et al.*, 2012). The callus initially was light brown which later turned to brown compact mass.

2,4-D helps in dedifferentiating the plant cells. Various workers have reported the significance of 2,4-D in callusing (Zafar *et al.*, 1995; Gharyal *et al.*, 1983). Harvey and Grasham (1969) while working on conifers reported the effectiveness of IAA, NAA and 2, 4-D for callus induction (Harvey and Grasham, 1969). Agrawal and Sardar (2006) observed induction of dark brown compact callus at the cut ends of the explants of *C. angustifolia* on MS medium augmented with 1 µM N⁻benzyladenine+1 µM 2, 4-D. Other auxins have also been reported to induce callus formation in different plants (Migas *et al.*, 2006; Maheshwari *et al.*, 2007).

The callus culture exhibited a gradual increase in Growth Indices (GI) up to 6 weeks and then it decreased showing a sigmoid pattern of growth index. The GI of *C. tora* calculated on fresh weight basis at different harvest times of the tissue showed maximum GI at 6 weeks (0.98) and minimum at 2 weeks (0.16) after fresh sub-culturing (Fig. 1).

In the present study kaempferol, quercetin and luteolin were identified and quantified both *in vivo* and *in vitro*. The best result was obtained using solvent system Benzene: Acetic acid: Water (data not shown). The melting point (mp) of the flavonoids was kaempferol (271-273°C); quercetin (309-311°C) and luteolin (328°C). Analysis of the IR spectra of the isolated compounds was compared with literature data (Harborne *et al.*, 1986) and co-comparison with authentic compounds run simultaneously for their characterization (Fig. 2-4).

Maximum content of flavonoids were found in leaves (1.88 mg g dw⁻¹). Minimum content was observed in stem (1.0 mg g dw⁻¹). The total flavonoid content (free+bound) in the callus tissue of

Table 1: Effect of different concentrations (mg L⁻¹) of phytohormones on callus response of *C. tora* nodal explant

2, 4-D	IAA	IBA	NAA	KN	Callus induction (%) using nodal explant
1.0	-	-	-	-	-
4.0	-	-	-	-	14.1±1.76
5.0	-	-	-	-	60.7±1.22
5.0	-	-	-	0.02	70.0±1.67
2.5	2.5	-	-	0.02	54.5±0.78
2.5	-	2.5	-	0.02	62.5±1.67
2.5	-	-	2.5	0.02	79.2±1.49

Values are Mean±SE, n = 10

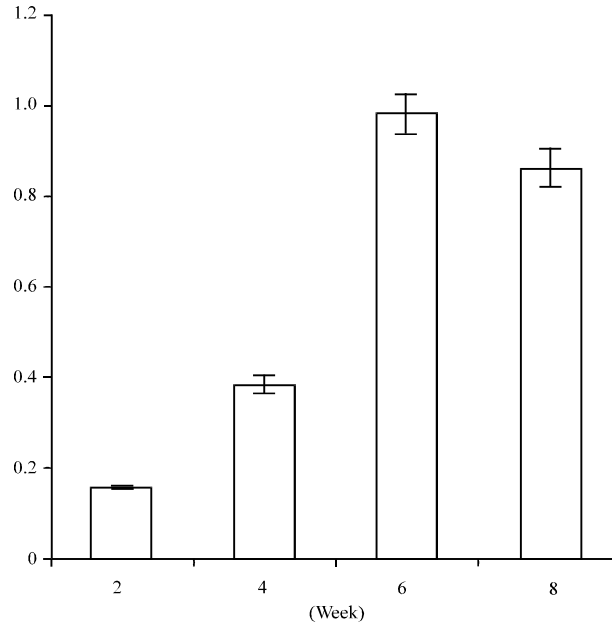


Fig. 1: Growth index (GI) of *C. tora* grown on modified MS medium, values are Mean±SEM, n = 10 (Repeated 3 times)

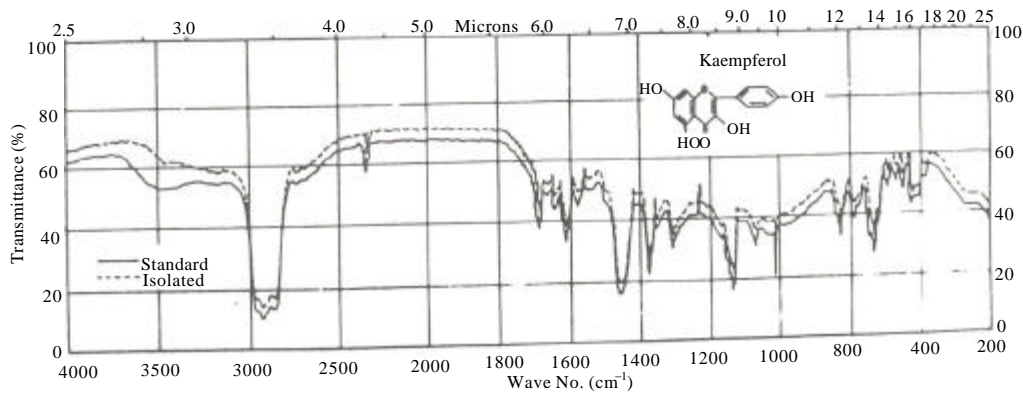


Fig. 2: IR spectra of isolated and standard kaempferol

C. tora it was $1.22 \text{ mg g dw}^{-1}$ (Table 2). Individually, in *C. tora* maximum content of all the isolated flavonoids, except kaempferol, was observed in leaves. Similar observations were made by Kamal and Yadav (1991) who reported increased flavonoid synthesis and recovery in tissue cultures of *Trigonella polycerata* as compared to the aerial parts. Overall the flavonoids in their free form were more than their bound form, except in the case of leaves of *C. tora*.

Presence of occidentalins has been identified by Hatano *et al.* (1999) in *C. occidentalis*. Iso-rhamnetin and kaempferol have been reported from *C. angustifolia*. Flavonols such as quercetin, kaempferol and the corresponding flavones, apigenin and luteolin have been well established as potent antioxidants that prevent oxidant of low-density lipoprotein and inhibit lipid peroxidation (Hertog and Hollman, 1996).

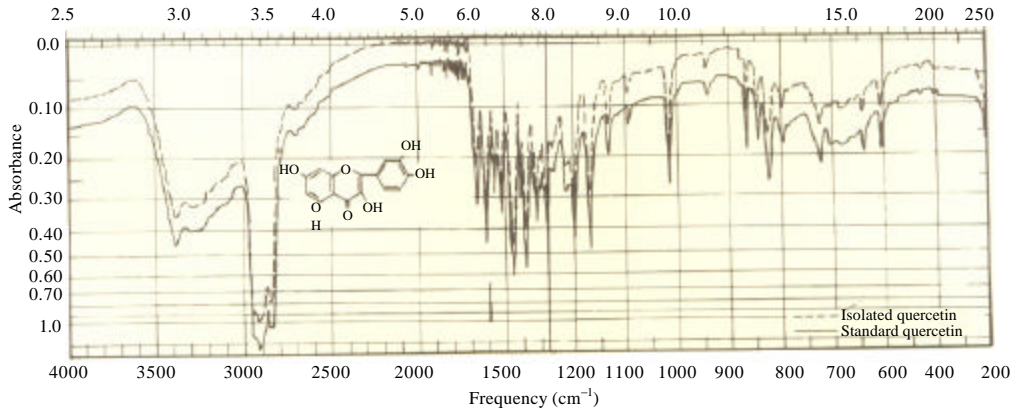


Fig. 3: IR spectra of isolated and standard quercetin

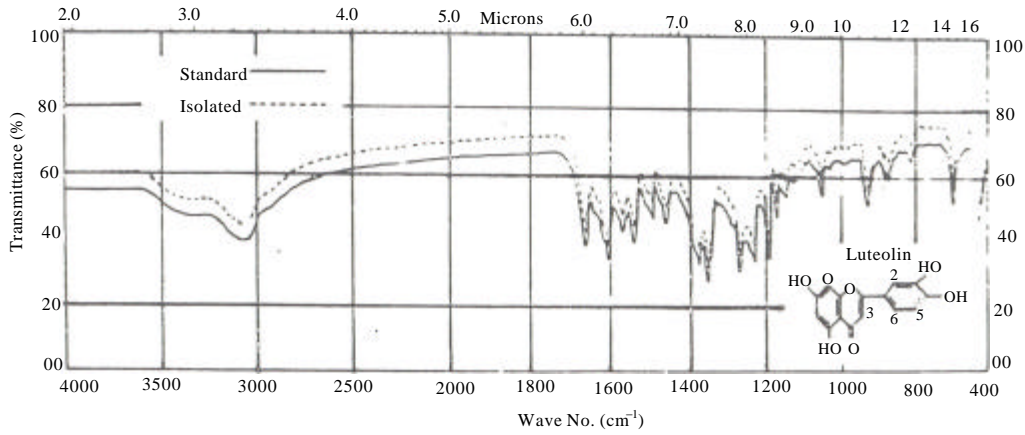


Fig. 4: IR spectra of isolated and standard luteolin

Table 2: Flavonoid content in various plant parts and callus tissue of *C. tora*

Plant parts	Flavonoids content											
	Isolated compounds (free)				Isolated compounds (bound)				Total isolated compounds			
	K	Q	L	Total	K	Q	L	Total	K	Q	L	Total
Stem	0.14 (±0.02)	0.19 (±0.07)	0.22 (±0.07)	0.55	0.12 (±0.01)	0.15 (±0.02)	0.18 (±0.01)	0.45	0.26	0.34	0.40	1.00
Leaves	0.17 (±0.08)	0.24 (±0.09)	0.26 (±0.09)	0.67	0.29 (±0.03)	0.48 (±0.09)	0.44 (±0.02)	1.21	0.46	0.72	0.70	1.88
Roots	0.22 (±0.07)	0.16 (±0.01)	0.21 (±0.02)	0.59	0.18 (±0.06)	0.14 (±0.05)	0.19 (±0.08)	0.51	0.40	0.30	0.40	1.10
Seeds	0.27 (±0.05)	0.36 (±0.01)	0.12 (±0.08)	0.75	0.21 (±0.09)	0.24 (±0.01)	0.09 (±0.07)	0.54	0.48	0.60	0.21	1.29
Callus	0.15 (±0.06)	0.28 (±0.05)	0.24 (±0.01)	0.67	0.12 (±0.04)	0.22 (±0.04)	0.21 (±0.05)	0.55	0.27	0.50	0.45	1.22

Values are Mean±SEM, n = 3

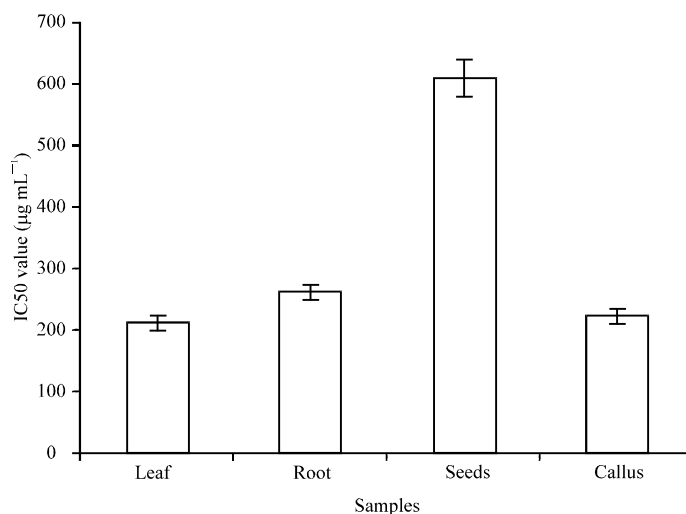


Fig. 5: DPPH scavenging activity of plant parts and callus of *C. tora* (Values are mean of 5 replicates)

In order to assess the antioxidant potential of the flavonoid extract DPPH assay was used. DPPH has an intense violet colour with a maximum absorbance at 517 nm which decreases to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule. Scavenging activities of DPPH were expressed as 50% Inhibitory Concentration (IC₅₀) value. The lowest IC₅₀ value was that of leaves of *C. tora* (210 µg mL⁻¹) followed by *in vitro* grown callus (220 µg mL⁻¹), which can be attributed to the higher flavonoid content (Fig. 5). Pigments and phenolic compounds contribute to the antioxidant potential of plants. Seeds of the experimental plant did not show significant antioxidant activity as compared to the other plant parts. The IC₅₀ value of the standard ascorbic acid was 10 µg mL⁻¹.

The present study reveals *C. tora* as a new source of flavonoids and antioxidants. The tissue culture system can be used for mass production of flavonoids as compared to the *in vivo* plant by using appropriate combinations of phyto-hormones.

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