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Antioxidant activity assay of stem and *in vitro* callusing of *Tinospora cordifolia* (Miers.)

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Abstract

The experiment was conducted to study the antioxidant potential among different hosts of *Tinospora cordifolia.* The results indicated that the among the different hosts of *T. cordifolia, Ficus religiosa* had highest phenol content (5.25 mg g^{-1}), inhibition activity (52.05%) and reducing capability as compared to others. *F. religiosa* showed the highest antioxidant activity of 41.73 µM Fe (II) g^{-1}. EC50 value for lipid peroxidation inhibitory activity was 0.1mg mL^{-1} for all the samples. EC50 value for DPPH radical scavenging activity was 0.6 mg mL^{-1} for *F. religiosa*. This results suggested that the active antioxidant compounds were higher in *F. religiosa* as compared to *Roystonea regia*. There was a direct correlation between the total polyphenols extracted and anti-oxidant activity. Among the various explants of *F. religiosa*, leaf explants were found to be superior for *in vitro* callus induction.

Keywords: antioxidant, *Ficus religiosa*, superoxide radical, *Tinospora*

Introduction

In recent years, there has been an upsurge in prevention of disease by free radicals and antioxidants. The role of medicinal plants in disease prevention and control has been attributed to the antioxidant properties of their constituents (Ivanova *et al.* 2005). The protective effect of plant products is due to the presence of several components such as enzymes, proteins, vitamins (Halliwell 1996), carotenoids (Edge *et al.* 1997), flavonoids (Zhang & Wang 2002) and other phenolic compounds (Angolo *et al.* 2004). In recent reviews, it has been underlined that the existing studies on humans demonstrated a convincing effect of antioxidant polyphenols on some aspects of health (Kroon & Williamson 2005).

T. cordifolia (Miers.) is an extensively spreading, glabrous, succulent, climbing shrub with several elongated twining branches belonging to the family Menispermaceace. The plant is distributed throughout the tropical region of India up to 1,200 m above MSL from Kumaon to Assam, in the north extending through West Bengal, Bihar, Deccan, Konkan, Karnataka and Kerala. The extracts of the various plant parts show various activities *viz.*, anti-inflammatory and anti-oxidant activity, anti-stress, anti-ulcer, digestive activities and hypolipedemic effect (Prince & Menon 1999). It is a traditional belief among the Ayurvedic practitioners that *Guduchi Satva* is bitter and efficacious than neem (Raghunathan & Roma 1982). The objective was to study the

antioxidant activities of stem collected from different hosts of *T. cordifolia* and standardization of *in vitro* callusing of superior host to regenerate elite host plant material. The findings from this work will help in mass cultivation of elite material to meet the demands of the growing pharmaceutical industry.

Materials and methods

Plant material: The fresh stem parts of the *T. cordifolia* from three different hosts: (a) *F. religiosa* (Ashwathya), (b) *Eucalyptus hybrid* (eucalyptus), and (c) *Roystonea regia* (royal palm) were collected from Indian Institute of Integrative Medicine, Jammu during 2010–11. The stem samples were dried in an oven at 40°C, powdered and used for extraction.

Preparation of extracts: The powdered form of the samples was passed through a sieve and defatting was done by using petroleum ether (60-80°C). Crude extraction was done using ethanol with the help of soxhlet apparatus. The extract was passed through a sterile Whatman filter paper into a clean conical flask and transferred into the sample holder of the rotary flash evaporator. The extracts were preserved at 4°C in airtight bottles for further analysis.

Phenol content: Folin-Ciocalteau (FC) reagent method (Malick & Singh 1980) was used for estimation of total phenol content using Gallic acid as standard. 100 mL stock solutions of different extracts were prepared (1 mg mL^{-1}). Different aliquots were pipette out in test tubes. Distilled water was added to each tube to make the final volume to 3 mL, followed by addition of 1.5 mL FC reagent in each tube. After three minutes, 1mL of sodium carbonate (20%) was added. The tubes were incubated at 40°C for 30 minutes. The absorption was measured at 765nm using a spectrophotometer.

Total reducing power: Various extracts (0.1-0.9 mg mL^{-1}) were mixed with phosphate buffer (500 µL, 20 mM, pH 6.6) and 1% potassium ferricyanide (500 µL), incubated at 50°C for two minutes, 500 µL of 10% trichloro acetic acid was added and the mixture was centrifuged at 2500 rpm for 10 minutes. Supernatant formed was mixed with 1.5 mL distilled water and 0.1% ferric chloride (300 µL) and absorbance was read at 700 nm (Yen & Duh 1993). An increase in the absorbance showed an increase in the reducing power (Fig. 1).

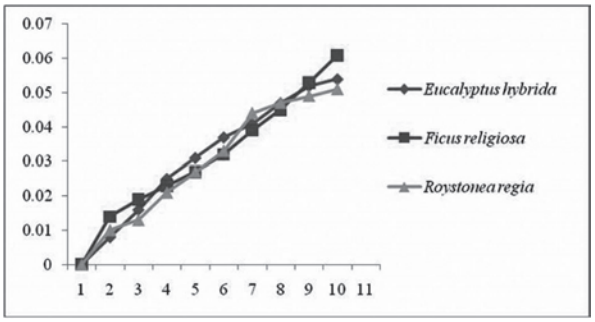

Fig. 1. Total reducing power assay as influenced by various hosts of *T. cordifolia*

Ferrous reducing antioxidant power assay (total antioxidant activity assay): The FRAP method (Benzie & Strain 1996) depends on the reduction of ferric tripyridyltriazine complex to the ferrous tripyridyltriazine by the reductant at low pH. The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-tri-azine solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3.6H_2O$. Plant extracts (150 µL) were allowed to react with 2.85 mL of the FRAP solution for 30 min in the dark condition. Readings of colored product (ferrous tripyridyltriazine complex) were taken at 593 nm.

Lipid peroxidation inhibitory activity (Duh & Yen 1997): Egg lecithin (3 mg mL^{-1} phosphate buffer, pH 7.4) was sonicated in an ultrasonic sonicator for 10 min to ensure proper liposome formation. Test samples (100 µL) of different concentrations (0.1-0.9 mg mL^{-1}) were added to the liposome mixture (1 mL) along with control. Lipid peroxidation was induced by adding ferric chloride (10 µL, 400 mM) and L-ascorbic acid (10 µL, 200 mM). After incubation for 1 h at 37°C the reaction was stopped by adding hydrochloric acid (2 mL, 0.25 N) containing trichloroacetic acid (150 mg mL^{-1}) and thiobarbutyric acid (3.75 mg mL^{-1}). The reaction mixture was subsequently boiled for 15 minutes, cooled and then centrifuged at 1000

rpm for 15 min. Absorbancy of the supernatant was measured at 532 nm. Percentage radical scavenging activity was calculated using the following formula:

$$\text{Inhibition (\%)} = [A_{control} - (A_{sample} - A_{sample\ blank} / A_{control})] \times 100$$

DPPH Radical Scavenging Activity: The free radical scavenging activity of stem extracts was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) (Moon & Terao 1998). Different aliquots (0.1-0.9 mg mL^{-1}) were mixed with 0.9 mL of tris-HcL buffer (pH 7.4), then 1 mL of DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and allowed to stand for 30 minutes. The absorbance of the resulting solutions was read at 517 nm and compared with that of ascorbic acid. The percentage of DPPH scavenging was calculated using the following formula:

$$\text{Scavenging (\%)} = [A_{control} - (A_{sample} - A_{sample\ blank} / A_{control})] \times 100$$

Superoxide Radical Scavenging Assay: Superoxide radical was generated in phenazine methosulphate–nicotinamide adenine dinucleotide (PMS-NADH) systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT) to a purple formazan (Fontana *et al.* 2001). The reaction mixture contained 1 mL NBT (50 µM prepared in 20 mM phosphate buffer, pH 7.4), 1mL NADH (73 µM prepared in 20 mM phosphate buffer, pH 7.4), and various concentrations of sample (0.1- 0.9 mg mL^{-1}). The reaction was started when 1 mL of phenazine methosulfate (PMS) solution (15 µM prepared in 20 mM phosphate buffer, pH 7.4) was added. The sample solution was incubated for 5 minutes at ambient temperature and absorbance was read at 562 nm against blank to determine the quantity of formazan generated. The inhibition of superoxide anion generation was calculated as that of DPPH Scavenging.

Results and discussion

Total Phenol Content: The preliminary phytochemical testing revealed that the level of polyphenols in the sample obtained from *F. religiosa* was higher. The amount of phenolic compounds in the sample obtained from *F. religiosa* had phenol content of 5.25 mg g^{-1}, while the sample obtained from *E. hybrida* had 5.00 mg g^{-1} and that of *R. regia* had 4.25 mg g^{-1}, (Table 1). It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant (Luo *et al.* 2004). An earlier study reported that the phenol content in the methanolic stem extracts of *T. cordifolia* was 7.2% w/w (Sivakumar *et al.* 2010). Phenol content was directly proportional to the antioxidant potential of different host plants of *T. cordifolia* (Table 1 & Fig. 2).

Table 1. Phenol content as influenced by various hosts of *T. cordifolia*

Sample	Host	Phenol content (mg g^{-1})
1.	*E. hybrida*	5.00
2.	*F. religiosa*	5.25
3.	*R. regia*	4.25

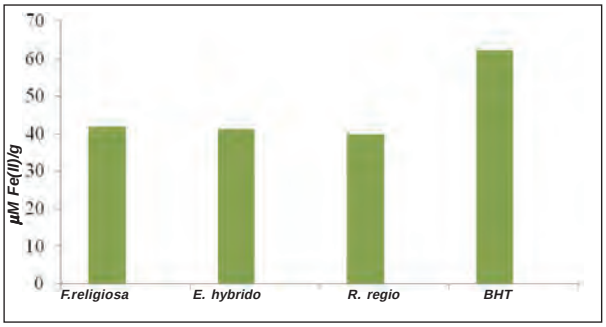

Fig. 2. Comparative antioxidant activity as influenced by various hosts of *T. cordifolia*

Total reducing power assay: The results showed that the reducing ability of the extracts increased with the concentration of reductant. Among the extracts tested, the ethanolic extract of *T. cordifolia* stem extract growing on the host *F. religiosa* showed better reducing ability (Fig. 1). The reducing ability of a compound generally depends on the presence of reductants, which exhibit antioxidant activity by breaking the free radical chain through

donation of a hydrogen atom (Oktay *et al.* 2003). Compounds having reduced abilities indicated that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen & Chen 1995).

Total antioxidant activity: It was observed that *F. religiosa* possessed higher antioxidant activity (41.73 µM) as compared to *E. hybrid* (41.15 µM) compared to other samples (Fig. 2), which is attributed to its higher polyphenol content. The results were compared with BHT, which had FRAP value of 62.15 ± 0.25. The method estimates the ability of antioxidants in the sample to reduce Fe^{3+} to Fe^{2+} in the presence of 2, 4, 6-Tri (2-pyridyl)-s-triazine (TPTZ) forming a complex that gives an intense blue color having absorption maxima at 593nm. The decrease in the absorbance is proportional to the antioxidant content (Benzie & Strain 1996).

Lipid peroxidation inhibitory activity: The results revealed that the ethanolic extract of the stem sample of *T. cordifolia*, climber on host *F. religiosa* had EC 50 value of 0.1 mg mL^{-1}, which showed an inhibition value of 52.05%, followed by *E. hybrida* (51.03%) and *R. regia* (50.06%) (Fig. 3). Lipid peroxidation inhibitory activity of

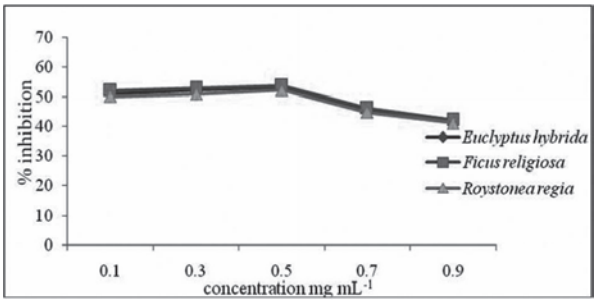

Fig. 3. Lipid peroxidation inhibitory activity of *T. cordifolia* at different concentrations (mg mL^{-1})

extracts gradually decreased with increasing concentration, which may be attributed to the degradation or peroxidation of the source lipid. The antioxidant properties of polyphenols (flavonoids) are attributed to the ability of phenolic compounds to quench the free radicals, which arises because of both their acidity (ability to donate protons) and the delocalized ð-electrons ability to transfer electrons while remaining relatively stable (Premanath & Lakshmidevi 2010; Hussain *et al.* 1987).

DPPH radical scavenging activity: DPPH radical scavenging activity of different stem extracts of *T. cordifolia* revealed that the sample obtained from the host *F. religiosa* had better scavenging activity (47.8), as compared to *E. hybrid* (42.2) and *R. regia* (40.09) (Fig. 4). The EC50 value of all extracts was achievable at concentration

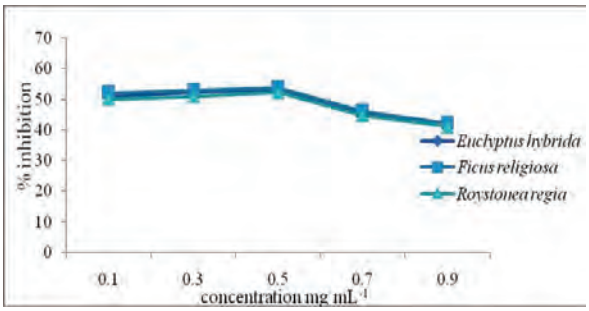

Fig. 4. DPPH radical scavenging activity of *T. cordifolia* stem extracts at different concentrations (mg mL^{-1})

between 0.5-0.6 mg mL^{-1}. The results were compared with ascorbic acid, which had quite high EC 50 value (7.03 µg mL^{-1}). The variation in scavenging activity may be because the plant has been reported to incorporate the virtues of its host (Raghunathan & Roma 1982).

Superoxide radical scavenging assay: The results revealed that quenching power was negligible with all the extracts. The 50% inhibition could not be achieved at any concentration from 0.1-0.9 mg mL^{-1}. Quercetin was found to achieve EC50 at169 µg mL^{-1}. The decrease in the absorbance at 562 nm with the plant extracts and reference compound quercetin shows their abilities to quench superoxide radicals in the reaction mixture. Lipid peroxidation generally showed a decrease in inhibition due to increasing concentration (Fig. 5). Superoxide anion gives rise to powerful and dangerous hydroxyl radicals as well as singlet O_2. Even though it is a weak oxidant, both the radicals contribute to oxidative stress (Meyer & Isaksen 1995). Our findings are supported by an earlier

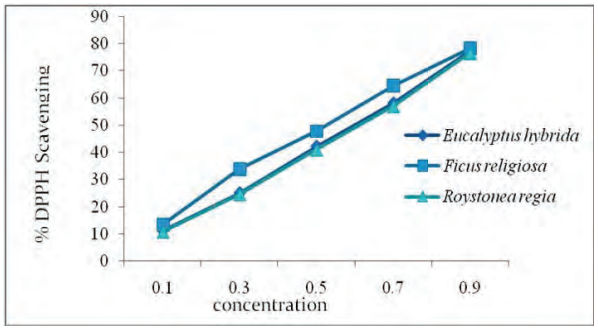

Fig. 5. Superoxide radical scavenging activity of *T. cordifolia* at varying concentrations (mg mL^{-1})

study which showed that the EC50 value for superoxide scavenging could be as high as 6 mg mL^{-1} (Mathew & Kuttan 1997).

MS medium showed significant effect on bud break (Kumar *et al.* 2003). NAA and 2,4 D alone started callusing from stem, leaf and nodal explants, but growth was slow (Nakano *et al.* 1994). The growth was also observed in various combinations of auxins and cytokinins.

Callus Induction: Based on the antioxidant activity, it was found that the *T. cordifolia* had higher antioxidant potential and therefore was selected for studying callus induction (Raghu & Geetha 2006). After 2-3 weeks of primary culture, explants showed growth response and callus formation occurred from the nodal, internodal and leaf explants, when inoculated in the medium containing different combinations of BAP, Kinetin, NAA and 2,4 D (Fig. 6, Table 2).

On the basis of different estimated parameters, the active antioxidant was higher in *F. religiosa* as compared to *R. regia*. Different hosts of *T. cordifolia* exhibited excellent antioxidant activities. All the hosts of *T. cordifolia* have considerably high amount of polyphenols, flavonoids and tannins which directly

Table 2. Effect of different hormone concentrations on callus induction in *T. cordifolia*

S.No.	Hormone concentration (mg L^{-1})			Explant type		
	NAA	BAP	2,4 D	Node	Internode	Leaf
1	0.5	0.25	-	-	-	-
2	0.75	0.50	-	-	-	-
3	1.0	1.5	-	+	-	+
4	1.5	2.0	-	++	+	-
5	2.0	2.5	-	++	++	++
6	2.5	3.0	-	++	++	+
7	-	0.25	0.5	-	-	-
8	-	0.50	1.0	-	-	-
9	-	1.0	1.5	+	+	+
10	-	1.5	2.0	++	++	++
11	-	2.0	2.5	++	++	+
12	-	2.5	1.0	++	+	+++
13	-	3.0	0.5	+	+	+++
14	-	5.0	2.0	-	-	-
15	-	6.0	3.0	-	-	-
16	0.5	3.0	-	+	+++	++
17	1.0	4.0	-	+	+	-
18	2.0	5.0	-	-	-	-
19	3.0	6.0	-	-	+	+

- =No response; + =Normal callus induction; ++ =Good induction; +++ =Best induction

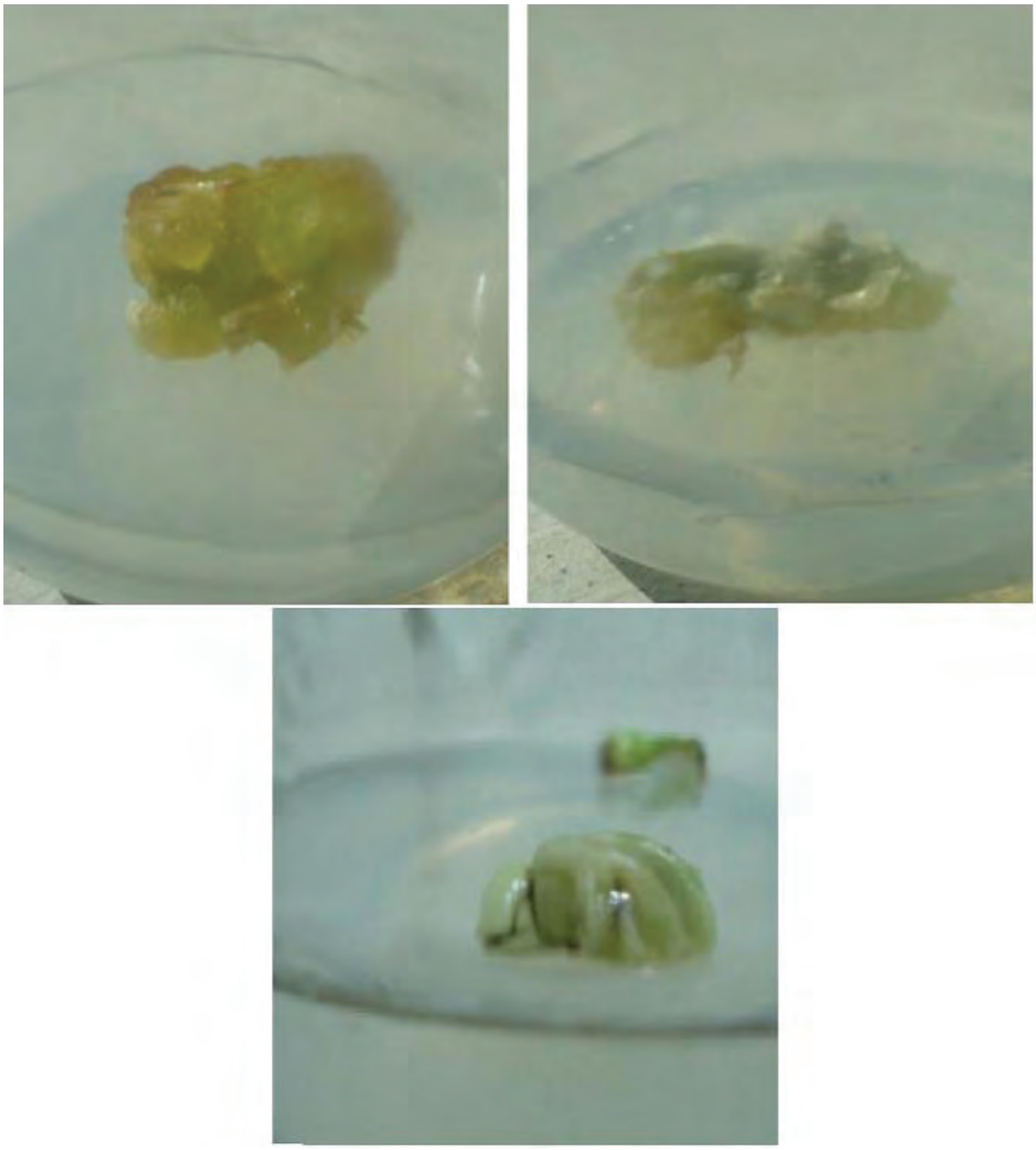

Fig. 6. Callus induction from nodal, internodal and leaf explants
(**a**) Callus from nodal explants using NAA 2.0 mg L^{-1} + BAP 2.5 mg L^{-1}; (**b**) Callus from internode using 2,4 D 1.0 mg L^{-1} + BAP 2.5 mg L^{-1}; (**c**) Callus obtained from the leaf explant using BAP 3.0 and 2,4 D 0.5 mg L^{-1}

correlated to strong free radical scavenging activity. The observed high antioxidant activity of the various hosts indicated the potential of plant as a source of natural antioxidants/ nutraceuticals to reduce oxidative stress. Among various explants of *F. religiosa*, leaf explants were found to be superior for *in vitro* callus induction.

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