Antioxidant activity and detection of (–)epicatechin in the methanolic extract of stem of *Tinospora cordifolia*

Preshita Pushp · Neha Sharma · G. S. Joseph · R. P. Singh

Revised: 24 March 2011 / Accepted: 25 March 2011 / Published online: 19 April 2011
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Abstract *Tinospora cordifolia* is known for its various medicinal and pharmacological properties. In this study, the antioxidant profile of the stem extract of *T. cordifolia* has been determined using various *in vitro* methods. An attempt was also made to identify phenolic compounds in *T. cordifolia* stem extract using silica gel column chromatography, high performance liquid chromatography (HPLC) and mass spectrometry (MS). The detection of (–) epicatechin has been reported for the first time in *T. cordifolia* stem extract.

Keywords *Tinospora cordifolia* · Antioxidant capacity · Epicatechin

Tinospora cordifolia is a plant of known and recognized medicinal value and is used as antibacterial, analgesic, antipyretic, for the treatment of jaundice, skin diseases, diabetes, anemia etc. (Iqbal et al. 2005). The major classes of compounds present in *T. cordifolia* are alkaloids and glycosides, diterpenoid lactone, steroids etc. (Singh et al. 2003). In the present study, the antioxidant activity of *T. cordifolia* extracts has been evaluated using various *in vitro* methods. Methanol is a solvent of choice for the extraction of antioxidant principles from plant sources (Abdille et al. 2005; Singh et al. 2002) and was used in the present study for the extraction of antioxidant rich fractions. The presence of (–) epicatechin in *T. cordifolia* stem extracts has also been reported for the first time.

Materials and methods

Plant material The fresh *T. cordifolia* creepers were collected in the month of January from a local garden in Mysore. The specimen of *Tinospora cordifolia* has been deposited in the Fruit and Vegetable Technology (FVT) department of Central Food Technological Research Institute, Mysore-570 020. The voucher specimen no. is FVTDH No. LGBCL-TC-7A and & B/2010.

Chemicals All solvents / chemicals used were of analytical grade. (–) epicatechin, Diphenyl picrylhydrazyl (DPPH) and BHA and have been procured from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of extracts The stem of *T. cordifolia* was separated from leaves and shade dried. The dried stem was reduced to size and was made to a coarse powder using a mixer grinder. This powder (50 g) was extracted with MeOH (150 ml) in a soxhlet extractor for 8–10 h. The extracts were concentrated *in vacuo* (Buchi, Switzerland) and stored at room temperature (28±3°C) in a desiccator.

Radical Scavenging Activity (RSA) The RSA of MeOH extract of *T. cordifolia* stem and butylated hydroxy anisole (BHA) were determined as described by Blois (1958). Different concentrations of extract and BHA were placed in different test tubes in a fixed volume of 100 μ L. 5 ml of 0.1 mM methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand at room temperature for 20 min in dark. Similarly, a control was prepared without any extract. MeOH was used for baseline correction. Changes in the absorbance of the samples were measured at 517 nm. RSA was expressed as the inhibition percentage and was calculated using the following formula:

P. Pushp · N. Sharma · G. S. Joseph · R. P. Singh (✉)
Human Resource Development,
Central Food Technological Research Institute (CSIR),
Mysore 570 020, India
e-mail: singhrp317@gmail.com

% radical scavenging activity = (control OD - sample OD / control OD) X100.

Reducing power The reducing power of MeOH extract of *T. cordifolia* stem was determined according to the method of Jayaprakasha et al. (2001). Different aliquots of the extract in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 500 C for 20 min. 2.5 ml of trichloroacetic acid (10%) was added to the mixture, and centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) and absorbance was measured at 700 nm. Increase in the absorbance of the reaction mixture indicated increased reducing power.

Antioxidant capacity The total antioxidant capacity (A-cap) of methanol extract of *T. cordifolia* stem was evaluated by the method of Prieto et al. (1999). The extract (0.1 ml) was mixed with 1.0 ml of phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Ascorbic acid was used as standard for comparison. In case of blank, 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in water bath at 95°C for 90 min. The samples were cooled to room temperature and the absorbance was measured at 695 nm in Spectronic 20 D (Spectronic Instruments, USA) spectrophotometer. The water-soluble antioxidant capacity of the extracts was expressed as equivalents of ascorbic acid (mmol/g of extract).

Deoxyribose degradation Assay The method of Halliwell et al. (1987) was used for determining non-site specific deoxyribose degradation assay. The extract was mixed with Haber-Weiss reaction buffer (10 mM FeCl₃, 1 mM EDTA, 10 mM H₂O₂, 10 mM deoxyribose and 1 mM ascorbic acid) and the final volume was made to 1.0 ml. The mixture was incubated at 37 °C for 1 h and then heated at 80 °C with 1 ml of TBA (0.5% TBA, in 0.025 M NaOH, 0.02% BHA) and with 1 ml of 10% trichloroacetic acid (TCA) in a water bath for 45 min. After cooling, absorbance of the mixture was measured at 532 nm. The % inhibition was calculated as described in DPPH method.

Determination of total phenolics The concentration of phenolic compounds in the extracts was determined according to the method of Heinonen et al. (1998), and results were expressed as tannic acid equivalents. The methanolic extract of *T. cordifolia* stem was dissolved in a mixture of MeOH and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of 10-fold-diluted Folin-Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution, the mixture was allowed to stand for 30 min at room temperature, The absorbance

was measured at 765 nm using Genesys-5 UV-visible (Milton Roy, USA) spectrophotometer.

Silica gel column chromatography Silica gel column chromatography was performed as described by Murray et al. (2004). 60 g silica gel (mesh size 60–120) was suspended in methanol for 2 to 3 h and packed in a glass column (id 2.0 cms, l 32 cms) and equilibrated with hexane. The MeOH extract was dissolved in MeOH (50 mg/ml) and 3 ml was loaded onto the column and eluted sequentially with 150 ml of each of the solvents in the following order; hexane, hexane: EtOAc (1:1), EtOAc, EtOAc:MeOH (1:1) and MeOH. Fractions of 50 ml each were collected from the column. The DPPH RSA of each fraction was determined, as described above. Based on DPPH RSA profile, three pools were made as follows: Pool A, mixture of fractions from 1 to 9; Pool B, mixture of fractions from 10 to 12 and Pool C, mixture of fractions from 13 to 15. The polyphenol content of each pool was carried out separately as described above. All the above parameters have been repeated thrice.

High performance liquid chromatography Based on the DPPH radical scavenging activity profile, pool B was further subjected for the identification of antioxidant compound (s) on an analytical RP HPLC (Agilent Technologies HP 1200, USA), integrated with Chemstation Rev. B.03.01 software, C-18 S3 ODS2 column from Waters Spherisorb, 4.6×250 mm, pore size 5 μ; VWD (variable wavelength detector). All solvents were filtered through 0.45 μ Millipore filter disk. 20 μl of the pool B solution (0.5 mg/ml) was injected to HPLC column. The following gradient elution (Bonoli et al. 2003) with total run time of 50 min. was carried out: Solvent A, water / methanol / formic acid (74.7/25/0.3; v/v); Solvent B, acetonitrile / formic acid (99.7/0.3; v/v). The linear gradient elution was performed as follows: 100% A for the first 8 min, and then reached 100% B in 24 min, held at 100% B for 6 min and returned to 100% A in 4 min and a final hold at 100% A for 6 min as post-time. The flow rate was 0.5 ml/min. Identification of compounds was carried out by comparing retention times of the peaks with that of standards at 270 nm. DPPH quenching experiments were performed as described by Yamaguchi et al. (1998). Pool B (3 parts) was reacted with DPPH solution (500 μM, 1 part) and injected onto HPLC. Spiking of pool B with standard (–) epicatechin was performed by coinjecting the two in 1:1 ratio.

Molecular weight determination by LC-MS MS analysis was done for MeOH extract, pool B and the HPLC eluents collected corresponding to peak of interest. The mass spectrum was recorded on a Waters Q-ToF Ultima instrument in the positive ion electron spray mode under the following conditions: Spray capillary voltage of 3.0 KV,

Fig. 1 The antioxidant profile of MeOH extract of *T. cordifolia* stem by different *in vitro* methods **a.** DPPH method **b.** Reducing power **c.** Antioxidant capacity **d.** Deoxyribose degradation method (Values are average of three repeats)

cone voltage :- 100 V, sheath gas N₂, source temperature - 120 °C, Desolvation temp. - 300 °C, Cone gas flow- 50 l/h, Desolvation gas flow- 500 l/h, Full scan acquisition, from 100 to 500 *m/z*. Spectra were achieved by injecting 10 µl of the sample into the electrospray ion source at flow rate of 0.1 µl/min by means of a syringe pump. The data acquisition was done using computer with the software program Mass Lynx 4.0.

Result and discussion

Yield of extract and polyphenol content The yield of the MeOH extract from the stem of *T. cordifolia* was 4.36 g (8.72%, w/w) and the phenolic content of the extract was determined to be 7.23% (w/w, with reference to dry wt of the extract) expressed as tannic acid.

Radical Scavenging Activity (RSA) DPPH radical is commonly used as a substrate to evaluate the radical scavenging activity of plant extracts and other materials. Figure 1A shows the RSA of *T. cordifolia* MeOH stem extract at different concentrations. The scavenging effect increased with increasing concentration of the extract. When compared with BHA at equivalent concentrations, it shows a lower RSA profile (15.6% RSA for the extract and 94.0% RSA for BHA at 25 ppm).

Reducing power Figure 1B presents the reducing power of the MeOH extract of *T. cordifolia* at different concentrations which indicates the concentration dependent increase in the reducing power of extract. It is often used as an indicator of electron donating activity, which is an important mechanism for testing antioxidant activity of plant extracts (Yildirim et al. 2001). Good correlation has been established between antioxidant capacity and reducing power (Yen et al. 2000), thus reducing power may be an indicator of potential antioxidant activity.

Antioxidant capacity The antioxidant capacity of the extract was measured spectrophotometrically by phosphomolybdenum method, which is based on the conversion of

Fig. 2 Polyphenol content and the % RSA of Pool A, B and C; values are average of three repeats

Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of MeOH extract of *T. cordifolia* stem is shown in Fig. 1C which indicates dose dependent response of the extract.

Deoxyribose degradation Assay When deoxyribose is incubated with H_2O_2 and an Fe_2^{+} -EDTA complex, the resulting

deoxyribose degradation is inhibited by an added scavenger of hydroxyl radical to an extent that depends only on the concentration of scavenger related to deoxyribose and on the scavenger's second order rate constant for reaction with free radical OH. When OH is generated, it escapes scavenging by the EDTA itself, enters free solution and is equally accessible to deoxyribose ribose and to any added scavenger.

Effect of methanol extract on deoxyribose degradation is shown in Fig. 1D. The extract was found to be effective in

Fig. 3 HPLC profile of *T. cordifolia* MeOH stem extract; **a**: Extract **b**: (–)Epicatechin standard **C**: Extract + (–)epicatechin

preventing the degradation of deoxyribose, showing an inhibition of 41.5% and 64.7% at 100 and 200 ppm concentrations of the extract respectively.

Silica gel column chromatography The profile of DPPH RSA of the fractions eluted from the silica gel column indicates that the activity is confined mainly to the fractions eluted with EtOAc and MeOH combination (fraction 10–12) with other fractions having lower activity than these fractions. Based on the activity profile, three pools were made namely Pool A, Pool B and Pool C. The yield of fractions A, B and C were 32.7 mg, 85.6 mg and 20.4 mg respectively. The RSA activity and polyphenol content of these pools are depicted in Fig. 2.

High performance liquid chromatography The pool B was found to be rich in antioxidant activity despite moderate polyphenol content when compared to other pools and subjected to HPLC analysis to identify the compounds responsible for antioxidant activity.

The HPLC pattern of the pool B showed a major peak at 20.869 min (Fig. 3A). This peak was substantially suppressed when coinjected with 500 μ M DPPH, confirming its radical scavenging property. This peak was found to elute at RT similar to that of (–) epicatechin (RT 20.658 min, Fig. 3B). This was confirmed by spiking pool B with standard (–) epicatechin which showed a clear increase in the peak height (Fig. 3C).

Detection of (–) epicatechin by LCMS Mass spectrometric analysis was done for pool B obtained from silica gel column chromatography and analysed in ESI/MS in positive ion mode and the MS spectra obtained is shown in Fig. 4. In ESI, samples give rise to singly charged molecular-related ions, usually protonated molecular ions of the formula $(M + H)^+$ in positive ionisation mode. The m/z spectrum shows dominant ion at m/z 291.34, which are consistent with the expected protonated molecular ions, $(M + H)^+$. These m/z ions are singly charged, so the m/z value is consistent with

the molecular mass, as the value of z (number of charges) equals 1. Hence the measured molecular weight is deduced to be 290.34 Da.

Figure 4 shows ESI/MS analysis of column chromatographic pool B in positive mode. The compound with high relative abundance is of molecular weight 290.34 Da. The molecular weight of both Epicatechin and catechin are 290.34 Da. However, in HPLC analysis, the RT of the peak of interest matched with that of standard (–) epicatechin.

Conclusion

In the present study, Methanol was used for the extraction of fractions rich in antioxidant properties from the stem of *T. cordifolia*. Previous studies from this laboratory have shown that for extraction of compounds, methanol is a better solvent of choice than other solvents (Abdille et al. 2005; Singh et al. 2002). The antioxidant activity of the methanolic extract by different methods has been determined which showed a dose dependent response in all the methods used. The antioxidant activity of various extracts of *T. cordifolia* has been described by several researchers. Antioxidant activity of feeding alcoholic extract of *T. cordifolia* at a dose of 100 mg/kg body weight has been shown by Prince et al. (2004) in alloxan induced diabetic rats. Mathew and Kuttan (1997) showed the inhibition of lipid peroxidation, superoxide and hydroxyl radicals *in vitro* and reduction of toxic effects of cyclophosphamide in mice by *T. cordifolia* extract. Similarly, Upadhyay et al. (2010) have described the elevation of glutathione levels, radical scavenging properties and other antioxidant parameters by *T. cordifolia* extracts.

An attempt has been made to separate the methanol extract of the stem of *T. cordifolia* on silica gel column which yields fractions (pool B) with high antioxidant activity as shown by DPPH method, even though it did not possess high polyphenol content. The HPLC pattern of this pool indicated

Fig. 4 MS Analysis of pool B in positive ESI / MS mode

the presence of (–) epicatechin as the peak obtained in the HPLC profile of this fraction which matched with the RT of standard (–) epicatechin not only showed the suppression when coinjected with DPPH but also the height of peak increased, when coinjected with standard (–)epicatechin. This was also confirmed by mass spectroscopy.

This is the first report of the presence of (–) epicatechin in *T. cordifolia*, it may be responsible along with other compounds in the prevention of various types of flu as the natural and semi-synthetic derivatives of (–) epicatechin are known to possess antiviral activity against influenza virus, including A/H1N1, A/H3N2 and B virus (Song et al. 2005, 2007).

Acknowledgement The authors thank Director, CFTRI and Head, Human Resource Development, CFTRI, for their keen interest and constant encouragement during the course of the work.

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