Antioxidant Potential of *Tinospora cordifolia* Extracts and their Protective Effect on Oxidation of Biomolecules

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ABSTRACT

Tinospora cordifilia is a medicinal plant, commonly known as guduchi or amritha. In the present study, the antioxidant potential of solvent extracts of leaf and stem were evaluated by various *in vitro* methods. Scavenging effects on DPPH, ABTS radical, hydroxyl radical and ferric reducing antioxidant power (FRAP) were found to be highest in methanolic extract of leaf and ethyl acetate extract of stem compared to all other extracts. These extracts also exhibited significant protection against radical induced protein (BSA) oxidation and plasmid DNA damage (pBR322). The extracts were further evaluated for their inhibitory properties on AAPH (2, 2′-azo (2-amidinopropane) dihydrochloride induced *ex vivo* oxidative stress in rat liver homogenates. The results again showed the potent antioxidant nature of methanolic extract of leaf and ethyl acetate extract of stem with respect to inhibition of lipid and protein oxidation. Overall, stem extracts showed to be the more effective antioxidant source than the leaf extracts with regard to all the radical scavenging activities. These protective properties of the extracts could be directly attributed to the presence of phytochemicals such as polyphenols, tannins etc. In conclusion, our results demonstrate the potential antioxidant activities of guduchi leaf as well as stem and therefore, it can be used as a source of antioxidant for health benefits through dietary supplementations.

Key words: *Tinospora cordifolia*; Polphenols; Tannins; Antioxidant activities; Protein oxidation; Lipid oxidation; DNA damage; Oxidative stress.

INTRODUCTION

Increasing evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in several degenerative diseases like cancer, asthma, arthritis, and cardiovascular problems.[1] Production of reactive oxidants such as superoxide, hydroxyl radicals and hydrogen peroxide in living cells is an inevitable process of normal oxygen metabolism. Mechanism responsible for the radical mediated injuries to cells and tissues mainly include lipid peroxidation, oxidative DNA damage and protein oxidation.[2] As a result, investigations on these biomolecules oxidation and their detrimental effects have been in focus for many years. Despite naturally occurring bodily antioxidant systems (enzymes and antioxidant nutrients) that are able to control the free radical mediated oxidative damage, its continuous exposure for a long time may lead to irreversible oxidative damage. Therefore, antioxidants particularly from dietary sources may have a great relevance in the prevention and therapeutics of such diseases for being safer and more effective in the context of their efficiency and non-toxicity than the synthetic antioxidants.[3-4]

Tinospora coridifolia is a well known medicinal plant and is widely used in folk medicine/ ayurvedic system of medicine. It is a large glabrous, succulent, climbing shrub belonging to the family of menispermaceae. Leaf, stem and roots of this plant have been shown to posses various therapeutic purposes. A range of pharmacological properties have been reported including immunomodulatory,[5-6] hypoglycemic,[7] anti-hepatotoxic,[8-9] antistress,[10] anti-inflammatory,[11] gastroprotective,[12] antioxidant,[13] radioprotective effects,[14] and memory enhancing properties.[15] Despite its long usage as testified in traditional folklore, the biological properties of various plant parts of *T. coridifilia* on free-radical scavenging ability and its effect on biomolecule oxidation are scanty. Hence, in the present study, the antioxidant properties of *T. coridifolia* leaf and stem were evaluated under *in vitro* and *ex vivo* conditions.

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MATERIALS AND METHODS

Folin-Ciocalteu reagent (FCR), Na_2CO_3, were purchased from Sisco Research Laboratory (SRL, Mumbai). Gallic acid, $FeCl_2$, BHT, Ferric cyanide and EDTA were procured from E-Merck, Mumbai, India. DPPH was purchased from Hi-Media. BSA, pBR322 plasmid were purchased from Genei, Bangalore. Agarose and ethidium bromide were purchased from Sigma-Aldrich (St.Louis.MO). All other reagents were of analytical grade.

Preparation of extracts

The leaves and stems of *T. coridifolia* were procured from local market and dried at 50°C in a hot air oven and then powdered. 200 g powder was used for the sequential extraction using different solvent systems with the increasing polarity viz hexane, chloroform, ethyl acetate, acetone and methanol which were used (1:10 ratio) in an orbital shaker for 12 hours. Extracts were filtered and dried by flash evaporation/ lyophilization as per the requirements. Finally, the crude extracts were stored in a deep freezer (–20° C) until further use.

Total polyphenols and Tannins

Total polyphenol content of the extract was determined using Folin-Ciocalteu reagent.[16] Gallic acid was used a standard compound and amount of total polyphenols content was expressed as mg gallic acid equivalent per mg extract (mg GAE/mg). Folin-Denis method was used for estimating tannin content of the extracts[17] and tannic acid was used as a standard compound.

In vitro Antioxidant activity

DPPH(1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the extract was determined as described by Braca *et al*.[18] ABTS (2, 2'-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) radical discoloration assay was performed as per Re *et al*.[19] The antioxidant capacity of the extract was expressed as% inhibition and IC_{50} value was calculated from regression analysis. Ferric reducing antioxidant power Assay (FRAP) was performed according to Benzie and Strain with slight modifications[20] and results were expressed in ferrous sulphate equivalent. Hydroxyl radical scavenging activity assay was performed following the method of Halliwell *et al* [21] using deoxyribose.

Protective effect on oxidation of biomolecules *in vitro*

a) Inhibition of protein oxidation

Protein oxidation was assayed as described by Kwon *et al* [22] with minor modifications. Oxidation of BSA (5 µg) in phosphate buffer was initiated by 20 mM AAPH and the inhibitory properties of extracts were measured at a fixed concentration (1 mg/ml). After incubation for 2 hours at 37°C, 0.02% BHT was added to prevent the formation of further peroxyl radical. The samples were then analysed by normal SDS-PAGE electrophoresis.

b) Inhibition of oxidative DNA damage

Conversion of the supercoiled form of plasmid DNA to open circular and further linear form has been used as an index of DNA damage. DNA strand breakage assay was performed using pBR322 plasmid DNA as per the method described by Lee *et al*.[23] A mixture of 10 µl of fixed concentration of extracts (1 mg/ml) and plasmid DNA (0.5 µg) was incubated at room temperature followed by the addition of AAPH (10 mM). The final volume of the mixture was made up to 20 µl and incubated for 30 min at 37°C. The DNA samples was electrophoresed on 1% agarose gel and band intensities were analyzed using Easy win 32 software from Herolab (Germany).

Protective effect on biomolecule oxidation *ex vivo*

Male Wistar albino rats weighing 200-220 g were housed under conventional conditions and were allowed free access to food and water, *ad libitum*. All experiments were carried out according to guidelines for the care and use of experimental animals approved by Institutional Animal Ethics Committee. The rats were anaesthetized using ether, their abdomen was opened and their liver was quickly removed. Liver was homogenized (10% w/v) in sodium phosphate buffer (pH, 7.4). Homogenate was then centrifuged at 5000 g for 15 min at 4° C. The protein concentration of the supernatant was determined by the total protein kit (Agappe diagnostics, India). Oxidative stress was induced as described previously[24] with a peroxyl radical inducer (AAPH). The reaction mixture was composed of liver homogenate (15 mg protein), 40 mm AAPH and a fixed concentration of various solvent extract of *Tinospora* stem and leaf (2 mg). The reaction mixture was incubated at 37° C for 2 hours and further analysed as below.

a) Inhibition of lipid peroxidation

The extent of lipid peroxidation of the liver homogenate was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS). After incubation, each reaction was terminated by adding 2% BHT followed by addition of 1 ml of TCA (20% w/v) to the mixture. After centrifugation at 3000 g for 15 min, the supernatant was incubated with 1 ml of thiobarbituric acid (TBA, 0.67%) at 100°C for 15 min. The color intensity of the TBARS/ TBA complex was measure at 532 nm. The amount of TBARS formed was calculated using absorbance coefficient of $1.56 \times 105\ cm^{-1}\ M^{-1}$. The data was expressed in terms of percentage inhibition.[25]

b) Inhibition of protein carbonyl formation

Protein carbonyl content of the rat liver homogenate in the presence and absence of various extracts was evaluated

by the method as described by Reznick and Packer.[26] One ml of 10 mM DNPH in 2N Hcl was added to the reaction mixture (2mg protein) and samples were incubated for 1 h at room temperature. Then, 1 ml of trichloroacetic acid, 10% was added to each reaction mixture and centrifuged at 3000 g for 10 min. the protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1,v/v) and dissolved in 1 ml of guanidine hydrochloride (6M, pH 2.3) and incubated for 10 min at 37°C. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4\ cm^{-1}\ M^{-1}$).The data were expressed in terms of percentage inhibition.

Statistical analysis

All the data were expressed as mean ± standard deviation. IC_{50} values were calculated using regression equation in excel programme. Statistical analysis was performed using student't' test. The *p* values less than 0.05 were considered as significant different.

RESULT AND DISCUSSION

In the present study various solvents namely hexane, chloroform, ethyl acetate, acetone, methanol and water were used sequentially for extracting antioxidant compounds from leaf and stem of *T. coridifolia*. Among the various leaf extracts obtained, water extract had highest amount of extractable compounds (17.0 ± 1.24%) whereas in case of stem, methanol extract had highest amount extractable compounds (6.0 ± 0.3%) as compared to other extracts (Table 1).

Total polyphenols and tannin content

It is well-known that plant phenolics, in general, are highly effective free radical scavengers and antioxidants.[27] Consequently the antioxidant activities of plant are often explained with respect to their total phenolics and tannin content. These natural antioxidants not only protect lipids from oxidation, but may also provide health benefits associated with preventing damage due to biological degeneration. As revealed by our data (Table 1), the total polyphenol was preferentially extracted in methanol extract of leaf (52.17 ± 0.52 mg/g extract) than other solvents and over all, the order of phenolic content was methanol > acetone > chloroform > water > ethylacetate > hexane. On the other hand in stem, ethyl acetate extracted highest amount of polyphenol (60.93 ± 03.21 mg GAE/g extract) and other extracts were in the decreasing order of ethyl acetate > chloroform > acetone > methanol > water > hexane. Similarly the tannin content also showed to be highest in methanolic extract of leaf (36.93 ± 1.52 mg/g extract) followed by acetone, chloroform, ethylacetae, water and hexane in the decreasing order. With respect to stem extracts, the ethylacetate extract had high amount of tannins (40.57 ± 0.23 mg/g extract) followed by acetone, chloroform, methanol, water and hexane in the decreasing order. Thus, methanolic extract of leaf and ethyl acetate extract of stem contained high levels of total polyphenols and tannins, which may account for its impressive antioxidant activity.

Antioxidant activities

a) DPPH and ABTS radical scavenging activity

DPPH and ABTS radical scavenging activities of leaf and stem extracts were evaluated (Table 2). The IC_{50} value was

Table1: Yield percentage of extractable compounds, total polyphenol and tannin content of various solvents extracts from leaf and stem of *T. coridifolia*

Solvent extracts	Extractable compounds (%)		Total Polyphenol mg GAE/g extract		Tannin mg/g extract	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
Hexane	3.8 ± 0.2	0.8 ± 0.06	7.62 ± 0.16	5.45 ± 0.23	4.10 ± 0.20	0.88 ± 0.13
Chloroform	5.0 ± 0.4	2.2 ± 0.2	20.93 ± 0.66	41.51 ± 0.38	17.00 ± 0.18	28.27 ± 0.09
Ethyl acetate	1.2 ± 0.31	0.4 ± 0.01	17.55 ± 0.21	60.93 ± 3.21	12.05 ± 0.48	40.57 ± 0.23
Acetone	0.8 ± 0.07	0.6 ± 0.02	46.35 ± 4.47	36.16 ± .044	33.54 ± 0.20	29.04 ± 0.12
Methanol	13.6 ± 0.97	6.0 ± 0.3	52.17 ± 0.52	33.93 ± 1.04	36.93 ± 1.52	25.92 ± 0.10
Water	17.0 ± 1.24	5.6 ± 0.42	18.67 ± 0.58	15.55 ± 0.05	15.69 ± 0.07	12.84 ± 0.07

Table 2: DPPH and ABTS radical scavenging activity of sequential extracts from *T. coridifolia*

Solvent extracts	DPPH radical scavenging activity		ABTS radical scavenging activity	
	Leaf (IC_{50}) mg	Stem (IC_{50}) mg	Leaf (IC_{50}) µg	Stem (IC_{50}) µg
Hexane	1.62 ± 0.05	3.95 ± 0.11	284 ± 11	695 ± 23
Chloroform	1.11 ± 0.04	0.64 ± 0.02	169 ± 09	62 ± 04
Ethyl acetate	1.58 ± 0.08	0.60 ± 0.02	252 ± 12	57 ± 03
Acetone	0.71 ± 0.02	0.79 ± 0.04	105 ± 08	97 ± 06
Methanol	0.54 ± 0.01	0.74 ± 0.03	95 ± 05	107 ± 10
Water	1.22 ± 0.06	1.79 ± 0.08	143 ± 08	298 ± 13

calculated and in general, the samples with the low IC_{50} are potent scavenger than the samples with high IC_{50} value. Methanolic extract was found to be the most effective radical scavenger of DPPH radical with the least IC_{50} value (0.54 ± 0.01 mg/ml) among all the leaf extracts followed by acetone, chloroform, water, ethyl acetate and hexane extracts. Ethyl acetate extract was found to be the most effective radical scavenger with the least IC_{50} value (0.54 ± 0.01mg/ml) among all other stem extracts followed by chloroform, methanol, acetone, water and hexane extracts. IC_{50} value for the BHA standard was at 9.77 ± 0.44 µg/ml.

The results of ABTS radical scavenging activity for leaf and stem extracts also showed that the methanol extract (leaf) was the most potent radical scavenger with the lowest IC_{50} of 95 µg/ml followed by acetone > water > chloroform > ethylacetate > hexane. Ethylacetate (stem) was the most potent ABTS radical scavenger with the lowest IC_{50} of 57 µg/ml followed by chloroform > acetone > methanol > water > hexane. IC_{50} value for the vitamin C standard was at 25.26 ± 0.32 µg/ml. Since phenolic compounds have been studied extensively earlier as important contributors to the antioxidant properties,[28] the highest radical scavenging ability of the potent extracts can be directly correlated to the presence of high content of polyphenols as well as tannins. The hydrogen donating ability of these compounds is responsible for their effective antioxidant property and used for protecting against cellular oxidative damage.

b) Hydroxyl radical scavenging activity

Hydroxyl radicals are most reactive ROS, capable of attacking most of the biological substrates.[29] The prevention of such deleterious effect is very necessary in terms of both human health and the shelf-life of food and pharmaceuticals. So, it is considered important to assess the protective ability of the sample extract against $OH^{\cdot}$ radicals. In the $OH^{\cdot}$ radical - mediated 2-deoxy-D-ribose degradation assay, radicals are generated by fenton chemistry using EDTA, Fe^{3+} ions, ascorbic acid and H_2O_2. The $OH^{\cdot}$ radicals degrade the 2- deoxy-D- ribose molecule into 2-thiobarbituric acid reactive substances (TBARS), which can be quantified spectrophotometrically. The *T.coridifolia* extract (1 mg/ml) added to the reaction mixture removed the radical from the sugar and prevented it from degradation and the results are shown in Figure 1. Amongst leaf extracts, the radical scavenging activity decreased in the order of methanol > acetone > chloroform > water > ethyl acetate > hexane and amongst stem extracts, the activity decreased in the order of ethylacetate > acetone > chloroform > methanol > water > hexane. Our study shows that methanol extract (88.44%) and ethyl acetate extracts (94.73%) were the most effective $OH^{\cdot}$ radical scavengers in leaf and stem respectively.

c) Ferric reducing antioxidant power (FRAP)

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity.[30] Higher absorbance indicates higher reducing/antioxidant power of the plant samples. Figure 2 clearly shows that methanol extract is the most potent reducing agent among leaf extracts (8.97 ± 0.11 µg ferrous sulphate equivalent) and ethyl acetate extract is the most potent reducing agent among the stem extracts (16.87 ± 0.07 µg ferrous sulphate equivalent) followed by other extracts at fixed sample concentration of 0.1 mg/ml. The antioxidant activity of typical antioxidants has been attributed by various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[31] The data on the ferric reducing power for the extracts suggest that it contributes significantly toward the observed antioxidant effect.

Protective effect on Biomolecules oxidation *in vitro*

a) Inhibition of Protein oxidation

Accumulation of macromolecular oxidative damage has been reported as a fundamental cause in many pathological

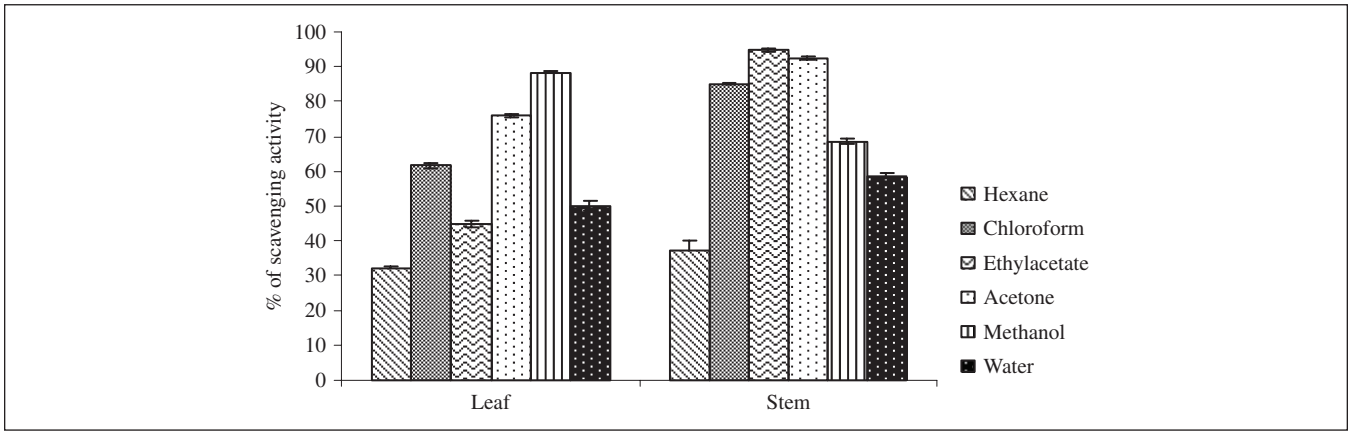

Figure 1: Hydroxyl radical scavenging activities of *T. coridifolia* extracts

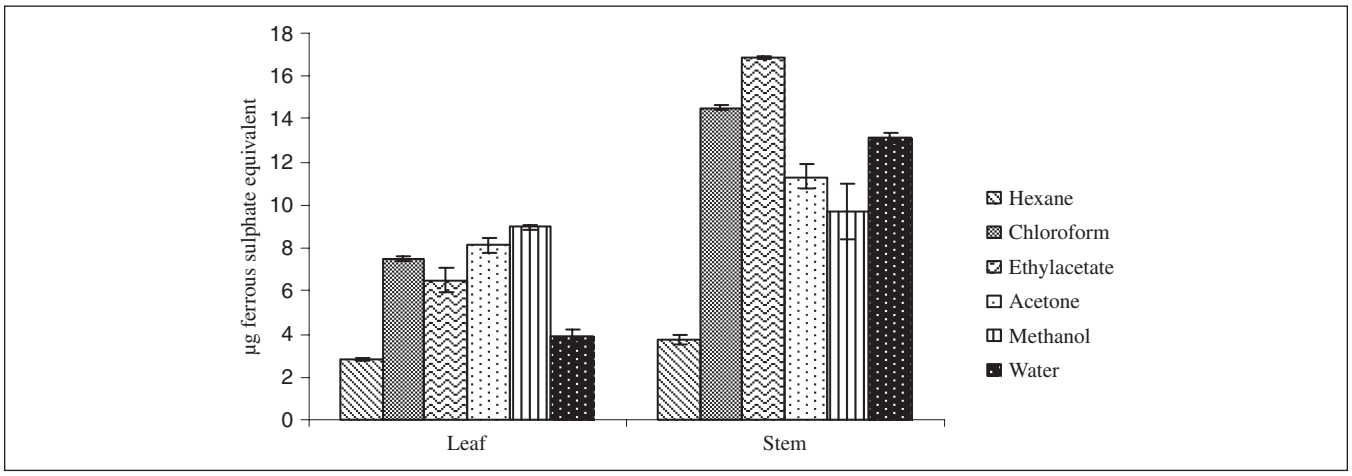

Figure 2: Ferric reducing antioxidant power (FRAP) of *T. coridifolia* extracts

conditions. Cellular proteins are one of the vulnerable targets for oxidation caused by reactive oxygen species. AAPH is a water soluble initiator, which decomposes at physiological temperature producing alkyl peroxyl radicals with oxygen to initiate the protein oxidation.[32] The protection by the *T.coridifolia* extract against protein oxidative damage was determined by the oxidation of BSA initiated by AAPH. Figure 3 shows that two hours after the incubation, the BSA was completely degraded by 20 mM AAPH in positive control as studied by SDS-PAGE electrophoresis (lane 2). Among all the leaf extracts, only methanol (lane 7) and ethyl acetate (lane 6) extract prevented the BSA oxidation significantly by 55 and 50% respectively compared to negative BSA control (lane 1) at the concentration of 1 mg/ml . Though all the stem extracts showed a significant protective effect, ethyl acetate was found to be the most potent extracts which completely prevented the oxidation (lane 8) whereas other extracts showed up to 50% protection only. Comparatively, the protective capacity of stem extracts was found to be more than leaf extracts might be due to the higher radical scavenging activity of the former.

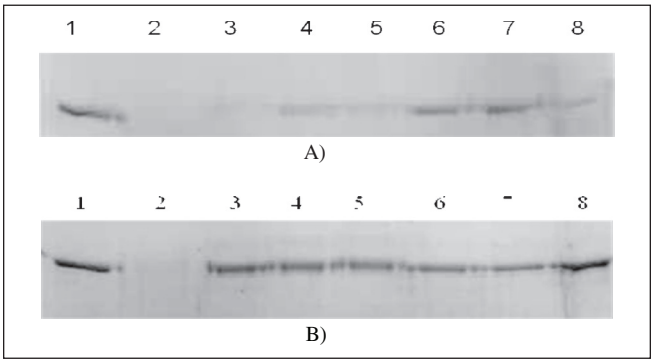

Figure 3: The protection of *T. coridifolia* extracts (1 mg/ml) on AAPH (40 mM) induced oxidative fragmentation of BSA. A) Lane 1, BSA alone; lane 2, BSA + AAPH (positive control); lane 3-8, BSA + AAPH + leaf extracts (hexane, chloroform, ethyl acetate, acetone, methanol and water). B) Lane 1-BSA alone; lane 2-BSA + AAPH (positive control); lane 3-8 BSA + AAPH + stem extracts (hexane, chloroform, acetone, methanol, water, ethyl acetate).

b) Inhibition of DNA damage

Oxidative modification of DNA has been suggested to contribute to aging and various diseases including cancer and chronic inflammation.[33] Therefore, the protective effect of extracts on oxidative DNA strand breakage was evaluated with pBR322 plasmid DNA. As shown in Figure 4 the plasmid DNA was mainly of the supercoild form (bottom band) and open circular form (top band) in the absence of AAPH (lane 1). With addition of 10 mM AAPH, the supercoild form decreased and converted into the open circular form (lane 2). Addition of extract significantly ($p < 0.05$) inhibited the formation of open circular form compared to positive control. The % of DNA protection offered by the leaf extracts (1 mg/ml) was in the decreasing order of methanol (79%) > ethylacetate (73%) > chloroform (71%) > acetone (68%) > hexane (50%) and water extract did not show any protection. With regard to stem, acetone and ethylacetate extracts were the most effective with 96% inhibition followed by chloroform (90%), methanol (81%), water (74%) and hexane (48%) extracts. Our results shows that methonolic extract of leaf, and acetone and ethylacetate extract of stem are the potent agents in preventing the oxidative DNA damage.

Protective effect on biomolecule oxidation *ex vivo*

a) Inhibition of lipid peroxidation

The AAPH model is a well-established system for investigating *in vivo* and *in vitro* lipid peroxidation. In this study, the potential of each of the extracts to inhibit lipid peroxidation in the rat liver homogenate induced by the AAPH was measured. The addition of AAPH to the liver homogenate for 2 hrs significantly increased the extent of TBARS formation relative to the control sample. Induction

of oxidative stress in rat liver homogenate uniformly resulted in an increase in lipid peroxidation levels. However, treatment with the plant extracts significantly decreased TBARS content. Figure 5 clearly demonstrates that the inhibition of lipid peroxidation in leaf extracts (2 mg/ml) was most effective in methanolic extract (69.19%) followed by acetone > ethylacetate > water > chloroform > hexane while among stem extracts, ethylacetate extract (80.23%) was the most effective one followed by acetone > chloroform > methanol > hexane > water extracts. As a positive control, catechin (50 μg/ml) showed high inhibitory effect (91.23%). Consequently, it can be suggested that plant extracts, may be effective in preventing lipid oxidation which is believed to occur during oxidation processes.

b) Inhibition of protein carbonyl formation

Proteins are known to be damaged by ROS directly and to be targets of secondary modifications by aldehydic products of lipid peroxidation or ascorbate autooxidation. All these processes can be collectively result in carbonyl modification of protein (PCO). The assessment of PCO is a widely-used marker for oxidative protein modification and it is reported to be a sensitive and early marker of oxidative stress to tissues as compared with lipid peroxidation.[34] Figure 6 shows the % of inhibition by the extracts (2 mg/ml) against the oxidation of proteins which is measured in terms of protein carbonyl content. Among leaf extracts, the methanol extract showed the highest inhibition (70.25%) followed by acetone, ethylacetate, water, chloroform and hexane in the decreasing order. Among stem extracts, ethylacetate showed

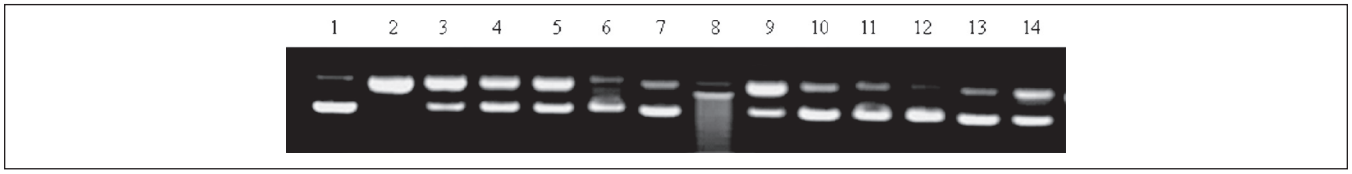

Figure 4: The protection of *T. coridifolia* extracts (1 mg/ml) on 10 mM AAPH induced pBR322 plasmid DNA strand breakage. Lane 1-plasmid DNA alone; lane 2-plasmid DNA + AAPH (positive control); lane 3-8, pBR322 plasmid DNA + AAPH + leaf extract (hexane, chloroform, ethyl acetate, acetone, methanol and water); lane 9-14, plasmid DNA + AAPH + stem extract (hexane, chloroform, ethyl acetate, acetone, methanol and water).

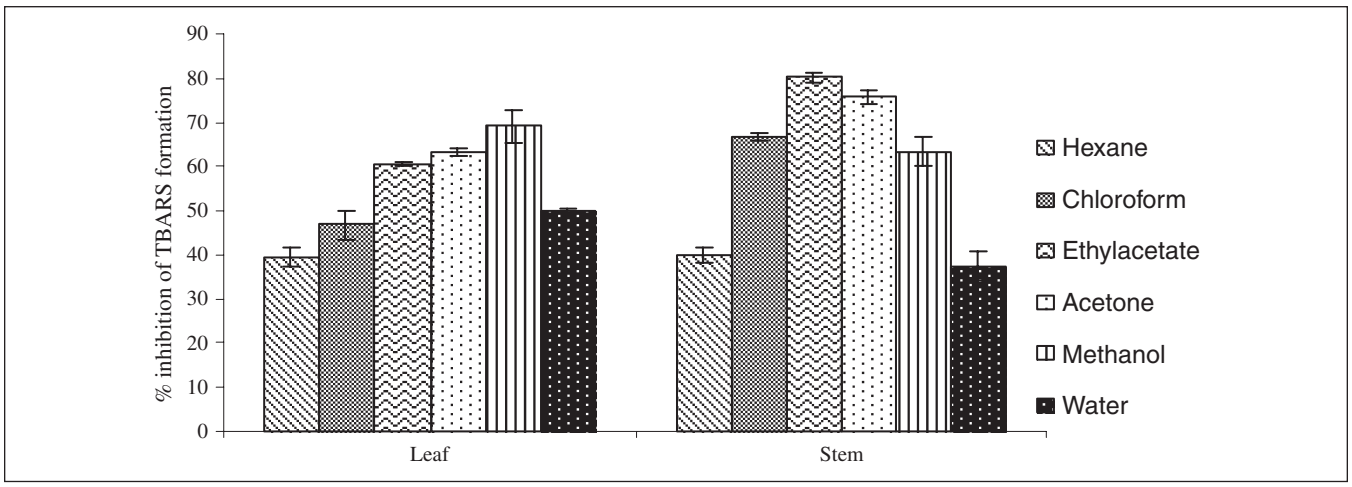

Figure 5: Inhibitory effect of *T. coridifolia* against AAPH reagent induced lipid peroxidation.

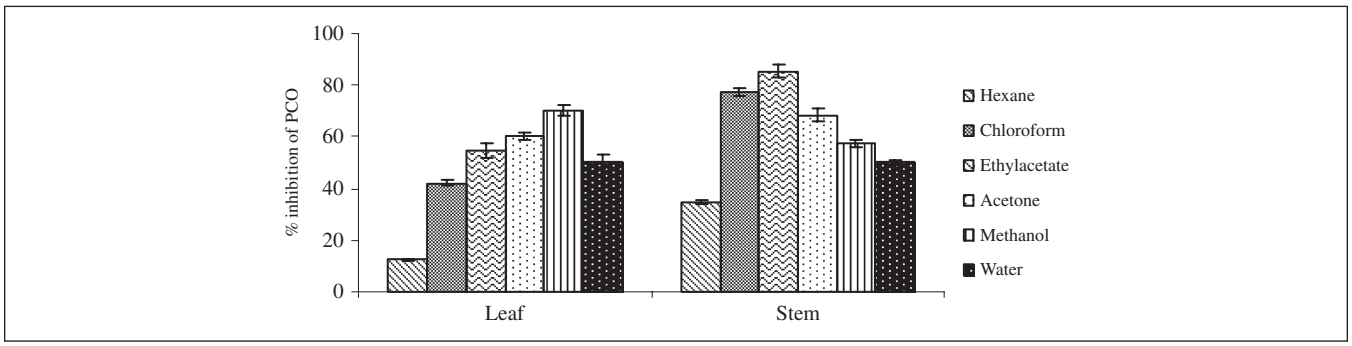

Figure 6: Inhibitory effect of leaf extract of *T. coridifolia* on AAPH reagent induced PCO formation.

highest inhibition (85.45%) followed by chloroform, acetone, methanol, water and hexane. Catechin standard (50 µg/ml) showed 96.72% inhibition against PCO formation. The inhibitory effect of extracts might operate by scavenging the peroxyl radical generated in the reaction mixture.

CONCLUSION

The results of present study indicate that the methanolic extract of leaf and ethyl acetate extract of stem are the most potent extracts compared to other solvent extracts towards various radical scavenging activities. These extracts also conferred significant protection against oxidation of biomolecules such as proteins, DNA and lipids. The radical scavenging ability of the extracts could be due to the presence of phenolic compounds. Overall, the stem extracts showed to be the more effective antioxidant source than the leaf extracts with regard to all the parameters analysed. Therefore stem of *T. coridifolia* can be considered as a potential source of natural antioxidants over the leaf for pharmaceutical use or dietary supplementations.

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