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Antioxidant activity of *Cinchona officinalis* stem bark extracts

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SUMMARY

Stem bark of *Cinchona* sp. (Rubiaceae) is one of the well known drugs for its therapeutic values in traditional as well as modern medicine. Even though a lot of work has been carried out on quinoline alkaloids of *Cinchona*, its phenolic constituents received very little attention. In the present study, we evaluated antioxidant properties of *C. officinalis* stem bark methanolic extract and water extract containing phenolic compounds (total phenolics 21.37, 5.18% w/w respectively in the two extracts) in different *in vitro* and *ex vivo* models *viz.*, antiradical activity by DPPH reduction, superoxide radical scavenging activity in riboflavin/light/NBT system, nitric oxide radical scavenging activity in sodium nitroprusside/Greiss reagent system and inhibition of lipid peroxidation induced by iron-ADP-ascorbate in liver homogenate and haemolysis of erythrocytes induced by phenylhydrazine in erythrocyte membrane stabilization study. Both the extracts exhibited very good antioxidant activity in all the models tested. The phenolic compounds including tannins present in the stem bark seem to offer protection from the oxidative damage.

Key words: *Cinchona officinalis*; Antioxidant activity; DPPH radical; Superoxide radical; Nitric oxide radical; Lipid peroxidation; Erythrocyte membrane stabilization; Phenolic compounds

INTRODUCTION

Cinchona sp. (Rubiaceae) is well known for the therapeutic values of its stem bark and it is also an important commercial source for quinine and quinidine. *Cinchona* stem bark and its preparations are mainly used as bitter tonic and stomachic in traditional medicine (Anonymous, 1950; Evans, 1996). Some of the chemical constituents of the stem bark are quinoline type of alkaloids, the important ones being, quinine, quinidine, cinchonine and cinchonidine. Apart from these, cinchona also contains tannins and small quantities of anthraquinones. The alkaloids are present in bark in combination with the tannins, especially quinic acid and cinchotannic acid. Quinic acid is present to an extent of 5 - 8% (Wallis, 1985; Evans, 1996). Tannins are the naturally occurring polyphenolic compounds widely present in plants. Polyphenols are known to posses various biological activities, including antioxidant properties (Hong *et al.*, 1995; Ravishankara *et al.*, 2002; Bagul *et al.*, 2003). Even though a lot of work has been reported regarding the biological activities of quinoline alkaloids of *Cinchona*, its phenolic constituents received very little attention. In the present study, we evaluated extracts of *C. officinalis* stem bark for antioxidant activity in different *in vitro* and *ex vivo* models. Efforts have been made to evaluate fractions containing tannins, which could be responsible for the antioxidant activity.

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MATERIALS AND METHODS

Plant material

C. officinalis stem bark was collected from Ooty, Tamilnadu, India and authenticated in Pharmacognosy and Phytochemistry Department and a voucher specimen (Number: PERD-PPH-01/98) is preserved.

Chemicals

Thiobarbituric acid (TBA), 1,1-diphenyl-2-picryl hydrazyl (DPPH) were purchased from Sigma Ltd. Trichloroacetic acid (TCA), ferrous sulphate, glacial

acetic acid, sodium dodecyl sulphate (SDS), ethylene diamine tetra acetate (EDTA), sodium nitroprusside, naphthalene diamine dihydrochloride, phenylhydrazine hydrochloride and Folin ciocalteu reagent were purchased from SD Fine Chemicals, India. Riboflavin, nitro blue tetrazolium (NBT), adenyl diphosphate (ADP) and pyrogallol were purchased from Hi-Media Ltd., India, Sulphanilamide, ascorbic acid and α-tocopherol acetate were obtained as gift samples from Cadila Pharmaceuticals Ltd., India. Gallic acid was a gift sample from Tetrahedron Ltd., India. All the reagents used for the experiments were of analytical grade.

Preparation of sample extracts of *C. officinalis* stem bark

Methanolic extract (ME): *C. officinalis* stem bark was dried in a hot air oven for 24 hours at 55°C and powdered to 40 mesh. 100 gms of the powder was extracted with methanol (3×300 ml), filtered and the solvent was removed under reduced pressure (extract obtained=24.8 gms dwb).

Water extract (WE): 100 gm of the powder of *C. officinalis* stem bark was moistened with ammonia (25%) solution and allowed to dry at room temperature. The dried powder was extracted with water (3×50 ml), filtered and the water was removed by heating on a water-bath at 60°C (extract obtained=18.3 gm dwb).

Estimation of total phenolics from ME and WE of stem bark of *C. officinalis*

The total phenolic content of the extracts was estimated according to the method described by Singleton and Rossi (1965). In brief the method is as follows: Stock solution (1 mg/ml) of the *ME* and *WE* was prepared in their respective solvents. From the stock solutions, suitable quantity aliquots were taken. 10 ml of water and 1.5 ml of Folin Ciocalteau reagent were added to it. After 5 min, 4 ml of 20% sodium carbonate solution was added and made up to 25 ml with double distilled water. The mixture was kept for 30 minutes and absorbance of blue colour developed was recorded at 765 nm. Percentage of total phenolics was calculated from calibration curve of gallic acid plotted (as given below) and total phenolics were expressed as % gallic acid.

Calibration curve of gallic acid

10 mg of accurately weighed gallic acid was dissolved in 100 ml distilled water in a volumetric flask (100 μg/ml of stock solution). From this stock solution 0.5 to 2.5 ml of aliquots were pipetted out in to 25 ml volumetric flasks and colour was developed as described above. A calibration curve was prepared by plotting absorbance *vs* concentration of gallic acid.

Free radical scavenging activity

A. Assay for antiradical activity with DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the sample (Navarro *et al.*, 1993; Vani *et al.*, 1997; Ravishankara *et al.*, 2002). A stock solution of DPPH (4.3 mg/3.3 ml methanol) was prepared such that 75 μl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of the sample extracts, ME and WE at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. Pyrogallol was used as positive control.

B. Assay for superoxide radical scavenging activity

The assay was based on the capacity of the sample extracts to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). The reaction mixture contains 50 mM phosphate buffer pH 7.6, 20 μg riboflavin, 12 mM EDTA, NBT 0.1 mg/3ml, added in that sequence. The reaction was started by illuminating the reaction mixture with different concentrations of samples extracts, ME and WE for 150 seconds. Immediately after illumination, the absorbance was measured at 590 nm and EC_{50} was calculated. Ascorbic acid was used as positive control.

C. Assay for nitric oxide scavenging activity

The assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent (Marcocci *et al.*, 1994). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of ME and WE dissolved

in methanol and water respectively and incubated at room temperature for 150 min. The same reaction mixture without the sample but with equivalent amount of solvent served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was recorded at 546 nm. Curcumin was used as positive control (Sreejayan and Rao, 1997).

D. Effect on lipid peroxidation in rat liver homogenate

Rat liver homogenate (10% w/v) was prepared according to the procedure described by Tripathi *et al.* (1996). Peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of liver homogenate (10% w/v), 100 μM $FeCl_3$, 1.7 μM ADP, 500 μM of ascorbate and different concentrations of the sample extracts, ME and WE in 2 ml of total incubation medium. The medium was incubated for 20 min at 37°C and the extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) formed. Results were expressed in terms of decrease in MDA formation by the sample extract. α-tocopherol acetate was used as positive control.

E. Assay for phenylhydrazine induced haemolysis of erythrocytes (membrane stabilization study)

Phenylhydrazine, an autooxidizable substance, under aerobic conditions may react with molecular oxygen to form superoxide radical, hydrogen peroxide, hydroxyl radical, and phenyl radical which initiate peroxidation of unsaturated fattyacids in the membrane phospholipids and bring about changes in erythrocyte membrane structure and function (Rice-Evans and Hochstein, 1981). The assay was carried out according to the procedure described by Cazana *et al.* (1990), with certain modifications. In brief the method is as follows: The reaction mixture comprises of 1 ml of phenylhydrazine hydrochloride (0.5 mM), different concentrations of the sample extracts, ME and WE and 0.1 ml of 20% erythrocyte suspension (from human blood, prepared according to the procedure described by Hill and Thornalley (1983)) made to a total volume of 3 ml with phosphate buffer saline (PBS) solution. The mixture was incubated at 37°C for 1 hour and centrifuged at 1000 g for 10 min. The extent of haemolysis was measured by recording the absorbance of the supernatant at 540 nm. Suitable controls were kept to nullify the effect of solvents and inherent haemolysis. α-tocopherol acetate was used as a positive control.

RESULTS

The antioxidant activity of ME and WE of *C. officinalis* stem bark was evaluated in different *in vitro* and *ex vivo* models.

Free radical scavenging activity

ME and WE showed a concentration dependent antiradical activity by inhibiting DPPH radical, with an EC_{50} value of 8.08 μg/ml and 64.19 μmg/ml respectively (Table 1). ME and WE also scavenged superoxide radicals in a concentration dependent manner, with EC_{50} of 6.92 μg/ml and 30.3 μg/ml,

Table 1. Antiradical activity of ME and WE of *C. officinalis* stem bark observed with DPPH

Sample	Concentration (μg/ml)	% inhibition[a]	EC_{50} (μg/ml)
ME	1.67	06.30 ± 0.12	
	4.17	28.72 ± 0.59	8.08
	8.33	60.71 ± 2.33	
	16.67	95.72 ± 2.65	
WE	16.67	16.40 ± 1.60	
	33.33	28.57 ± 1.07	
	66.67	51.72 ± 1.24	64.19
	100.00	64.55 ± 3.04	
	133.33	76.46 ± 2.37	
	166.67	87.30 ± 0.64	
Pyrogallol			1.67

[a]Mean±SD; n=3

Table 2. Superoxide anion scavenging activity of ME and WE of *C. officinalis* stem bark observed with riboflavin-light-NBT system

Sample	Concentration (μg/ml)	% inhibition[a]	EC_{50} (μg/ml)
ME	2.08	17.29 ± 1.49	
	4.17	40.00 ± 2.23	
	8.33	63.75 ± 5.79	6.92
	12.5	78.96 ± 2.61	
	16.67	97.92 ± 1.85	
WE	16.67	30.63 ± 4.75	
	33.33	54.29 ± 3.23	
	66.67	75.17 ± 6.39	30.31
	100	85.15 ± 2.96	
	133.33	100.0 ± 4.37	
Ascorbic acid			15

[a]Mean±SD; n=3

Table 3. *In vitro* nitric oxide scavenging activity by ME and WE of *C. officinalis* stem bark

Sample	Concentration (μg/ml)	% inhibition[a]	EC_{50} (μg/ml)
ME	1	10.32 ± 3.20	
	5	39.61 ± 2.53	
	10	60.81 ± 4.78	9.93
	20	76.99 ± 5.48	
	40	80.06 ± 1.17	
WE	5	12.41 ± 4.71	
	10	33.89 ± 1.93	
	20	63.46 ± 0.41	15.68
	40	81.73 ± 4.77	
	50	89.82 ± 1.47	
	100	93.17 ± 2.01	
Curcumin			17.47

[a]Mean±SD; n=3

respectively (Table 2). ME showed a better activity than ascorbic acid (EC_{50}=15.3 μg/ml) which was used as positive control in this model. ME and WE also showed efficient NO scavenging activity, with an EC_{50} of 9.9 μg/ml and 15.7 μg/ml, respectively (*cc.* the positive control curcumin EC_{50} 17.17 μg/ml) (Table 3).

Protection from damage due to lipid peroxidation

ME and WE protected liver cells from damage due to lipid peroxidation induced in rat liver homogenate by iron-ADP and ascorbate, with an EC_{50} of 16 μg/ml and 83.05 μg/ml respectively (Table 4), evaluated in terms of malondiadehyde produced. The activity of ME was comparable with that of α-tocopherol, the positive control in this model.

In erythrocyte membrane stabilization study, ME and WE inhibited the haemolysis of erythrocytes induced by phenylhydrazine in a dose dependent manner, with increased protection from 0.3 μg/ml to 53.3 μg/ml and 16.7 μg/ml to 133.3 μg/ml respectively (EC_{50}, 2.99 μg/ml and 30.65 μg/ml respectively) (Table 5). ME showed better protection when compared to α-tocopherol (EC_{50}=12.5 μg/ml). This reveals the ability of the extracts to scavenge most of the free radicals generated, since the mechanism for haemolysis of erythrocytes in this model was due to the generation of different free radicals. ME showed better protection when compared to the positive control α-tocopherol (EC_{50}=12.5 μg/ml).

DISCUSSION

The role of free radical oxidative stress in various

Table 4. Inhibition of lipid peroxidation by ME and WE of *C. officinalis* stem bark induced by iron/ADP/ascorbate system in rat liver homogenate

Sample	Concentration (μg/ml)	% inhibition[a]	EC_{50} (μg/ml)
ME	5	23.51 ± 8.18	
	10	43.91 ± 9.27	
	20	52.97 ± 0.77	16
	40	95.18 ± 2.11	
	80	92.35 ± 1.57	
WE	25	38.92 ± 4.75	
	100	56.31 ± 3.23	
	150	72.44 ± 6.39	83.05
	200	83.81 ± 2.96	
	250	98.86 ± 4.37	
α-tocopherol			13.68

[a]Mean±SD; n=3

Table 5. Erythrocyte membrane stabilizing activity of *C. officinalis* stem bark measured in terms of % inhibition of haemolysis induced by phenylhydrazine

Sample	Concentration (μg/ml)	% inhibition[a]	EC_{50} (μg/ml)
ME	0.33	07.79 ± 2.11	
	1.67	28.29 ± 1.53	
	3.33	55.70 ± 7.80	
	6.67	66.58 ± 4.89	2.99
	13.33	73.56 ± 7.91	
	26.67	78.79 ± 5.78	
	53.33	82.95 ± 4.07	
WE	16.67	19.61 ± 1.65	
	33.33	55.84 ± 6.38	
	66.67	70.76 ± 0.72	30.65
	100	84.36 ± 4.82	
	133.33	86.04 ± 5.53	
α-tocopherol			12.50

[a]Mean±SD; n=3

diseases conditions has been well established. Superoxide radical, hydroxyl radical, peroxy radical, singlet oxygen are some of the important reactive oxygen species that cause damage to the biological systems (Devasagayam *et al.*, 1996). Herbal drugs containing radical scavengers are well known for their therapeutic activity. Certain plants exhibit efficient antioxidant properties due their phenolic constituents (Larson, 1988; Toda *et al.*, 1991). The biological activities of the phenolics include anti-tumor, anti-viral, anti-HIV activities and also inhibition of lipid peroxidation (Haslam, 1996; Ravishankara *et al.*, 2002; Bagul *et al.*, 2003). *C. officinalis* stem bark is known to contain tannins and other phenolic components. In the present experiment, the total phenolic content of the ME and WE was found to be 21.37, 5.18% w/w respectively (6.49, 0.70% w/w respectively, with respect to the powdered stem bark).

In the present study, the antioxidant properties of methanolic extract and water extract of *C. officinalis* stem bark were evaluated, basing on the response obtained in the five different *in vitro* and *ex vivo* models, especially to find if the extracts containing tannins and other phenolic compounds have any role in affording protection. The study was mainly concentrated on three major radicals *viz.*, superoxide, hydroxyl and nitric oxide radicals, since these radicals are mainly responsible for the oxidative damage of the cellular components of our body (Halliwell and Gutteridge, 1985; Marletta, 1989;

Moncada, 1991; Miyake and Shibamoto, 1997), while DPPH is one of the common free radicals used for testing preliminary radical scavenging activity of a compound or a plant extract.

Methanolic extract and water extract of *C. officinalis* stem bark showed very good superoxide and NO scavenging activity along with antiradical activity. The extracts were also found to inhibit lipid peroxidation of liver and heamolysis in erythrocytes efficiently revealing their ability to scavenge most of the free radicals generated in the models used.

The antioxidant activity could be mainly due to the phenolic components of the extracts. In the models used, it was observed that methanolic extract showed about five or more times higher antioxidant activity than that of the water extract, except in NO model, where it is twice that of the water extract. The difference in the amount of total phenolics present in the two extracts correlates fairly with the above difference in the extent of antioxidant activity of the two extracts.

CONCLUSIONS

In conclusion stem bark of *C. officinalis* exhibited very good antioxidant properties. The phenolic compounds in the extracts seem to offer protection from the oxidative damage. This could possibly be the reason that though *Cinchona* stem bark has been used in traditional medicine including homeopathy as febrifuge, bitter stomachic and tonic, no toxic side effects have so far been reported while quinine, when given in pure form, is known to have some side effects.

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