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Research Article

In Vitro Evaluation of Antioxidant Activity of *Colebrookea oppositifolia Smith*.

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Abstract

The present study was carried out to evaluate the antioxidant activities of methanolic extract of *Colebrookea oppositifolia* Smith. (Lamiaceae) leaf in various systems. The free radical scavenging potential was studied by using different antioxidants models of screening using vitamin C (5mM) as standard. About 200, 400, 600 & 800 µg/ml methanolic extract inhibited the FeSO4 induced lipid peroxidation in a dose dependent manner and showed IC50 value 480 ± 1.87µg/ml. The methanolic fraction at 800 µg/ml exhibited significant antioxidant activity in ferrous sulphate induced lipid peroxidation and Superoxide scavenging models with simultaneous improvement in hepatic glutathione (9.16 ± 0.2161µg GSH/mg of wet tissue) and catalase levels (117.22 ± 0.8668 µM of H_2O_2 consumed /min/mg tissue) compared to standard group. The results suggest that the methanolic extract of *Colebrookea oppositifolia* Smith. leaf play an important role in the modulation of oxidative stress.

Key Words: *Colebrookea oppositifolia* Smith., antioxidant activity, Lipid peroxidation, Superoxide scavenging, glutathione, catalase.

Introduction

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions[1]. Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an efficient antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases[2]. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury[3]. Many plant extracts and phytochemicals have been shown to have antioxidant/freeradical scavenging properties[4] and it has been established as one of the mechanisms of their action. Some of the non-nutritive antioxidants of plants are phenolic compounds, flavonoids, coumarins, benzyl isothiocyanate3 etc.[5].

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Colebrookea oppositifolia Smith belonging to the family Lamiaceae are commonly known as Indian Squirrel Tail (English), Binda (Hindi), Pansara (Bengali), Bosiki (Oriya), Dhusure (Nepali), Jolidi (Telegu)[6]. The plant grows wild on hills & plains throughout India mainly subtropical Himalaya, Madhya Pradesh and Deccan Peninsula. Also it is found in Burma, Bhutan, China, Nepal, Myanmar and Thailand[7]. *Colebrookea oppositifolia* Smith is a branched shrub, growing upto 1-3 m tall. There are many pale branches which are hairy when young. Oppositely arranged light green leaves are crowded at branch ends, leaf blade 10-20 × 3-7 cm, base broadly cuneate to rounded, margin crenulate-serrulate, apex long acuminate, adaxially rugulose and puberulent, abaxially densely-tomentose to lanate-tomentose. The leaves are oblong, lancelike, finely serrated, 10-15 cm long.Light colored stems are stout. Numerous tiny white flowers occur in panicles of upright spikes, 5-10 cm long[8].

Material and Methods

Plant material

The leaves of *Colebrookea oppositifolia* Smith were collected from local areas of Sikkim Himalayan region at a high altitude, in the month of July, 2010 and was authenticated at Botanical survey of India, Gangtok, Sikkim. A voucher specimen was deposited at the Department of Pharmacology, Himalayan Pharmacy Institute, Sikkim. The leaves of the plants were thoroughly washed in running water to remove the earthy material and/or adherent impurities and dried in shade.

Preparation of extract

About 700 gm of the air-dried and powdered plant material was extracted by continuous hot percolation method in Soxhlet apparatus with methanol. The extract was concentrated by distilling off the solvent and evaporating to dryness on water bath. On removal of the methanol by evaporation, a sticky dark brown mass was obtained. The percentage yield was found to be 14.9 % w/w. Phytochemical investigations showed the presence of alkaloids, glycosides, flavonoids and other phenolic compounds.

ANTIOXIDANT ASSAYS

1) Lipid Peroxidation Assay

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of

thiobarbituric acid reactive substances (TBARS) by using standard method[9] with minor modifications[10] with the help of spectrophotometer.

Tissue sample preparation for Lipid peroxidation assay[11]

Goat liver was collected from slaughter house;the liver lobes were washed with 0.9% Sodium Chloride solution. (Toremove excess blood). The lobes were dried by blotting papers and were cut into small pieces with a heavy-duty blade. From that 1gm tissue were then homogenized with 10 ml of cold phosphate buffer (pH-7.4) to get 10% homogenate in glass-Teflon homogenizing tubes and filtered to get clear solution. The solution was centrifuged at 3000 r.p.m at 4° C for 10 min. The supernatant was diluted with phosphate buffer to obtain final concentration of protein equal to 8.0-15.0 mg/ml. This solution was taken for lipid peroxidation assay.

Protein concentration was measured by using standard method of Lowry[12].

Assay procedure

Liver homogenates (3.0ml) were taken in six 10 ml test tubes. The first two test tubes were treated as control and standard where buffer and Vitamin C (5mM) were added. In the third to sixth test tubes different concentrations of extract were added. Lipid peroxidation was initiated by adding 100 µl of 15mM ferrous sulphate solution to 3.0 ml of liver homogenate[13]. After 30 min, 200µl of this reaction mixture was taken in a tube containing 3.0 ml of 10% trichloroacetic acid. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 3.0 ml of 0.67% thiobarbituric acid in acetic acid. The mixture was heated in a water bath at 85^0C for 30 minutes, followed by heating in boiling water bath to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm in a spectrophotometer. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of control as per the following formula:

%Inhibition =

$$\frac{(Control\ Absorbance - Test\ Absorbance)}{Control\ Absorbance} \times 100$$

The TBARS concentration was calculated by using the following formula and expressed as nM/mg of tissue[14].

nM of TBARS/mg of tissue =

$$\frac{OD \times Volume\ of\ homogenate \times 100 \times 10^5}{(1.56 \times 10^5) \times Volume\ of\ extract\ taken}$$

Tissue sample preparation for Catalase, GSH, SOD assay

Liver tissue was collected from slaughter house, washed in normal saline and soaked in filter paper. 1gm tissue was then homogenized in 2.0ml M/15 phosphate buffer (pH-7.0) and centrifuged at 3000 r.p.m at 4° C for 1 hr. The supernatant collected was taken for the assay[15].

2) Catalase Assay (Cat) [16]

Catalase activity was measured based on the ability of the enzyme to break down H_2O_2. 10 µl sample was taken in tube containing 3.0ml 30% (w/v) of H_2O_2 in phosphate buffer (M/15 phosphate buffer; pH-7.0). Time required for 0.05 units change in absorbance was observed at 240 nm against blank containing the enzyme source inphosphate buffer free from H_2O_2. The absorbance was noted at 240 nm after the addition of enzyme; Δt was noted till absorbance was 0.45. If Δt was longer than 60 seconds, the procedure was repeated with more concentrated enzyme sample. Reading was taken at every 5 seconds interval. One unit catalase activity is the amount of enzyme that liberates half the peroxide oxygen from H_2O_2 solution of any concentration in 100 seconds at 25^0C which is determined by CAT activity expression:

Moles of H_2O_2 consumed/min (units/mg of tissue) =

$$\frac{2.3}{\Delta t} \times \ln\left(\frac{E_{initial}}{E_{final}}\right) \times 1.63 \times 10^{-3}$$

Where E= optical density at 240nm,

Δt = time required for a decrease in the absorbance.

3) Reduced Glutathione Assay [17]

Reduced glutathione (GSH) activity was assayed according to the method of Ellman. Reduced Glutathione in the liver homogenate was estimated spectrophotometrically by determination of 2-nitro 5-thiobenzoic acid (yellow colour) formed as a result of reduction of DTNB (Dithiobis-(2-nitrobenzoic acid) by GSH, expressed as µg/mg of tissue.

To 0.1 ml of different tissue samples, 2.4 ml of 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then 2.0 ml of distilled water and 0.5 ml of 50 %w/v TCA were added. This mixture was kept on ice for 10-15 min, then centrifuged at 3000 r.p.m. for 15 min. To 1.0 ml of supernatant, 2.0 ml of Tris buffer (0.4M) was added. Then 0.05 ml of DTNB solution (Ellman's reagent; 0.01M DTNB in methanol) was added and vortexed thoroughly. OD was read (within 2-3 min after the addition of DTNB) at 412 nm in spectrophotometer against a reagent blank. Different concentrations (10-50µg) of standard glutathione were taken and processed as above for standard curve. The amount of reduced glutathione was expressed as µg of GSH/mg of wet tissue.

4) Superoxide Dismutase (Sod) Assay [18]

Superoxide dismutase (SOD) activity was assayed according to the method of Marklund and Marklund. The liver homogenates were prepared in Tris (ethylenediamine tetraacetic acid) buffer centrifuged for 40 min at 10000 r.p.m at 4°C, the supernatant was used for the enzyme assay. 2.8 ml Tris-EDTA and 100µl Pyrogallol (2mM) were taken in the cuvette and scanned for 3 min at 420 nm wavelength. Then 2.8 ml Tris-EDTA buffer (pH -8.0),

100µl Pyrogallol and 50µl tissue homogenate were taken and scanned for 3 min at the same wavelength. One unit of SOD activity is the amount of the enzyme that inhibits the rate of auto oxidation of pyrogallol by 50% and was expressed as Units/mg protein/min. The enzyme unit can be calculated by using the following equations:

$$\text{Rate(R)} = \frac{\text{Final OD} - \text{Initial OD}}{3\ min}$$

$$\% \text{ of inhibition} = \frac{\text{Blank OD} - R}{\text{Blank OD}} \times 100$$

Enzyme unit (U) = (% of inhibition/50) x common dilution factor

[50% inhibition = 1 U]

Results and discussion

The results presented in Table-1 showed that the methanolic extract of the ***Colebrookea oppisitifolia*** **leaf** inhibited FeSO4 induced lipid peroxidation in a dose dependent manner. The extract at 800µg/ml exhibited maximum inhibition (61.07 ±2.34%) of lipid peroxidation nearly to the inhibition produced by Vit. C. The IC50 value was found to be 480 ± 1.87µg/ml. The inhibition could be caused by the absence of ferryl-perferryl complex or by changing the ratio of Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by changing the iron itself or combination thereof[19].

Catalase is an enzymatic antioxidant widely distributed in all animal tissues including RBC and liver. Catalase decomposes hydrogen peroxide and helps protect the tissues from highly reactive hydroxyl radicals[20]. 800µg/ml methanolic extract shows higher rate of H_2O_2 consumption and provide the protection highly reactive hydroxyl radicals.

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C[21]. The tissue glutathione levels were significantly elevated in the 800µg/ml extract. Therefore, it clearly demonstrates that the extract at 800µg/ml have protective role against oxidative damage in the liver tissue.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome[22]. The results indicate that methanolic extract of the Colebrookea oppisitifolia leaf is an effective scavenger of superoxide anions and this may be due to the presence of multiple antioxidants with relatively high superoxide scavenging activity.

The main finding of this work is the fact that methanolic extract of the Colebrookea oppisitifolia leaf, shows antioxidant potential. With respect to this, it is possible to consider the methanolic extract of the Colebrookea oppisitifolia leaf as a potential source of natural antioxidants. Thus cyto-protective role of the methanolic extract of the Colebrookea oppisitifolia leaf is justified from the results , and its use in traditional medicine as a mediator of cellular protection receives force.

Table 1: Effect of methanolic extract of *Colebrookea oppisitifolia* leaf on ferrous sulphate induced lipid peroxidation on goat liver homogenate.

Test tube no.	Treatment	% Inhibition	IC_{50} value and confidence interval (µg/ml)	TBARS (nM/mg of tissue)
1	Control	-	-	-
2	Vitamin C (5mM)	85.34 ± 2.35		0.1902 ± 0.046
Concentration of methanolic extract (µg/ml)				
1	200	23.12 ± 2.98	480 ± 1.87	0.9979 ±0.037
2	400	37.16 ± 2.12		0.8157 ± 0.026
3	600	52.79 ± 2.00		0.6128 ± 0.076
4	800	61.07 ±2.34		0.5053 ± 0.056

Values are expressed as Mean ± SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the *P* value was found to be *P*<0.01 when compared with control group.

Table 2: Effect of methanolic extract of *Colebrookea oppisitifolia* leaf on catalase assay on goat liver homogenate.

Test tube no.	Treatment	Initial OD	Final OD	Time (min)	µM of H_2O_2 consumed /min/mg tissue
1	Control	0.7857± 0.0022	0.7688 ± 0.0020	0.30	54.07 ± 0.4860
2	Vitamin C (5mM)	0.8876 ± 0.0006	0.8367 ± 0.0006	0.30	147.58 ± 0.4455
Concentration of methanolic extract (µg/ml)					
1	200	0.8413 ± 0.0082	0.8164 ± 0.0083	0.30	58.99 ± 0.6126
2	400	0.8628 ± 0.0029	0.8378 ± 0.0025	0.30	73.18 ± 0.9034
3	600	0.8684 ± 0.0013	0.8217 ± 0.0012	0.30	98.55 ± 0.4210
4	800	0.8685 ± 0.0019	0.8287 ± 0.0021	0.30	117.22 ± 0.8668

Values are expressed as Mean ± SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the *P* value was found to be *P*<0.01 when compared with control group.

Table 3: Effect of methanolic extract of *Colebrookea oppisitifolia* leaf on glutathione assay on goat liver homogenate.

Test tube no.	Treatment	Absorbance	Reduced glutathione (µg GSH/mg of wet tissue)
1	Control	0.2590 ± 0.0584	4.21 ± 0.9569
2	Vitamin C (5mM)	0.9223 ± 0.0360	14.94 ± 0.5897
Concentration of methanolic extract (µg/ml)			
1	200	0.2773 ± 0.0173	4.36 ± 0.2868
2	400	0.3203 ± 0.0092	5.06 ± 0.1530
3	600	0.4930 ± 0.0199	7.89 ± 0.3227
4	800	0.5703 ± 0.0130	9.16 ± 0.2161

Values are expressed as Mean ± SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's testand the *P* value was found to be *P*<0.01 for all, except test tube no. 6 where *P* > 0.05 when compared with control group

Table 4: Effect of methanolic extract of *Colebrookea oppisitifolia* leaf on superoxide dismutase assay on goat liver homogenate.

Test tube no.	Treatment	Rate of absorbance change	% Inhibition	SOD (U/mg tissue wet)
1	Control	0.0387 ± 0.0096	28.37 ± 0.688	0.57 ± 0.0120
2	Vitamin C (5mM)	0.061 ± 0.0140	60.47 ± 0.663	1.21 ± 0.0145
Concentration of methanolic extract (µg/ml)				
1	200	0.1613 ± 0.0269	32.78 ± 0.653	0.66 ± 0.0145
2	400	0.1727 ± 0.0064	38.22 ± 0.291	0.77 ± 0.0058
3	600	0.165 ± 0.0085	47.01 ± 0.305	0.94 ± 0.0058
4	800	0.1636 ± 0.0107	55.69 ± 0.696	1.11 ± 0.0145

Values are expressed as Mean ± SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the *P* value was found to be *P*<0.01 when compared with control group.

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