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In-vitro and in-vivo antioxidant activity of different extracts of the leaves of *Clerodendron colebrookianum* Walp in the rat

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Abstract

The in-vitro antioxidant activities of different concentrations of the water, alcoholic, petroleum ether and ethyl acetate extracts of the dried leaves of *Clerodendron colebrookianum* Walp, and in-vivo antioxidant activity of the water extract was studied in experimental rat models. The results obtained from in-vitro lipid peroxidation induced by $FeSO_4$-ascorbate in rat liver homogenate showed a significant inhibition of lipid peroxidation by different extracts of *C. colebrookianum* leaf. Water extracts at concentrations (w/v) of 1:30, 1:50, 1:200 and 1:1000 showed the strongest inhibitory activity over the other organic extracts, suggesting maximum antioxidant effect. Chronic feeding of the water extract to Wistar albino rats (both sexes, 150–200 g) in 1 or 2 g kg^{-1}/day doses for 14 days significantly increased the ferric reducing ability of plasma by 19% and 40% on the seventh day, and by 45% and 57% on the fourteenth day of treatment, respectively. Thiobarbituric acid reactive substances (TBARS), as a marker of lipid peroxidation, and some cellular antioxidants (superoxide dismutase, catalase and reduced glutathione) were estimated in heart, liver and kidney. There was a significant reduction in hepatic and renal TBARS with both the doses, without any change in myocardial TBARS. There was no change in the level of antioxidants in heart, liver and kidney, except for the hepatic superoxide dismutase. The findings of this study showed that the leaf extract of *C. colebrookianum* increased the antioxidant capacity of blood and had an inhibitory effect on the basal level of lipid peroxidation of liver and kidney. This lends scientific support to the therapeutic use of the plant leaves, as claimed by the tribal medicine of North-East India.

Introduction

Clerodendron colebrookianum Walp (Family Verbenaceae) is distributed widely in South and South-East Asia (Wang et al 2000). In India, it mainly grows in the north-eastern region up to an altitude of 1700 m (Nath & Bordoloi 1991; Goswami et al 1996). In Indian traditional medicine, the leaves of *C. colebrookianum* (locally known as "Nefafu") are mostly used in the treatment of hypertension (Nath & Bordoloi 1991, 1988). The roots are used as an anthelmintic (Banerjee 1936), antibacterial (Hosozowa et al 1974), and to cure bronchitis, asthma, fever, stomach troubles, syphilis and gonorrhoea (Singh et al 1995). In China, it is used to induce diuresis (Yunnn Medical Material Corporation 1993). Gupta et al (1994) documented the hypotensive effect of *C. colebrookianum* leaf extract in rats. The leaves and roots of *C. colebrookianum* contain many chemical compounds, such as flavones and their glycosides (Jacke & Rimpler 1983; Lin et al 1989), which may have significant antioxidant activity.

Excessive production of reactive oxygen species plays an important role in the pathogenesis and progression of various diseases involving different organs (Visioli et al 2000). Lipid peroxides, produced from unsaturated fatty acids via free radicals, cause toxic effects and promote the formation of additional free radicals in a chain reaction. If the in-vivo activity of enzymes or scavengers is not adequate to neutralize these radicals, oxidative stress develops and leads to various diseases such as atherosclerosis, ischaemic heart disease, liver disease, diabetes, renal dysfunction, or accelerated ageing may result (Niki 1995). The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major

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aetiopathological role. Various population studies support that consumption of fruits and vegetables, rich in antioxidant compounds, are associated with a lower incidence of oxidative stress-induced diseases (Keys 1995; Vayalil 2002). For a very long time the tribal people of the north-east region of India have used the leaves of the plant of *C. colebrookianum* to keep away many degenerative diseases (Singh 1995). The scientific basis of such a beneficial effect of plant leaves is not clear. It is possible that the compounds present in *C. colebrookianum* might contribute to its overall protective effect. Therefore, this study was designed to investigate the in-vitro antioxidant activity of the different extracts of *C. colebrookianum* leaf in the rat to establish its potential therapeutic value.

Materials and Methods

Animals

The study was approved by the Institute Animal Care Ethics Committee. Laboratory bred Wistar albino rats of both sexes (150–200 g), maintained under standard laboratory conditions ($25 \pm 2\,^{\circ}C$, relative humidity $50 \pm 15\%$, normal 12-h dark/light photoperiod), were used for the experiment. A commercial pellet diet and water were freely available. The commercial pellet diet (Ashirwad, India) contained: protein 24%, fat 5%, fibre 4%, carbohydrate 55%, calcium 0.6%, phosphorous 0.3%, moisture 10% and ash 9% w/w.

Plant material

The aerial part of *C. colebrookianum* was collected from Guwahati (Assam, India) during April to August, identified and authenticated in the Department of Botany, Gauhati University, Assam, using some references (Nath & Bordoloi 1988, 1991).

Preparation of extracts

Water extracts

The leaves were dried in shade, and 84 g dried leaves were extracted with warm water. Briefly, 84 g dried powdered leaf was mixed with 840 mL double distilled water, slightly heated (60 °C) and filtered off. The filtrate was lyophilized, weighed (yield 3%) and stored at 4 °C to evaluate the in-vitro and in-vivo antioxidant activity.

Alcoholic extract

Powdered dried leaves (10 g) were soaked with 100 mL absolute alcohol and kept for 72 h at room temperature. The filtrate was separated by filtration, lyophilized and weighed (yield 3%). Four different concentrations were made from this lyophilized powder and used for evaluation of in-vitro antioxidant activity.

Petroleum ether and ethyl acetate extracts

Shade-dried finely powered leaves (1 kg) were exhaustively extracted by a cold maceration process with petroleum ether (60–80 °C, yield 3%) and ethyl acetate (yield 2.8%) sequentially. The extract was lyophilized, weighed and four different concentrations from this lyophilized powder were made for in-vitro antioxidant activity.

Chemicals used

All chemicals were of analytical grade and chemicals required for all biochemical assays were obtained from Sigma Chemicals (St Louis, MO). Double distilled water was used in all biochemical assays.

Assay of in-vitro lipid peroxidation

Preparation of rat liver homogenate

Adult Wistar albino rats (150–200 g) were anaesthetized with sodium pentobarbitone ($35\,mg\,kg^{-1}$), one lobe of the liver was excised and washed with 0.9% NaCl solution. Tissue homogenate was prepared in a ratio of 1 g wet tissue to 10-times (w/v) 0.05 M ice-cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. The homogenate was used for estimation of thiobarbituric acid reactive substances (TBARS).

TBARS assay (Okhawa et al 1979)

Liver homogenate (0.25 mL) was mixed with 0.1 mL Tris-HCl buffer (pH 7.2), 0.05 mL 0.1 mM ascorbic acid, 0.05 mL 4 mM $FeCl_2$ solution and 0.05 mL of the test extracts. All extracts were tested in four different concentrations (w/v) 1:30, 1:50, 1:200 and 1:1000. The mixture was incubated at 37 °C for 1 h and then 1.5 mL 0.8% (w/v) 2-thiobarbituric acid, 1.5 mL 20% acetic acid, and 0.2 mL 8.1% (w/v) sodium dodecyl sulfate were added to the reaction mixture. The mixture was made up to 4.0 mL with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 mL distilled water and 5.0 mL of a mixture of n-butanol and pyridine (15:1, v/v) were added. The mixture was shaken vigorously and centrifuged at 5000 *g* for 10 min. After centrifugation, the optical density of the butanol layer was measured at 532 nm in a spectrophotometer (Beckman, UK).

In-vivo experiment

Groups

Eighteen Wistar albino rats of either sex were divided into three groups of six animals each. Rats of group A served as control. Groups B and C were orally fed water extract of *C. colebrookianum* leaf dissolved in distilled water ($1\,g\,kg^{-1}$/day or $2\,g\,kg^{-1}$/day) for 14 days. After 14 days, rats were killed by decapitation and heart, liver and kidney were excised, washed in cold saline and stored in liquid nitrogen for biochemical study.

Preparation of rat heart, liver and kidney homogenate

Tissue homogenate was prepared in a ratio of 1 g of wet tissue to 10-times (w/v) 0.05 M ice cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. A 0.2-mL sample of homogenate was used for estimation

of TBARS. The remaining part of the homogenate was divided into two parts. One part was mixed with 10% trichloroacetic acid (1:1), centrifuged at 5000 *g* (4 °C, for 10 min) and the supernatant was used for reduced glutathione (GSH) estimation. The remaining part of the homogenate was centrifuged at 15 000 *g* at 4 °C for 60 min, and the supernatant was used for superoxide dismutase (SOD), catalase and protein estimation (Jiankang et al 1990; Bruce & Baudry et al 1995).

Ferric reducing ability of plasma (FRAP) assay (Benzie & Strain 1996)

Total plasma antioxidant capacity was measured according to the FRAP method. This test measured total antioxidant capacity determined by nonenzymatic antioxidants; the main contributors in this test were water soluble antioxidants, whereas plasma proteins and low molecular weight SH group-containing compounds, such as glutathione, had very low activity in this method.

For FRAP assay blood samples were collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 7 and 14 days of treatment. Briefly, 3 mL freshly prepared and warm (37 °C) FRAP reagent was mixed with 375 μL distilled water and 25 μL test sample. The FRAP reagent contained: 1 mL 10 mM TPTZ (2,4,6 tripyridyl-s-triazine) solution in 40 mM HCl, plus 1 mL 20 mM $FeCl_2$ $6H_2O$, plus 10 mL 0.3 mol L^{-1} acetate buffer (pH 3.6). Readings at the absorption maximum (593 nm) were taken for 5 min using a Beckman spectrophotometer. The temperature was maintained at 37 °C. The readings at 180 s were selected for the calculation of FRAP values. $FeSO_4$. $7H_2O$ was used for calibration.

Biochemical parameters

TBARS were measured as a marker of lipid peroxidation by using the procedure described by Okhawa et al (1979), while GSH (Ellman et al 1959), SOD (Kakkar et al 1984) and catalase (Aebi 1974) were estimated as levels of cellular antioxidants.

Statistical analysis

All values are expressed as mean ± s.d. One way analysis of variance was applied to test for significance of biochemical data of the different test groups. Significance was set at $P < 0.05$.

Results

There were no differences in weight gain, food intake and water intake patterns between the control and treated groups. There was no mortality in any group after 14 days feeding of *C. colebrookianum* extract.

In-vitro lipid peroxidation study

All extracts showed inhibition of lipid peroxidation (LPO) in rat liver homogenate in the in-vitro study (Table 1). The relative antioxidant potencies for different extracts of the dried leaves of *C. colebrookianum* were water extract > alcoholic extract > petroleum ether extract > ethyl acetate extract. The water extract showed LPO inhibition in all concentrations (up to 105%), followed by alcoholic extract which showed 99% and 95% inhibition. The petroleum ether extract and ethyl acetate extract (1:30 and 1:50) lowered lipid peroxidation by 86.7% and 36.3%, respectively, but only with the higher concentration (1:30). No antioxidant activity was observed at the lower concentrations for the alcoholic, petroleum ether and ethyl acetate extracts. However, there was an increase in lipid peroxidation with the petroleum ether extract at concentrations of 1:200 and 1:1000, and with the ethyl acetate extracts at 1:50, 1:200 and 1:1000.

In-vivo study

As maximum lipid peroxidation inhibition was found with the water extract of *C. colebrookianum* in the in-vitro study, this was used for the in-vivo study.

FRAP assay

The total antioxidant capacities measured as FRAP in the plasma of rat after administration of *C. colebrookianum* leaf extract over a period of 14 days is shown in Figure 1. In group A there was no change in FRAP value on days 7 and 14 as compared with 0 day. However, in groups B and C on

Table 1 Antioxidant activity of *Clerodendron colebrookianum* leaf extracts. Inhibition of the production of thiobarbituric acid reactive substances (TBARS) in rat liver homogenate. The signs in the parentheses indicate antioxidant activity (+), oxidant activity (−) or no change (0).

Concn of extract	Percent inhibition			
	Water extract	Alcoholic extract	Petroleum ether extract	Ethyl acetate extract
1:30	105.42 ± 3.97 (+)	99.2 ± 12.7 (+)	86.75 ± 8.51 (+)	36.30 ± 11.43 (+)
1:50	104.21 ± 5.44 (+)	95.06 ± 2.15 (+)	(0)	77.51 ± 19.4 (−)
1:200	103.35 ± 5.33 (+)	(0)	114.6 ± 30.57 (−)	106.85 ± 10.06 (−)
1:1000	84.72 ± 11.78 (+)	(0)	304.2 ± 34.42 (−)	117.34 ± 25.12 (−)

*Inhibition percentage calculated by $(1-P_{tm}/P_{cm}) \pm 100$, where P_{tm} is production of TBARS of test samples and P_{cm} is production of TBARS in control samples.

days 7 and 14 there was a significant ($P < 0.05$) increase in FRAP value as compared with 0 day. Maximum enhancement was obtained in group C after 14 days of treatment.

Biochemical parameters

The effect of various doses of water extract of *C. colebrookianum* on basal lipid peroxidation and endogenous antioxidants of liver, heart and kidney are shown in Figures 2, 3 and 4, respectively. A significant ($P < 0.01$ and $P < 0.05$) decrease in TBARS concentration in liver tissue in groups B and C (281.5 ± 27.3 and 303 ± 38.3 nmol (g wet wt)$^{-1}$, respectively) compared with group A (407.8 ± 83.7 nmol (g wet wt)$^{-1}$) was observed. There was no significant change in GSH and catalase activity in liver, but SOD activity was reduced significantly ($P < 0.01$) in groups B and C (22.7 ± 4.8 and 12.7 ± 2.5 U (mg protein)$^{-1}$, respectively) as compared with group A (35.7 ± 5.0 U (mg protein)$^{-1}$).

There was no change in TBARS and endogenous antioxidant levels in heart tissue in the treated groups (B and C) compared with control group (A).

In kidney, there was a significant ($P < 0.05$) decrease in TBARS concentration in groups B and C (208.4 ± 26.7 and 222.4 ± 31.1 nmol (g wet wt)$^{-1}$, respectively) compared with the control level (259.3 ± 14.2 nmol (g wet wt)$^{-1}$. However, there was no change in the endogenous antioxidant levels (GSH, SOD and catalase) in groups B and C compared with group A.

Discussion

Oxidative stress has been implicated in the pathogenesis of many cardiovascular diseases (Harrison 2003). It may be

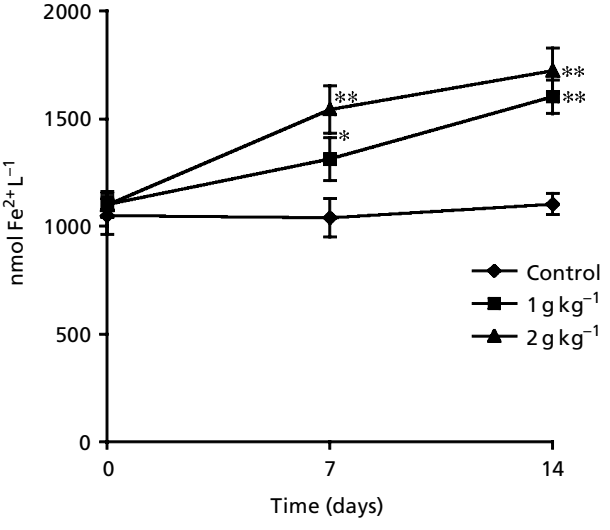

Figure 1 Changes in rat total antioxidant capacity of plasma (FRAP) measured by Fe^{2+} equivalent after administration of water extracts of *Clerodendron colebrookianum*. Values are mean ± s.d. (n = 6). $^{*}P < 0.01$, $^{**}P < 0.001$.

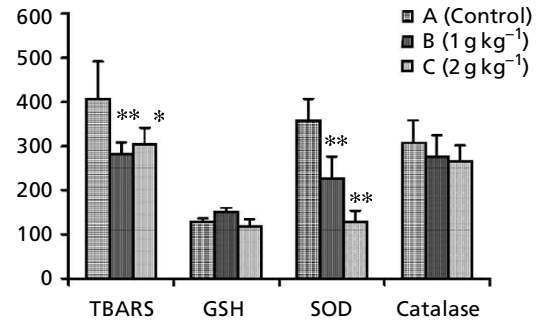

Figure 2 Changes in rat liver TBARS (nmol (g wet wt)$^{-1}$), GSH (10^{1} μg (g wet wt)$^{-1}$), SOD (10^{-1} U (mg protein)$^{-1}$) and catalase (10^{-1} U (mg protein)$^{-1}$) following oral administration of water extracts of *Clerodendron colebrookianum*. Values are mean ± s.d. (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ (one-way analysis of variance) compared with control.

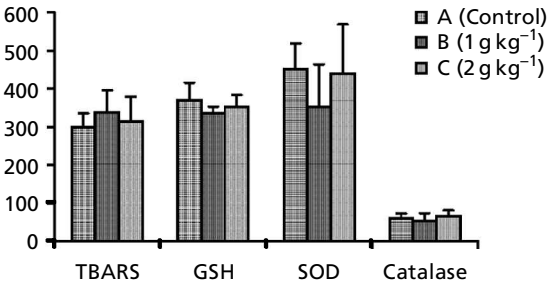

Figure 3 Changes in rat myocardial TBARS (nmol (g wet wt)$^{-1}$), GSH (μg (g wet wt)$^{-1}$), SOD (10^{-1} U (mg protein)$^{-1}$) and catalase (10^{-1} U (mg protein)$^{-1}$) following oral administration of water extracts of *Clerodendron colebrookianum*. Values are mean ± s.d. (n = 6).

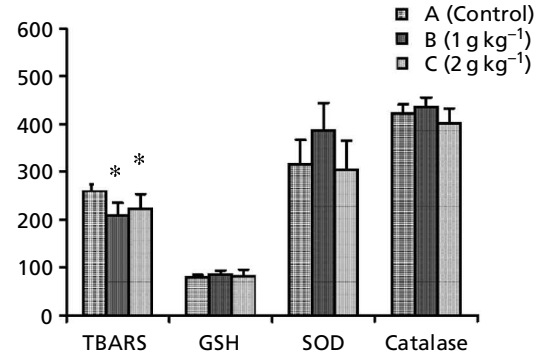

Figure 4 Changes in rat kidney TBARS (nmol (g wet wt)$^{-1}$), GSH (10^{-1} μg (g wet wt)$^{-1}$), SOD (10^{-1}U (mg protein)$^{-1}$) and catalase (10^{-1} U (mg protein)$^{-1}$) following oral administration of water extracts of *Clerodendron colebrookianum*. Values are mean ± s.d. (n = 6). $^{*}P < 0.05$ (one-way analysis of variance) compared with control.

possible to limit oxidative stress-induced tissue damage and, hence, prevent or ameliorate disease progression by favouring the balance towards lower oxidative stress. An increase in the consumption of foods rich in antioxidant nutrients may decrease the risk of developing many disease including coronary heart disease and cancers (The Scottish Office 1993). The antioxidant systems in living organisms include various enzymes, macromolecules and an array of small molecules. Several methods have been developed to assess the total antioxidant capacity of serum or plasma because of the difficulty in measuring each antioxidant component separately in the serum or plasma (Cao & Prior 1998). One of these is the ferric reducing ability of plasma (FRAP), which measures the reduction of ferric to ferrous iron in the presence of water soluble exogenous antioxidants (Benzie & Strain 1996).

Oral administration of *C. colebrookianum* leaf extract increased the antioxidant capacity of plasma, as evidenced by the increase in FRAP. The reducing activity of the different doses of water extract varied markedly. The significant increase in FRAP after oral administration of water extract indicated the presence of bio-available antioxidants in *C. colebrookianum* leaf. Previous studies indicated that the change in FRAP reflected an increase in plasma concentration of both ascorbate and phenolics (Pederson et al 2000), which are readily bioavailable and can efficiently scavenge a range of oxidizing species in cells and plasma (Sies et al 1992). Our result suggested that *C. colebrookianum* leaves had antioxidant compounds, which were responsible for the increase in FRAP in plasma following oral administration. Increased total antioxidant capacity in plasma following consumption of the *C. colebrookianum* extract was also associated with the decreased lipid peroxidation in in-vitro and in-vivo treatment.

The liver, the largest organ in the body, has many complex functions. Due to its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target of the toxicity of drugs, xenobiotics and oxidative stress, which is why the liver was chosen for our in-vitro antioxidant experiment. The in-vitro lipid peroxidation in liver homogenate proceeds in enzymatic and non-enzymatic processes. The former process is NADPH-dependent, but the latter process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, even with boiled liver homogenate. Lin et al (1998) reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. In this in-vitro study, all of the extracts inhibited the $FeCl_2$–ascorbic acid stimulated lipid peroxidation in rat liver homogenate (Table 1). The water extract at the concentrations (w/v) of 1:30, 1:50, 1:200 and 1:1000 exhibited the best response against lipid peroxidation in comparison with the other organic extracts.

From a practical viewpoint, biological membranes, richly endowed with highly peroxidizable, polyunsaturated fat, are very susceptible to severe oxidative damage during oxidative stress. The ideal defense against this is the suppression of free radical reactions in-vivo. This form of inhibition is, in effect, the function of primary or preventive antioxidants, which act by removing free radicals (Burton & Ingold 1984). Thus the basal level of endogenous antioxidant has an important role to play to reduce oxidative stress.

In this in-vivo study, water extract of *C. colebrookianum*, after chronic oral administration, caused a significant reduction of lipid peroxidation (TBARS) in liver and in kidney, but there was no change in lipid peroxidation in heart compared with control. It was interesting to find no change in the endogenous antioxidants SOD, catalase and GSH; however, there was a change in SOD in liver tissue. SOD is a key enzyme for dismutation of superoxide to hydrogen peroxide, and thus provides the first step in metabolizing this radical (Steare & Yellon 1995). The lowering of the level of SOD in liver tissue indicated that there was a reduced need for SOD for trapping the superoxide radical. In liver, the lowering of TBARS with concomitant lowering of SOD indicated that there was reduced basal oxidative stress after administration of *C. colebrookianum* extract.

Conclusion

This study suggested that the *C. colebrookianum* leaf extract had significant antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases of major organs such as the liver, kidney and heart.

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