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Protective Effect of Leaf Extract of *Trichilia connaroides* on Hypercholesterolemia Induced Oxidative Stress

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Abstract: The current study envisaged evaluation of potential antioxidant role of chloroform and methanol extracts of leaves of *Trichilia connaroides* in hypercholesterolemia induced oxidative stress. Male Albino Wistar rats were rendered hypercholesterolemic by feeding them with high fat diet. Hypercholesterolemic animals were then treated orally, each day with chloroform and methanol extract of leaves of *Trichilia connaroides* (CETC and METC) (100 mg kg⁻¹ b.wt.) for a period of eight weeks. At the end of the eight week, biomarkers (in hepatic tissue) of oxidative stress viz., products of lipid peroxidation (MDA), catalase, superoxide dismutase (SOD) and reduced glutathione (GSH). Hypercholesterolemic rats had significantly elevated levels of products of lipid peroxidation, LPO (p<0.001), 0.2184±0.0071 nmol mg⁻¹ of protein, compared to normal animals (0.1610±0.0083 nmol mg⁻¹ of protein). Significantly lower levels of catalase (p<0.01) 2.054±0.2234 units mg⁻¹, normal 2.726±0.1236 units mg⁻¹), SOD (p<0.001, 3.373±0.1653 units mg⁻¹ of protein, (normal 4.906±0.0780) and GSH (p<0.001, 8.498±0.4805 μmoles mg⁻¹ of protein (normal 12.69±0.63910) were observed in such animals indicating the development of pro-oxidant status in these animals. CETC and METC extract treated animals recorded significantly reduced levels of LPO (p<0.001, 0.1725±0.0094, 0.1744±0.0032 respectively) and significantly elevated levels of SOD (p<0.001, 4.705±0.1632, 4.752±0.1220) and GSH (p<0.01, 11.71±0.4930, p<0.001, 12.92±0.5890), respectively. Levels of LPO and of endogenous antioxidant enzyme levels were restored closer to that of normal animals, signifying reversal of oxidative stress. CETC and METC protected the animals against hypercholesterolemia induced oxidative stress.

Key words: *Trichilia connaroides*, pro-oxidant state, antioxidant, lipid peroxidation, hypercholesterolemia

INTRODUCTION

Free Radicals (FR) can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic and molecular orbits and this unpaired electron usually gives a considerable degree of reactivity to the free radicals. Oxygen free radicals/Reactive Oxygen Species (ROS) and reactive nitrogen species are products of normal cellular metabolism and are well recognized to play a dual role as deleterious and beneficial species. The harmful effects causing potential biological damage is termed as Oxidative Stress (OS), likely to occur when there is overproduction of ROS on one side and deficiency of enzymatic and non-enzymatic antioxidants on the other side. In other words, OS results from metabolic reaction that represents a disturbance in equilibrium status of pro-oxidant/antioxidant reactions in living organisms. Excess of ROS can damage cellular lipids, proteins, or

DNA, thus inhibiting normal functions, therefore, implicated in number of human diseases; cardiovascular, cerebrovascular (stroke), cancer, diabetes, aging-to mention few. The delicate balance between beneficial and harmful effects of FR is important and achieved by mechanism-Redox regulation, which protects the living organisms from various oxidative stresses and maintains redox homeostasis by controlling redox status *in vivo* (Valko *et al.*, 2007). While considering the role of FR in diseases, its generation, is one aspect and more important being its control, containment and safe disposal. Free radicals are continuously generated and physiologically need to be closely controlled (since they are chemically reactive) to avoid damage to vital components. To maintain cell and tissue integrity the 'antioxidant system' maintains a check and balance with the production of reactive free radicals regarding their use in essential pathways and effective clearance

(Crowther, 2005). is now well-established fact that majority of cardiovascular diseases-the leading cause of death and disability all over the world results from the complications of atherosclerosis. An important initiating event for atherosclerosis may well be the transport of oxidized cholesterol (Ox-LDL) across endothelium into artery wall (which is) likely to occur at the sites of endothelial damage, caused by Ox-LDL itself (Madamanchi *et al.*, 2005). The accumulated evidence that oxidative modification of low-density lipoprotein playing an important role in the pathogenesis of atherosclerosis in animal model is very strong (Steinberg, 2009). A large number of studies in experimental animals have shown that the common risk factor for atherosclerosis being increased production of free oxygen radicals, not only by endothelial cells, but also by vascular smooth muscles and adventitious cells. Hypercholesterolemia, diabetes, hypertension, ageing-all increasing the production of ROS and these have been shown to initiate several processes involved in atherogenesis, including expression of adhesion molecules, stimulation of vascular smooth cells proliferation and migration, apoptosis in endothelium, oxidation of lipids and vasomotor activity (Harrison *et al.*, 2003).

Ever since the role of FR in the pathophysiology was proposed and substantiated, antioxidants-especially those derived from herbal sources have been increasingly gaining universal acceptance as a potential strategy for the management of cardiovascular diseases. In the recent past, several herbs have been subjected to extensive examination-at clinical and laboratory animal models mimicking human atherosclerosis. Such herbs include those popular in folklore for the treatment of cardiovascular diseases or with well defined chemistry, especially rich in bioactive flavonoids, terpenoides etc. and for obvious reasons have drawn the attention of investigators. Majority of the reported finding had assessed the endogenous antioxidant enzymes levels to assess the degree of protection against FR or hypercholesterolemia induced oxidative stress (Ochami and D'Mello, 2009; Kusku-Kiraz *et al.*, 2010; Kucukgergin *et al.*, 2009; Yazdanparast *et al.*, 2008).

Trichilia connaroides (Wight and Arn.) Benth. [(Syn: *Zanthoxylum connaroides* (Wight and Arn.) Benth, *Heynea trijuga* (Roxb.ex sims) (Family: Meliaceae)] found in moist forests through out greater part of India and is referred locally as *Karai*, *karavilangu*, *korakudi*, *kuravatti*, *Kora*, etc., in local/vernacular language. etc. (Shastri, 1959), is a rich source of terpenoides (Puroshothaman *et al.*, 1983; Puroshothaman *et al.*, 1987; Mathuram and Kundu,

1990) and reported to possess significant biological activities (Ashok *et al.*, 2003, 2005, 2006). Literature review revealed paucity of study on effect of this herb on pro-oxidant status. The current study envisaged evaluation of potential antioxidant role of chloroform and methanol extracts of leaves of *Trichilia connaroides* in hypercholesterolemia induced oxidative stress.

MATERIALS AND METHODS

Collection and extraction: Fresh leaves of *Trichilia connaroides* were collected in the month of November 2009 from Chaurla ghat section of Jhamboti village of Belgium district of Karnataka and were authenticated. A voucher specimen of the same has been deposited at Regional research Centre, Indian Council of Medical Research, Belgium and also in the Department of Pharmacology of our institution (H1/Tc/2007-8). Shade dried leaves were coarse powdered, and around 400 g of leaves was defatted with n-hexane (2.5 L), by leaving the powder with the solvent overnight, with occasional shaking and successively extracted with chloroform and methanol (2.5 L of each), in a soxhlet extractor. The Chloroform Extract (CETC) (around 8% yield,) Methanol Extract (METC) (around 10% yield) were stored in airtight containers in cool, dry place, away from sunlight till further use.

Chemicals: Solvents like n-hexane, n-butanol, chloroform, methanol were of analytical grade (M/s Qualigens, India), and ingredients for high fat diet were locally procured and cholesterol and bile acid were of analytical grade (M/s Spectromed and M/s LOBA Chemie, Mumbai). Thiobarbituric acid (M/s Spectrochem, Mumbai, Folin Folin-ciocalteu reagent, phenazine methosulphate, Nitroblue tetrazolium chloride and sulfosalicylic acid and others were from M/s SDFC, Mumbai, India.

Preparation of high fat diet: High fat diet was prepared according to formula standardized in our laboratory containing cholesterol upto 6%, bile salts 2% and adequate amount of carbohydrate and vitamin mixture. This high fat diet was prepared in our laboratory at regular intervals during the study and suitably stored. Usually the quantity required for a week is prepared and stored.

Animals: Healthy, male, Wistar albino rats (130-150 g) were purchased from a registered breeder and maintained in the animal house facility of this

institution, in accordance with guidelines of Committee for Purpose of Control and Supervision of Experimental Animals, Ministry of Environment and Forestry, Government of India. During the study, the test animals either received commercial pelleted diet (M/s Gold Mohr, India) or specially formulated high fat diet and water *ad libitum*. Animals were allowed for a week for acclimatization. Ethical Clearance was taken from the Institutional Animal Ethics Committee of this institution prior to experimentation (626/02/a/CPCSEA).

Induction of hypercholesterolemia: Healthy, male, Wistar albino rats weighing 130-150 g, (whose initial serum cholesterol level was recorded) had free access to high fat diet for 6 weeks. At the end of 6th week, animals with serum cholesterol level above 150 mg dL⁻¹ were selected for study.

Treatment protocol: Animals with serum cholesterol above 150 mg dL⁻¹ were randomly assigned to three groups of eight animals each (n = 8) and simultaneously, age/weight and sex matched normal animals, served as control.

Group I (Normal control) were administered 0.2 mL of Tween 80 in water.

Group II was a hypercholesterolemic control and received no treatment.

Group III and IV animals were treated with CETC (100 mg kg⁻¹ b.wt.) and METC (100 mg kg⁻¹ b.wt) respectively.

Treatment was for a period of eight weeks (starting from June 2010) when test animals received respective treatment orally, every day and continued to have free access to high fat diet. At the end of treatment, animals of all groups were sacrificed by cervical dislocation, liver was removed, homogenized and used for the biochemical estimation of total protein content, TBARS/lipid peroxides, catalase, superoxide dismutase, and reduced glutathione.

Total protein estimation: Modified procedure of Lowry described by Pomory (2008) was followed.

Measurement of lipid peroxides: The determination of Thiobarbituric Acid Reactive Substances (TBARS) i.e., Malondialdehyde (MDA) as an index of lipid

peroxidation was estimated as described by Wills (Wills.,1966)

Measurement of Catalase: Catalase activity in homogenate was measured according to method described by Sinha (1972).

Measurement of Superoxide Dismutase (SOD): SOD activity in homogenate was estimated according to method described by Kakkar *et al.* (1984), a modified version of Nishikimi *et al.*

Estimation of Reduced Glutathione: Reduced glutathione in the homogenate was estimated as described by Ellman (1959).

RESULTS

Levels of various biomarker enzyme levels in normal animals, hypercholesterolemic control, and extracts treated animals are as shown in Table 1.

Effect of extracts on lipid peroxidation: Hypercholesterolemic control animals, (at the end of the study), recorded a significantly elevated levels of LPO (p<0.001) compared to age, sex and weight matched normal animals treated with vehicle and maintained on normal pelleted diet. However, hypercholesterolemic animals treated with CETC and METC (100 mg kg⁻¹ b.wt.) recorded significantly (p<0.01) reduced level of the same, restoring the level closer to untreated normal animals.

Effect of extracts on catalase: Hypercholesterolemic control animals, recorded a significantly lower level of catalase (p<0.01) compared to age, sex and weight matched normal animals treated with vehicle and maintained on normal pelleted diet. Hypercholesterolemic animals treated with CETC and METC (100 mg kg⁻¹ b.wt.) animals recorded significantly elevated levels (p<0.01 and p<0.001, respectively) of the catalase indicating restoration of levels of catalase closer to normal, untreated animals.

Effect of extracts on Superoxide Dismutase: Hypercholesterolemic control animals, recorded a

Table 1: Effect of extracts treatment on oxidative stress biomarkers

Group	LPO (nmol mg ⁻¹ of protein)	Catalase (units mg ⁻¹)	SOD (units mg ⁻¹ of protein)	GHS (μ moles mg ⁻¹ of protein)
I-Vehicle control	0.1610±0.0083	2.726±0.1236	4.906±0.078	12.69±0.6391
II- Hypercholesterolemic control	0.2184±0.0071*** ¹	2.054±0.2234** ¹	3.373± 0.1653*** ¹	8.498±0.4805*** ¹
III- CETC treated (100mg/kg)	0.1725±0.0094*** ²	3.009± 0.0808** ²	4.705± 0.1632 *** ²	11.71±0.4930** ²
IV- METC treated(100mg/kg)	0.1744 ± 0.0032*** ²	3.221± 0.1911*** ²	4.752± 0.1220 *** ²	12.92± 0.5890*** ²

CETC= Chloroform extract of leaves of *Trichilia connaroides*. METC= Methanol extract of leaves of *Trichilia connaroides*. ¹elevated level, ² lowered/reduced. ***¹ p< 0.001, **¹ p<0.01, when compared to vehicle control group of animals. ***² p<0.001, **² p< 0.01, when compared to hypercholesterolemic control animals

significantly lower level of superoxide dismutase ($p < 0.001$) compared to age, sex and weight matched normal animals treated with vehicle and maintained on normal pelleted diet during the month long study. However, treatment with CETC and METC (100 mg kg^{-1} b.wt.) to hypercholesterolemic animals resulted in significantly elevated levels ($p < 0.001$) of the superoxide dismutase.

Effect of extracts on reduced glutathione:

Hypercholesterolemic control animals, at the end of the study, recorded a significantly lower level of reduced glutathione ($p < 0.001$) compared to age, sex and weight matched normal animals treated with vehicle and maintained on normal pelleted diet during the month long study. CETC and METC (100 mg kg^{-1} b.wt.) treatment restored the level that is closer to vehicle control animals, as indicated by significantly elevated levels ($p < 0.01$ and $p < 0.001$, respectively) of reduced glutathione in hypercholesterolemic animals.

DISCUSSION

In the current study, we have investigated the protective role of leaf extracts of TC, in hypercholesterolemic/high fat diet fed animals-a condition that mimics the risk factor for atherosclerosis. Using hypercholesterolemic animals was justified in the light of reports of elevated oxidative stress parameters in plasma, plasma lipoproteins, erythrocytes and several other tissues as observed in experimental animals fed with high cholesterol diet (Mahfouz and Kummerow, 2000; Kumar *et al.*, 2006; Shih *et al.*, 2008). An increase in oxidative stress parameters have also been detected in hypercholesterolemic patients (Lavy *et al.*, 1991; Balkan *et al.*, 2004).

Since hypercholesterolemia and oxidative stress are involved in the pathogenesis of atherosclerosis, the current study examined the protective role of these extracts in altering OS markers due to its rich bioconstituents, therefore, likely to be beneficial as a prophylactic measure in preventing consequences of atherosclerosis. The current study is similar to the one that's been reported by several authors, investigating the potential role of herbs for antioxidant effect (Brown and Evans, 1998; Balkan *et al.*, 2003; Sudhadar *et al.*, 2007).

Feeding leads to elevated plasma and tissue cholesterol level in experimental animals and can be used to study the pathogenesis of atherosclerosis, as well to study the protective effect of herbs and synthetic drugs. In this current study, rats were fed

with diet containing upto 6% cholesterol and 2% bile acid to ensure adequate absorption, for 6 weeks to ensure development of hypercholesterolemia, as evidenced by significantly elevated plasma total cholesterol level and reduced HDL-c. In our study, we found that hypercholesterolemic animals, had elevated hepatic levels of oxidant biomarkers levels (MDA) and depressed antioxidant enzyme levels (Catalase, SOD, and GSH). Hypercholesterolemia disturbed the oxidant-pro-oxidant balance in favor of pro-oxidation as observed in several studies (Mahfouz and Kummerow, 2000; Balkan *et al.*, 2004).

The efficiency of this defense system is apparently weakened in hypercholesterolemia, resulting in ineffective scavenging of free radicals, which lead to tissue damage (Aruoma, 1994; Halliwell, 1994). There is growing evidence that excess generation of highly reactive free radicals, largely due to hypercholesterolemic or hyperlipidemic producing OS, which further exacerbates the development and progress of atherogenesis (Penn and Chisolm, 1994). MDA is an end product of LPO and is a measure of free radical generation and an elevated level of the same in high fat diet fed rats suggests that hypercholesterolemia could enhance the process of lipid oxidation-the possible explanation is that hypercholesterolemia could elevate the cholesterol content in the platelets, polymorphonuclear neutrophils, lymphocytes and endothelial cells, which initiates series of reaction, leading to generation of oxygen free radicals, thus speeding up the course of lipid peroxidation (Stuart *et al.*, 1980; Ismail *et al.*, 1999). MDA-most frequently used indicator of lipid peroxidation (Nur Azlina and Nafeeza, 2007; Sabina and Rasool, 2007; Yassa *et al.*, 2008) and the attenuated levels of this in extracts treated animals is suggestive of the antioxidant nature of them. Logically, any decrease in LPO is likely to reduce the chances of development of atherosclerosis in hypercholesterolemic patients.

SOD catalyses dismutation of superoxide anions into hydrogen peroxide. CAT and GPX, which detoxify H_2O_2 and convert lipid peroxides to non-toxic alcohols. The important hepatic detoxification elements are GPX, GSH and glutathione reductase (Shaw *et al.*, 1983). SOD and CAT are the major enzymes dealing with reactive oxygen species in most cells and both of them deal with reactive oxygen species in most cells. Both these enzymes play an important role in the elimination of reactive oxygen species derived from the redox processes of xenobiotics in the liver tissues. Studies have shown that hypercholesterolemia diminishes the antioxidant defense system and decreases the activity

of SOD and CAT, thereby elevating the lipid peroxide content. Further, CAT and SOD are easily inactivated by lipid peroxides or reactive oxygen species, thus accounting to lower SOD and CAT activities in the liver of high fat diet fed rats (Anila and Vijayalakshmi, 2003; Halliwell and Gutteridge, 1984; Daniel *et al.*, 1998). Extracts treated hypercholesterolemic animals had significantly elevated levels of SOD and CAT, reversing the ill effects of hypercholesterolemia.

GSH, a reactive, intracellular, non-protein (tripeptide) thiol in living organisms, performs a key role in coordinating innate antioxidant defense mechanism. It is involved in the maintenance of normal structure and function of cells, probably through redox and detoxification reactions (Guerra and Grisolia, 1980). In the current study, hypercholesterolemic rats had significantly lower levels of GSH and extracts treated hypercholesterolemic animals, a significantly elevated level of GSH (hepatic) and it is possible that extracts might have reduced the extent of oxidative stress, leading to lesser GSH degradation or increase in the biosynthesis of GSH.

Biological antioxidants are natural compounds, which can prevent the uncontrolled formation of free radicals and activated oxygen species and or inhibit their reaction with biological structures-which includes above discussed antioxidant enzymes and non-enzymatic components. Role of natural products or antioxidant principle rich herbs producing beneficial effects in disease processes and strengthening the endogenous antioxidant defenses against reactive oxygen species and restoring optimal balance by neutralizing the free radicals is well established. The observed beneficial effects of extracts in hypercholesterolemic animals is likely to be due to presence of several chemical (antioxidant) constituents-suppressing lipid peroxidation, modulating antioxidant enzymes and non-enzymatic components.

CONCLUSION

Oral treatment of chloroform and methanol extract of leaves of *Trichilia connaroides* in the dose of 100 mg kg⁻¹ b.wt. significantly altered the levels of biomarkers of oxidative stress in hypercholesterolemic animals, thus eliciting a protective role against deleterious/harmful effects of oxidative stress. Thus, the use of such extracts is likely to be beneficial in the management of disease/disorder, where, oxidative stress plays a significant role-especially in hypercholesterolemia or hyperlipidemia.

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