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Role of Phenolics in Anti-Atherosclerotic Property of *Thuja occidentalis* Linn.

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Abstract

The present study was carried out to evaluate the Lipid peroxidation activity and related hypolipidaemic activity of an (EFTO) ethanol fraction of extract of aerial part of *Thuja occidentalis* Linn. (Cupressaceae). Lipid peroxidation activity was carried out to evaluate the antioxidant potential, and hypolipidaemic activity on cholesterol fed rats. The antioxidant activity of ethanol fraction was increased in a concentration dependent manner. About 100, 150, 200, 250 & 300 µg EFTO (ethanol fraction of extract of aerial part of *Thuja occidentalis*) inhibited the $FeSO_4$ induced lipid peroxidation in a dose dependent manner and showed IC_{50} value 195.60µg/ml. in hypolipidaemic activity

EFTO at the dose of 200 mg and 400mg/kg body weight significantly reduced serum cholesterol (77 and 92%), LDL (53 and 84%), triglycerides (27 and 46%). The increase in HDL to total cholesterol ratio and reduction in atherogenic index in EFTO treated groups strongly supports anti-atherosclerotic property of *Thuja occidentalis.* The results obtained in this study indicate that EFTO can be a potential source of natural antioxidant and activities related to this.

Keywords: *Thuja occidentalis*; Antioxidant; hypolipidaemic activity.

Introduction

Thuja occidentalis, commonly known as Arbor vitae or white cedar, is indigenous to North America and is grown in Europe as an ornamental tree. In folk medicine, *Thuja occidentalis* has been used to treat bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea

and rheumatism (1). Extract of this plant has shown anti oxidant, anti viral, anti diarrhoeal activity (2, 3). It has been reported to increase the proliferation of spleen cells as well as increase in TNF-α, IL-6 and IL-1 activity in serum and also have protective effect against radiation-induced toxicity (4). Today it is mainly used in homeopathy as mother tincture or dilution. The aim of the present investigation was to evaluate the possible hypolipidaemic activity of *Thuja occidentalis* aerial part.

Materials and Methods

Plant material

Fresh aerial part (twigs) of *Thuja occidentalis* were collected from Jaipur, Rajasthan, India, in October-2007 and were authenticated by experts of Deptt. Of Botany University of Rajasthan, Jaipur. The voucher specimen is preserved for further research in our laboratory.

Preparation of extract

Shade dried and powdered twigs (40-mesh size, 1kg) were soxhlet extracted with 90% EtOH (Dept. of Botany), the solvent was removed and the residue was triturated with hot (65^0C) petroleum ether ($60\text{-}80^0$C). Solvent was evaporated from the petroleum ether soluble portion and the residue dissolved in ethanol. On removal of the ethanol by evaporation, a semi solid reddish brown mass (12.76g) was obtained. Phytochemical investigations showed the presence of flavonoids (quercetin, kaempherol), tannic acids, polysaccharides and proteins.

Animals

Wistar albino rats of either sex, weighing 175-220 g, provided by the Sanjeevan college of Pharmacy, Dausa, Rajasthan, India, were used. Animals were maintained under standard environmental conditions and had free access to standard pellet food (Hindustan lever, India) and water. The animals were maintained as per the norms of IAEC and the experiments were cleared by IAEC and the local institutional ethical committee. After vacuoe vaporation crude extract was suspended in 0.5%carboxymethyl cellulose (CMC).

Chemicals

Thiobarbituric acid was obtained from Loba Chemie, India. Ferrous sulphate, trichloro acetic acid, Potassium dihydrogen phosphate, Potassium hydroxide, were of analytical grade and obtained from Ranbaxy fine chemicals.

Determination of Anti oxidant activity

Assay of lipid per oxidation

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 (5)

with minor modifications (6) with the help of spectrophotometer (Shimadzu model 1601).

Goat liver was purchased from local slutter house. Its lobes were dried between blotting papers (to remove excess blood) and were cut into small pieces with a heavy-duty blade. They were then homogenized in glass-Teflon homogenizing tubes in cold phosphate buffer saline (pH 7.4). It was centrifuged at 2000rpm for 10 min, and supernatant was diluted with phosphate buffer saline up to final concentration of protein 0.8-1.5 mg/0.1ml. protein concentration was measured by using standard method of Lowery *et.al.* (7) . To study the comparative response, the experiments was divided into nine groups. Liver homogenates (5%, 3ml) was aliquoted to nine different 35mm glass Petri dishes. The first two groups were treated as control and standard where buffer and Vit. E were added. In the third to seventh group, different concentrations of EFTO were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3 ml of liver homogenate (8). After 30 min, 100µl of this reaction mixture was taken in a tube containing 1.5ml of 10% trichloro acetic acid. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% thio barbituric acid. The mixture was heated in a water bath at 85^0C for 30 min, and in boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535 nm.

The percentage of inhibition of lipid peroxidation was calculated by compring the results of the test with those of controls as per the following formula i.e. Eq. (1);

Inhibition(%) = (Control Absorbance- Test Absorbance) X 100/Control absorbance.

Determination of total phenolic compound

Total soluble phenolic compound in the EFTO were determined with the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (9). A 0.1 ml aliquot of a suspension of 1mg of EFTO in water was totally transferred to a 100ml Erlenmeyer flask and the final volume was adjusted to 46 ml by the addition of distilled water. Next 1 ml Folin – Ciocalteu reagent was added to this mixture, followed by 3 ml 2% Na_2CO_3 3 min latter. Subsequently, the mixture was shaken for 2h at room temperature and absorbance was measured at 760 nm. All tests were performed in triplicates. The concentration of total phenolic compounds in EFTO was determined as µg pyrocatechol equivalents using the following equation obtained from a standard pyrocatechol graph;

Determination of hypolipidaemic activity

Experimental design

Acute toxicity was determined for EFTO and minimum and maximum cut off dose, which found, is respectively 200 and 400 mg /kg body weight (10).
Twenty four wistar rats were divided in following 04 groups of 06each.
Group I (-ve Control): Normal diet (Standard chow diet)
Group II (+veControl): High Fat Diet (HFD)
Group III: HFD+EFTO 200mg/kg b.w
Group IV: HFD+ EFTO extract 400mg/kg b.w
The compositions of the two diets were as follows:

Control Diet (Normal)
Wheat flour 100g
Hydrogenated vegetable oil 5ml
Casein 20g
Cellulose 4g
Salt mixture(NaCl, KCl, $CaCl_2$) 1.5g
Citric acid 0.5ml
Vitamin B complex composition

High fat Diet
Wheat flour 100g
Casein 20g
Hydrogenated vegetable oil 10ml
Butter 10g
Cellulose 4g
Salt mixture (NaCl, KCl, CaCl2): 1.5g
Cholesterol (dried egg yolk) 0.5g
Citric acid 0.5ml
Vitamin B complex composition

Procedure
Group I served as normal control and was given normal saline only along with normal diet. Group II-IV received high fat diet plus cholesterol for induction of hyperlipidaemia. In, addition to this, groups III and IV were given ethanolic fraction of *Thuja occidentalis* at the dose of 200 and 400

mg/kg body weight (11).

Group III and Group IV were given once daily in the morning over a period of 8 days the EFTO in doses ranging from 200mg/kg b.w and 400 mg/kg b.w.

Body weight of each animal was registered at the beginning and at the end of the experiment. During the whole period, the animals have free access to food and water. Twenty hours prior the end of the experiment, food was withdrawn and blood samples were taken by retro orbital puncture. The blood samples were centrifuged for 2 min at 16 000 *g*. Serum Total Cholesterol, Serum HDL, Serum LDL, Serum VLDL, Serum Triglycerides, were determined in each blood sample.

These parameters were estimated by using Span Diagnostic & Erba Diagnostic Kits.

The LDL, VLDL and Atherogenic index were calculated by using the following formulae

LDL = TC – HDL – VLDL (where VLDL = TG/5)

Atherogenic index = (LDL+VLDL)/HDL

Statistical analysis

All data are expressed as mean ±SEM. For comparison amongst different groups, one-way ANOVA was performed. A *P* value less than 5% ($P<0.05$) was considered to be statistically significant.

Results

Antioxidant activity

Assay of lipid peroxidation

The results presented in Table-1 showed that the ethanol fraction of the *Thuja occidentalis* inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. The extract at 300µg/ml exhibited maximum inhibition (61.516±0.131 %) of lipid peroxidation nearly equal to the inhibition produced by Vit.E. The IC_{50} value was found to be 195.60µg/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 (12).

Table 1. Effect of Ethanol Fraction of *Thuja occidentalis* (EFTO) on Lipid peroxidation.

Concentration (µg/ml)	% inhibition (Lipid peroxidation)
300	61.52±0.13
250	58.17±0.33
200	57.39±0.65
150	42.14±0.37
100	33.95±1.80
Vitamin E (5mM)	68.32±0.42
IC_{50} (µg/ml)	195.60

Values are mean ±SEM of 3 replicates.

Amount of total phenolic compound

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. In the EFTO (1.0 mg) 123µg/ml, pyrocatechol equivalents to phenols were detected. The phenolic compound may contribute directly to the anti oxidative action (13). The results indicate a strong association between antioxidant activities and phenolic compounds are probably responsible for the ant oxidative property of *T. occidentalis*. Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants (14). Similarly, Shahidi and Naczk (15) reported that naturally occurring phenolic compound exhibit antioxidant activities. Thus, the therapeutic property of *T. occidentalis* may be possibly attributed to the phenolic compounds present.

Hypolipidaemic activity

A significant ($p<0.05$ and $p<0.001$) reduction in body weight of rats was observed in EFTO treated Groups III and IV respectively (Table 2).

A 65% increase in serum total cholesterol (TC) was noticed in rats fed high fat diet plus cholesterol (Group II) in compared to rats fed normal diet (group I). Administration of EFTO lowered the serum total cholesterol by 77 to 92% in Group III and Group IV respectively (Table 3).

Table 2. Body weight of high fat diet / *Thuja occidentalis* fraction treated rats.

S.No.	Treatment groups	Initial body weight (g)	Final body weight (g)
1.	Group I	213.33±3.57	215.83±3.97
2.	Group II	215.0±2.58	235.0±5.32
3.	G roup III	215.83±4.16	215.0±3.65
4.	Group IV	217.5±2.14	208.33±3.07

(Values are Mean ± SE from 06 animals in each groups).

A significant reduction in LDL cholesterol level was observed in EFTO treated groups and lowering percentage were 53% and 84% in Group III and IV respectively. The HDL cholesterol level was significantly reduced in Group II due to high fat diet, which became normal in-group III and Group IV with the effect of EFTO. The atherogenic index has shown the same effect. The serum triglycerides (TG) were decreased significantly ($p<0.001$) after treating with ethanolic extract of *Thuja occidentalis* and lowering percentage were 27% and 46% in Group III and Group IV respectively(Table 3).

Table 3. Lipid profile of high fat diet /*Thuja occidentalis* fraction treated rats
Values are Mean ± SE from 06 animals in each group.

Groups	Diet	Dose	Total cholesterol mg/dl	HDL mg/dll	LDL mg/dl	VLDL mg/dl	Triglycerides mg/dl	Atherogenic index	HDL/Total cholesterol ratio
(1)Control –ve	Normal diet	25 ml/kg N.S	107.0 ± 0.36	64.33 ± 1.12	28.83±1.24	13.83±0.09	69.16± 0.47	0.663	0.601
(II)Control +ve	High fatty diet	25 ml/kg N.S	177.33 ± 1.38	41.0± 0.36	117.0±1.63	19.33 ±0.33	96.66± 1.66	3.325	0.231
(III)Ethanol extract	High fatty diet	200mg/kg b. w	100.83 ± 0.94***	59.5± 0.42***	27.13±1.14***	14.2±0.07	71.0 ± 0.36***	0.694	0.590
(IV)Ethanol extract	High fatty diet	400mg/kg b. w	92.83± 0.79***	63.0 ±0.36***	19.3±0.92***	10.53±0.04	52.66 ±0.21***	0.473	0.678

Discussion

Lipids are widely involved in oxidation reactions and these reactions, can be induced by so called Reactive Oxygen Species (ROS). Oxidative stress caused by ROS in the living cell is associated with numerous diseases, like coronary heart disease, atherosclerosis, inflammation, cancer, anemia, and age related macular degeneration and ageing. Use of anti oxidants (substances that when present in low concentrations with those of an oxidisable substrate, significantly retard oxidation of that substance) can postpone problems caused by ROS and they retard oxidation process. Enzyme modifying actions of anti-oxidants could account for their pharmacological

activities. In our present study EFTO (ethanolic fraction of *Thuja occidentalis*) was evaluated for free radical scavenging activity and showed potent anti-oxidant activity and evidenced that with free radicle scavenging potential helps in ameliorating disease process. In the evaluation of hypolipidaemic activity Significant reduction in body weight in extract treated groups also suggest that certain enzymes are secreted in quantity involved in bile acid synthesis and its excretion and this may have caused decrease in serum cholesterol and triglycerides (16).

A rise in LDL may cause deposition of cholesterol in the arteries and aorta and hence it is a direct risk factor for coronary heart disease. LDL carries cholesterol from the liver to the peripheral cells and smooth muscle cells of the arteries (17, 18 and 19). HDL promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Therefore, increased levels of HDL are desirable. On the contrary, high levels of VLDL and LDL promote arteriosclerosis. LDL, especially in its oxidized form, is taken up by macrophages via a scavenger mechanism. Therefore, anti-arteriosclerotic drugs should reduce VLDL and LDL and/or elevate HDL.The search for hypolipidaemic drugs follows that high level of serum cholesterol are associated with an increased incidence of coronary heart diseases. Reduction in LDL cholesterol and increase in HDL cholesterol concentration are significantly related to lipid lowering therapy (20,21).

In the present study, ethanolic extract of *Thuja occidentalis* resulted in significant reduction in total cholesterol and LDL cholesterol level.

A significant fall in HDL cholesterol to total cholesterol ratio was observed in Group II (high fat diet). Low level of HDL cholesterol is associated with high risk of coronary artery disease (22). Ethanolic extract of *Thuja occidentalis* feeding back this ratio to normal by increasing HDL concentration high.

The decrease in serum TG level and reduction in atherogenic index in plant extract treated groups is an important finding of this experiment. Most of the hypolipidaemic drugs do not decrease serum triglycerides level but *Thuja occidentalis* extract showed it significantly. Reverse back of atherogenic index provides strong additional benefits in the prevention and treatment of atherosclerosis.

Conclusion

It can be concluded from the present study that the potent free radicle scavenging effect of *Thuja occidentalis* extract interfere in adsorption, degradation and excretion of cholesterol. However, this possibility remains to be investigated in detail.

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