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Anti-atherogenic effects of seabuckthorn (*Hippophaea rhamnoides*) seed oil

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Abstract

Seabuckthorn (SBT) seed oil is a rich source of unsaturated fatty acids, phytosterols, carotenoids and flavonoids, which are known to have significant anti-atherogenic and cardioprotective activity. The anti-atherogenic activity of supercritical CO_2 extracted SBT seed oil was evaluated in white albino rabbits fed on high cholesterol diet for 60 days. The study was performed on 20 male healthy rabbits divided into four groups of 5 animals each. Group I – control, group II – SBT seed oil, group III – cholesterol (1%) for 60 days, group IV – cholesterol + SBT seed oil. After 30 days of high cholesterol diet, group IV rabbits received 1 ml of SBT seed oil daily for 30 days. Blood total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and triglyceride (TG) levels were measured before and after the administration of SBT seed oil. The vasorelaxant activity of the seed oil was studied in vitro using aortic ring model technique and changes in isometric force were recorded using a polygraphic recording system. Accumulation of cholesterol in the aorta was studied using Sudan-IV staining technique.

SBT seed oil feeding to normal rabbits for 18 days caused a significant decline in plasma cholesterol, LDL-C, atherogenic index (AI) and LDL/HDL ratio. The HDL-C levels, HDL-C/TC ratio (HTR) and vasorelaxant activity of the aorta were significantly increased. In cholesterol-fed animals the TC, TG, LDL-C and AI were significantly increased and showed a decline following seed oil administration. The increase in HDL-C was more marked in seed oil treated hypercholesterolemic animals. The acetylcholine-induced vasorelaxant activity was significantly decreased in cholesterol-fed animals and could be restored to that of normal values by seed oil administration. These observations suggest that supercritical CO_2 extracted SBT seed oil has significant anti-atherogenic and cardioprotective activity.

Keywords: Atherosclerosis; Seabuckthorn; *Hippophaea rhamnoides*; Atherogenic index; Vasorelaxation; Hypercholesterolemia

Introduction

Atherosclerosis leading to coronary artery disease (CAD) has assumed a pandemic proportion the world over and has become the most common cause of death in developed as well as developing nations (WHO, 2003). In CAD, the arteries that supply blood to the heart are partially or fully blocked due to deposition of cholesterol in the endothelium of the arteries. Besides increased dietary intake of cholesterol, free radical-induced oxidative stress has been also implicated as one of the major contributory factor in the pathogenesis of plaque formation in CAD patients (Berliner et al., 1995; Libby et al., 2002). Cellular exposure to exogenously or endogenously generated oxidants causes macromolecular damage, including protein oxidation, lipid peroxidation, nucleic acid instability and mutations (Ames and Shigenaga, 1992; Halliwell, 1998) and has been implicated

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not only in the onset of the atherosclerotic process but also in aggravation of the disease process. Therefore, lowering of blood cholesterol using drugs like statins and antioxidant therapy has become a treatment of choice for the management of CAD patients.

In recent years, the clinical importance of the herbal drugs has received considerable attention as statins and synthetic antioxidants have been shown to have one or the other side effects (Bradford et al., 1991; Musk et al., 1994; Nocentini et al., 2001). Recent investigations from our laboratory have demonstrated that seabuckthorn (SBT) (*Hippophaea rhamnoides* L. Elaegnaceae) fruits and leaves have significant cytoprotective, immunomodulatory and antioxidant activity when studied in vitro using immune cells like rat spleenocytes, murine macrophages (J-774), human lymphocytes and C6 glioma cells and in vivo in animals fed on heavy metal pro-oxidant like chromium or following exposure to cold-hypoxia-restrain stress (Geetha et al., 2002, 2003a, b; Narayanan et al., 2005). All parts of this plant are considered to be a good source of a large number of bioactive substances thereby having high medicinal and nutritional properties. Many medicinal effects of SBT against flu, cardiovascular diseases, mucosal injuries and skin disorders have been suggested to be due to the high contents of antioxidant substances present in this plant (Beveridge et al., 1999; Yang et al., 1999; Eccleston et al., 2002; Yao and Tigerstedt, 1992). SBT berry oil is one of the most versatile natural oil and is rich in bioactive substances like carotenoids, tocopherols, omega-3 and omega-6 fatty acids and phytosterols, which have not only high antioxidant activity but can also inhibit cholesterol deposition in the arteries (Frank and Walter, 2002). However, whether SBT seed oil can curtail the process of atherosclerosis remains unknown. In the present investigation, anti-atherogenic potential of SBT seed oil was studied in rabbits fed on high cholesterol diet for 2 months.

Materials and methods

Seabuckthorn seed oil

Well-ripened SBT (*Hippophaea rhamnoides*) berries were collected from the hilly regions of Western Himalayas in the month of September where the plant grows wildly under natural conditions. The supercritical CO_2 extraction of seed oil was carried out using a pilot model supercritical extraction unit (SFE-2L, Thar Designs, Inc, USA) at 60 °C and at 450-bar pressure with a gas flow of 60 g/min for 3 h. The seed oil was collected in the cyclone separator. Fatty acid composition, β-carotene, tocopherols/tocotrienols and sterols in the seed oil were measured as described previously (Ranjith et al., 2006) and shown in Table 1.

Fatty acid methyl esters (FAME) of lipids of berries were prepared according to IUPAC method (Paquot and Hautfenne, 1987). FAME was quantified using Hewlett Packard 5890 series II model gas chromatograph equipped with flame ionization detector. β-Carotene in the seed oil was estimated using the technique of Gao et al. (2000). Tocopherol and tocotrienols were estimated using HPLC binary system (Shimadzu LC-10A) (Devi et al., 2000). Sterols in the oils were hydrolyzed and analyzed as aglycones using HPLC system (Holen, 1985).

Animals and diet

Adult New Zealand white rabbits (2.50 ± 1.0 kg body weight) were housed in the animal research facility of our institute. They were individually caged in stainless-steel wire-bottomed cages in a room maintained at $20° \pm 2$ °C temperature, 50 ± 10% humidity and a 12 h light/dark cycle with food and water ad libitum.

Atherogenesis was induced by feeding the animals with cholesterol-rich diet for 60 days. To prepare cholesterol rich diet, rabbit chow obtained from M/S Lipton India Ltd. was grinded and mixed with 1% cholesterol (E Merck, India). After thorough mixing, the chow was palletized and dried prior to feeding the animals. Both food consumption and weight gain were measured periodically. The study protocol was

Table 1. Chemical composition of supercritical CO_2 extracted seabuckthorn seed oil

(A) Fatty acids composition (wt%)	
Myristic acid ($C_{14:0}$)	00.32
Palmitic acid ($C_{16:0}$)	17.68
Palmitoleic acid ($C_{16:1}$)	04.55
Stearic acid ($C_{18:0}$)	03.11
Oleic acid ($C_{18:1}$)	30.09
Linoleic acid ($C_{18:2}$)	26.18
Linolenic acid ($C_{18:3}$)	17.77
(B) Carotenoids (mg kg^{-1})	350
(C) Tocopherols/tocotrienols (mg kg^{-1})	
α-Ta	806.1
α-T3b	38.9
γ-Ta	480
γ-T3b + δTa	42.4
δT3b	22.5
Total	1389.9
(D) Sterols (mg kg^{-1})	
β-sitosterol	13,774
Stigmasterol	1235
Campesterol	1878
Total	16,888

approved by Institute's animal ethical committee and confirms to national guidelines on the care and use of laboratory animals.

Experimental design

Twenty healthy male rabbits were randomly divided into four groups of five animals each. Group I served as control, Group II received 1 ml of SBT seed oil orally for 18 days, Group III animals received high cholesterol diet for 60 days whereas Group IV animals received high cholesterol diet initially for 30 days followed by high-cholesterol diet + 1 ml of SBT seed oil orally daily for additional 30 days.

Plasma lipid analysis

Blood samples were obtained after an overnight fast in heparin-coated tubes from the ear vein of all the animals on days 0, 15, 30, 45 and 60 of high cholesterol diet feeding. Plasma was obtained by centrifuging the blood at 2000*g* for 15 min at 4 °C. TC, TG, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) were determined enzymatically using kits obtained from M/S Randox, India.

Vascular reactivity

At the end of the experimental period, the rabbits were anesthetized with ketamine hydrochloride (80 mg/kg bw) and xylazine (20 mg/kg bw). The thoracic cavity was opened and the lungs enbloc with aorta were removed quickly and placed in cold Kreb's Ringer solution. The abdominal aorta was dissected free and cleaned of fat and adventitia. The aorta was cut into rings of 2 mm long. Each ring was mounted with two L-shaped stainless steel hooks in jacketted tissue chambers containing Kreb's Ringer solution at 37 °C and gassed with 95% O_2 and 5% CO_2. The upper hook of each aortic ring was attached to a force displacement transducer (BIOPAC) through a silk suture and changes in isometric force were recorded on a polygraphic system (BIOPAC software, USA). Phenylephrine and acetylcholine were used as standard vasocontractile and vasodilatory agents.

Initially, the rings were allowed to equilibrate for 1 h under tension of 2*g* (0.0196 N) and rinsed with Kreb's buffer 3–4 times. All the rings were tested with higher molar concentration of phenylephrine to assess the viability of the endothelium. The rings were then pre-contracted with phenylephrine (10^{-12}–10^{-4} M) and optimal contraction was observed at a concentration of 10^{-6} M phenylephrine. Therefore, for future experiments the rings were then pre-contracted with 10^{-6} M phenylephrine followed by exposure to acetylcholine (10^{-12}–10^{-4} M) after stabilization. Percentage change in the tension during exposure to acetylcholine against the tension of phenylephrine was calculated and compared with various groups for assessing vascular reactivity.

Statistical analysis

All data are expressed as mean ± SEM and the statistical significance was analyzed with two-way ANOVA and student '*t*'-test using SPSS package program. The results were only considered as significant if the value of p was less than 0.05.

Results

Body weight

The body weight did not show any significant change in cholesterol or SBT seed oil treated animals.

Lipid profile

The mean ± SE total cholesterol (TC), triglycerides (TGs), LDL-C and atherogenic index (AI) in all the groups at day 0 were not significantly different from each other (Table 2).

Fig. 1 shows alterations in TC, TG, LDL-C, HDL-C, LDL/HDL ratio, HTR and AI in normal animals, which received SBT seed oil for 18 days. TC and TG levels did not show any significant change ($p > 0.05$) following administration of SBT seed oil in these animals. The LDL-C levels were significantly decreased ($p < 0.01$) after SBT seed oil administration, whereas the HDL cholesterol levels were found to be significantly higher than the pre-treatment values. The HTR showed a significant rise ($p < 0.01$) after seed oil administration whereas LDL/HDL ratio and AI were significantly decreased ($p < 0.05$).

Feeding of high cholesterol diet to the animals of groups III and IV resulted in a significant increase in TC, TG, LDL-C, HDL-C, AI and decline in HTR after 30 days of cholesterol administration and the levels were maintained till the entire duration of cholesterol feeding (Table 2) (Badimon et al., 1989; Jeon et al., 2004). Administration of SBT seed oil 30 days after cholesterol feeding restricted further rise of TC on days 45 and 60 of treatment and caused a significant decline in TG levels on day 60 of observations. Although HDL levels were also increased following cholesterol administration, the rise in HDL over the basal values in seed oil treated animals was significantly higher than the non-treated animals. Administration of cholesterol caused a significant decline in HTR and increase in AI in cholesterol and cholesterol + seed oil treated animals. However, the

Table 2. Lipid profile of rabbits fed on high cholesterol diet (Gp III, $n = 5$) and in animals we fed on both cholesterol and SBT seed oil (Gp IV, $n = 5$)

Parameter	Groups	Days of treatment			
		Basal	Day 30	Day 45	Day 60
Total cholesterol (mg/dl)	Cholesterol	47.48 ± 0.75	963.60 ± 27.48***	1200.40 ± 52.06***^{aa}	1323.20 ± 45.21***^{aaa}
	Cholesterol + seed oil	50.00 ± 1.64	1200.40 ± 42.71***^{bb}	1239.60 ± 73.56***^{b}	1260.00 ± 41.36***
Triglyceride (mg/dl)	Cholesterol	49.99 ± 1.55	68.88 ± 1.87***	74.86 ± 3.22**	77.58 ± 4.63**
	Cholesterol + seed oil	51.61 ± 0.97	71.33 ± 1.14***	70.00 ± 2.34***	66.01 ± 3.02**^{a}
LDL-cholesterol (mg/dl)	Cholesterol	23.20 ± 0.23	47.80 ± 0.32	50.48 ± 0.73**^{a}	52.88 ± 1.15**^{a}
	Cholesterol + seed oil	24.52 ± 0.46	57.10 ± 1.80**^{bb}	36.08 ± 1.61***^{aaabbb}	36.80 ± 0.58***^{aaabbb}
HDL-cholesterol (mg/dl)	Cholesterol	21.70 ± 0.42	40.06 ± 0.43***	42.26 ± 1.64***	50.02 ± 0.65***^{aaa}
	Cholesterol + seed oil	19.44 ± 0.39^{b}	37.07 ± 0.19***^{bb}	43.72 ± 0.41***^{aaa}	52.54 ± 0.28***^{aaabb}
HTR (%)	Cholesterol	45.73 ± 0.97	4.17 ± 0.10***	3.56 ± 0.25***^{a}	3.79 ± 0.10***^{a}
	Cholesterol + seed oil	39.12 ± 1.91^{b}	3.10 ± 0.11***^{bbb}	3.58 ± 0.23***	4.19 ± 0.16***^{aaab}
Atherogenic index	Cholesterol	1.19 ± 0.05	23.05 ± 0.61***	27.63 ± 1.86***^{a}	25.43 ± 0.65***^{a}
	Cholesterol + seed oil	1.58 ± 0.12	31.39 ± 1.18***^{bbb}	27.37 ± 1.75***	22.99 ± 0.86***^{aaa}

The seed oil feeding was started after 30 days of high cholesterol diet.
vs. Basal * < 0.05; ** < 0.01; *** < 0.001.
vs. 30 Day ^{a} < 0.05; ^{aa} < 0.01; ^{aaa} < 0.001.
vs. cholesterol ^{b} < 0.05; ^{bb} < 0.01; ^{bbb} < 0.001.
HTR% = HDL cholesterol/total cholesterol ratio, atherogenic index (AI) = total cholesterol−HDL cholesterol/HDL cholesterol.

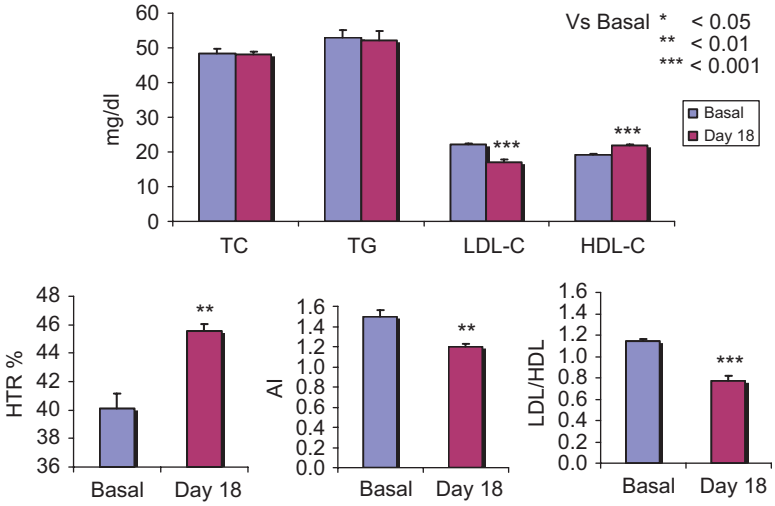

Fig. 1. Plasma total cholesterol (TC), triglyceride (TG), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), HTR% (HDL cholesterol/total cholesterol ratio), atherogenic index (AI = total cholesterol – HDL cholesterol/HDL cholesterol) and LDL/HDL ratio in control animals after 18 days of seed oil administration (Gp II, $n = 5$).

rise in AI in cholesterol + seed oil treated animals on day 60 of observations was significantly lower than the group, which received cholesterol only.

Vascular reactivity

Fig. 2 shows the vascular reactivity of aortic segments in groups I–IV. Addition of phenylephrine 10^{-6} M caused a significant vasoconstriction which could be relieved by addition of Ach in a concentration-dependent manner. Maximum relaxation response in control untreated animals was about 17%. In SBT seed oil treated normal animals the vasorelaxant response of 35% was significantly higher ($p < 0.01$) as compared to untreated animals. In cholesterol treated animals the vasorelaxant response was decreased ($p < 0.05$) to about 6% whereas in animals treated with cholesterol + seed

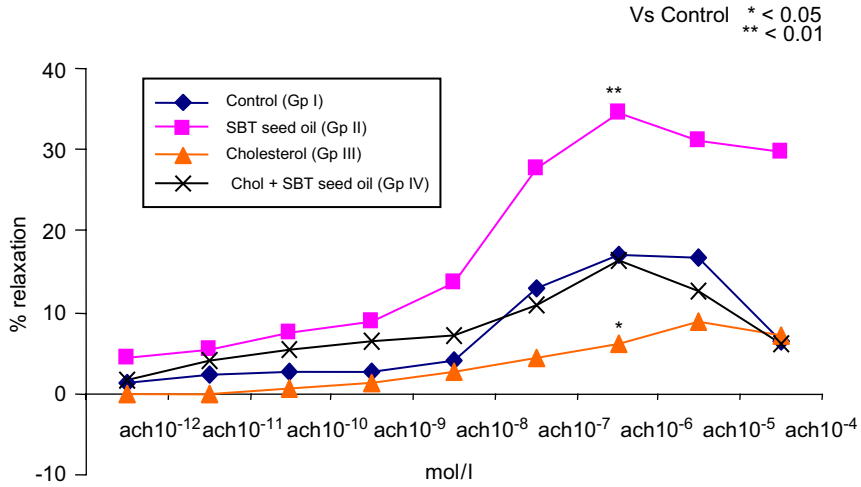

Fig. 2. Vascular reactivity of aortas from control rabbits (Gp I, $n = 5$), rabbits fed on seabuckthorn (SBT) seed oil only (Gp II, $n = 5$), hypercholesterolemic rabbits (Gp III, $n = 5$) and hypercholesterolemic rabbits fed on SBT seed oil (Gp IV, $n = 5$).

oil the vasorelaxant response (16%) was not significantly different ($p > 0.05$) than the control values.

Discussion

The experimental model of non-genetic rabbits is widely accepted in studies of induction of hypercholesterolemia atherosclerosis as their lipid metabolism exhibits a number of similarities to that of human (Kritchevsky, 1991; Kroon et al., 1986). The results from the present study demonstrate that administration of SBT seed oil to normal rabbits resulted in reduction of LDL-C by about 24% and increased HDL-cholesterol by about 13%. The AI was also markedly decreased besides causing a significant reduction in LDL/HDL ratio. In animals fed on high cholesterol diet, seed oil curtailed gradual increase in plasma TC and caused a significant reduction in TG levels besides decreasing LDL-C. The LDL-C following seed oil administration was also decreased in hypercholesterolemic animals. Similarly, the rise in HDL-C after high cholesterol diet in SBT seed oil treated animals was more pronounced as compared to non-treated animals (170% vs. 130%). Besides decreasing TC, LDL-C and the AI, the seed oil administration also inhibited accumulation of cholesterol in the aorta and was found to have significant vasorelaxant activity.

Increase in HDL or HTR ratio is one of the most desirable criteria of an ideal anti-hypercholesterolemic agent, since higher the HDL, the lower the atherosclerotic risk. Numerous studies have demonstrated that high levels of HDL-C are associated with a lower incidence of CAD and low levels of HDL-C are associated with a higher incidence of CAD (Young, 2005). Epidemiological studies and investigations in experimental animals have suggested that the raising of HDL-C may retard the development of atherosclerosis. In humans, each increase in baseline HDL-C of one mg per deciliter is associated with 6% decrease in the risk of death from CAD or myocardial infraction (Gorden et al., 1986). Studies in animals have demonstrated that over expression of the apolipoprotein A-1 gene (the major apolipoprotein in HDL-C) prevents the development or progression of atherosclerosis (Benoit et al., 1999; Brewer, 2004). HDL-C transports excess cholesterol from peripheral tissues to the liver for conversion to bile acid and inhibits oxidation of LDL-C besides curtailing expression of cellular adhesion molecules and monocyte recruitment which may reduce the risk of thrombosis by inhibiting platelet activation and aggregation (Shali et al., 2001). Although the exact mechanism for increase in HDL-C after seed oil administration remains unknown, the increase in HDL-C may be due to stimulation of pre-β HDL-C and reverse cholesterol transport (Gupta et al., 1993).

AI indicates the deposition of foam cells or plaque or fatty infiltration or lipids in heart, coronaries, aorta, liver and kidney. The higher the AI, higher is the risk of above organs for oxidative damage. The AI was significantly reduced in SBT seed oil treated normal and hypercholesterolemic animals.

The SBT seed oil treatment also caused a significant vasorelaxation of the phenylephrine-induced pre-contracted aorta in response to acetylcholine in normal animals. However, in hypercholesterolemic animals, acetylcholine-induced vasorelaxation was markedly impaired which could be restored to control values when SBT seed oil was administered alongwith the high cholesterol diet. Some atherosclerotic arteries in humans and various hypercholesterolemic animal

models of atherosclerosis exhibit increased vasoconstriction (Ginsburg et al., 1984; Vrints et al., 1990). The pathophysiological basis of this increased vasoconstriction remains speculative. The endothelium dependent relaxation has been reported to be impaired in vessels from atherosclerotic patients (Bossaller et al., 1987; Forstermann et al., 1988) and in hypercholesterolemic animal models (Chappell et al., 1987; Shimokawa and Vanhoutte, 1989) suggesting modification of the endothelium derived relaxing factor, which is assumed to be nitric oxide (NO) in hyperlipidemia. Whether SBT seed oil activates NO synthase actively remains to be investigated.

These anti-atherogenic and cardioprotective effects of SBT seed oil may be due to the presence of polyunsaturated fatty acids, phytosterols, tocopherols and β-carotene in the supercritical CO_2 extracted seed oil. Infact, the seed oil used in the present study appears to be the best single source of these cardioprotective agents. About 80% of the total fatty acids present in the seed oil are unsaturated fatty acids. The seed oil is very rich in polyunsaturated fatty acids like oleic acid (30%), linoleic acid (26%) and linolenic acid (18%). Oleic acid is a unsaturated fatty acid that is difficult to oxidize and is involved in the fluidity of lipoproteins and as of a consequence in generation of HDL (Sola et al., 1990). Dietary linoleic acid serves as a precursor for biosynthesis of arachidonic acid, the substrate for eicosanoid synthesis through activity of the enzyme cyclo-oxygenase and 5-lipoxygenase. The linoleic acid derivative, particularly γ-linolenic acid is more potent in reducing blood cholesterol in humans and rats (Takada et al., 1994). The major phytosterols present in the seed oil were β-sitosterol, campsterol and stigmasterol. Higher β-sitosterol in the liver can result in an increase in cholesterol-7, α-hydroxylase and a decrease in the activity of HMG – COA reductase in the liver (Gylling and Miettinen, 2005) causing reduction in the synthesis of hepatic cholesterol and an increase of bile acid excretion. The cholesterol lowering effects of these phytosterols may be due to the inhibition in re-absorption of cholesterol from endogenous sources in association with a simultaneous increase in its excretion into faeces in the form of neutral steroids. Other compounds present in SBT seed oil that could be altering aortic cholesterol deposition and preventing lipoprotein structural alterations are tocopherol and β-carotene. α-Tocopherol (the most potent vitamin E) is the major form of vitamin E in SBT oil. The vitamin E contents in the SBT seed oil is two times higher than wheat oil, nine times more than corn oil and 35 times higher than the soyabean oil (Aluokumofu, 1992).

In conclusion, the present study suggests that seabuckthorn (SBT) seed oil has significant anti-atherogenic activity when administered to normal or hypercholesterolemic animals. The cardioprotective effects of SBT seed oil may be due to presence of Omega-3 and Omega-6 fatty acids, tocopherols, phytosterols and β-carotene which in combination may have synergistic effects on cardiovascular health.

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