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Research Article

ANTIOXIDANT IMPACTS OF *BOERHAAVIA DIFFUSA* & BLACK CARAWAY OIL ON CONJUGATED DIENE, LIPID HYDROPEROXIDATION & MDA CONTENT IN DMBA-INDUCED HYPERCHOLESTEROLEMIA IN RATS

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ABSTRACT

The root extract of *Boerhaavia diffusa* and Black Caraway Oil have potent antioxidant & hypolipidemic effect, on plasma lipid (TL, TGs and TC), FFA, plasma total antioxidants, conjugated diene, lipid hydro-peroxide, MDA content in DMBA-Induced rats. The present study was carried out to investigate antioxidant & hypolipidemic impact of *Boerhaavia diffusa* and Black Caraway Oil using as a drugs. Plasma total antioxidants, conjugated diene, lipid hydroperoxide and MDA content were evaluated in normal and DMBA-Induced rats. After supplementation (2ml/Kgb.w for 16-week) of this extract (*Boerhaavia diffusa* and Black Caraway Oil) significantly increases the antioxidant activity level in Plasma as compare to infected control. Elevated antioxidant properties (Plasma) total antioxidants, conjugated diene, lipid hydroperoxide and MDA content were diminished significantly by the treatment of *Boerhaavia diffusa* and Black Caraway Oil in respect to infected group. All the above mentioned parameters were significantly restored to the control level. In addition, daily use of dietary *B. diffusa* and Black Caraway Oil will be efficacious, cost effective, no side effects and a good source of hypolipidemic/antiatherogenic, antihypercholesterolemic, antioxidant actions and anticarcinogenic.

Keywords: Total Antioxidant, MDA, Lipid hydroperoxide, Conjugated diene, *Boerhaavia diffusa*, Black Caraway Oil.

INTRODUCTION

The term "antioxidant" refers to any molecule capable of stabilizing of deactivation free radicals before they attack cells. These are in particular the primary 'antioxidant'. There are also molecules deserving the "antioxidant" team, because they act as chelating agents binding metal ions (redox activity). To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly complex antioxidant protection system. Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing[1-4]. These diseases fall into two groups: (i) the first group involves diseases characterised by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance-the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) the second group involves disease characterised by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (implicated in ischemia and reperfusion injury)[5].

Boerhaavia diffusa (B. diffusa) commonly known as rakta punarnava[6] of family Nyctaginaceae[7]. The plant possesses diuretic and cardio tonic activity[8], hypotensive[9], immunomodulating[10], antioxidant[11], cytotoxic[12], and anticancerous activity[13]. The preliminary photochemical studies of ethanolic root extract of *B. diffusa* showed the presence of alkaloids, flavonoids and saponins. Any of these phytoconstituents may be responsible for this pharmacological activity, however detail study yet to be undertaken in order to confirm the clear mode of the pharmalogical action. The purpose of this work was to study antidiabetic and antioxidant activity of the ethanolic root extract of *B. diffusa*. Its English name is Black Cumin or Black Caraway. It should be noted that the latter two names bear no relation to the plants Cumin (*Cuminum cyminum*, Linne) and Caraway (*Caram carvi*, Linne) that belong to the botanical family *Umbelliferae*. It was first identified and described by Linnaeus in 1753. The detailed taxonomy of the plant was described by Muschler[14]. Protective effect of liver damage[15], Chronic cyclosporine nephrotoxicity[16], Oxidative stress [17], Antioxidative and antihistaminic[18], Hepatotoxicity and antioxidant[19], Proliferation and biochemical marker levels of Hep-2 cells[20], Heart rate, some haematological values and pancreatic beta-cell[21], Hepatoprotective effects[22], Antimicrobial activity[23], Antiepileptogenic and antioxidant [24], Hypolipidemic and hypoglycemic properties [25], Immunomodulatory [26], diabetic neuropathy [27].

Lipids are transported through plasma compartment in lipoproteins, which are complex water soluble molecules consisting of a core of cholesteryl esters and TG covered by a surface monolayer of phospholipids, free cholesterol. In the last two decades, there have been major advances in our understanding of the role of plasma lipoproteins, lipolytic enzymes, and lipoprotein receptors in cholesterol and lipoprotein metabolism. This new information has provided major insights into the role of cholesterol and lipoproteins in the pathogenesis of premature atherosclerosis. It is mainly intended to provide a comprehensive knowledge regarding our existing knowledge of the pharmacological and toxicological actions of this plant. It is hoped that the provided knowledge will generate a real clinical appraisal and evaluation of the effectiveness of at least the *B. diffusa* and Black Caraway Oil in the treatment of some cardiovascular diseases (hypercholesterolemia), cancer and regulatory effect on Hepatic, Lung and Kidney antioxidants enzymes and malondialdehyde content in DMBA-Induced rats.

MATERIALS AND METHODS

Plant material and extraction procedure

Fresh root of *B. diffusa* were collected from Srinagar (Garhwal) and its adjoining areas. The collected plant was identified by Dr. R. L. Painuly, Taxonomist, Department of Botany and Microbiology, H.N.B. Garhwal University, Srinagar, India and the voucher specimen (GUH- 20434) has been preserved in our research laboratory for future reference. The plant root dried in shade, coarsely powdered and subjected to soxhlet extraction using 70% hydro-alcoholic solvent (70% ethanol : 30% distilled water), at 48^{0}C for 24 h. The final extract was allowed to evaporate yielded a 7.78% dark brownish solid residue. Black Caraway Oil was purchased local market from Srinagar (Garhwal).

Animals

White male albino rats weighing 150-180 gm were used for the present study, maintained on animal house under normal condition having natural photoperiod (12 hours light/dark cycle) at temperature 25±1^{0}C and 50-60% humidity. Animal experimentation protocols confirm to the Institutional Animal Ethics Committees guidelines. They were provided with standard feed and tap water *ad libitum*.

Experimental Design

Animals were divided into four groups and for each group fifteen animals were taken. Group I (Normal control) (0.9% NaCl; 5 ml/kg. body weight orally (b. w. o.)) and hypercholesterolemia was induced to other rats by intraperitoneal injection of 7,12-Dimethylbenz[α]anthracene (DMBA) [Sigma-Aldrich Inc., St. Louis USA] (65 mg/kg. body weight). After 3 weeks, animals showing plasma lipid profile level increase 510.36 ± 2.23 mg/dl were considered hypercholesterolemic. The hypercholesterolemic animals were stabilized for 3 weeks and the next day experiment was started. Group II served as hypercholesterolemic infected control, Group III and IV received *B. diffusa* and Black Caraway Oil 2 ml/kg. b. w. o two equal does) regulatory effect on plasma total antioxidants, conjugated diene, lipid hydroperoxide and MDA content in DMBA-Induced rats. At the 16 week all the animals were sacrificed and evaluated for the antioxidant activity.

Group I- Normal control (N-C)

Group II- DMBA-Induced infected control (I-C)

Group III- Infected *B. diffusa* treated (I-BdT) [2 ml/Kg b.w]

Group IV- Infected Black Caraway Oil treated (I-BCOT) [2 ml/Kg b.w]

Determination of free radical scavenging activity (antioxidant capacity) of *B. diffusa* and Black Caraway Oil

The procedure of Mellors and Tappel (1966) as modified by Khanduja and Bhardwaj[28] was used for determining the free radical scavenging activity of *B. diffusa and* Black Caraway Oil. The assay was carried out in a medium containing 40 mM tris buffer, pH 7.4 and 125 μM ethanolic solution of 2, 2-diphenyl-1-picryl hydrazyl (DPPH). The reaction was started by the addition of ethanolic solution of *B. diffusa and* Black Caraway Oil (25-200 μM) in a total volume of 2 ml. The samples were mixed thoroughly and the absorbance was recorded in dark at 517 nm (27 ± 2^{0}C) at 1 min time interval up to 10 min against absolute ethanol. A control blank containing all the above ingredients except the test compounds was used in order to monitor the absorption of DPPH. The percent inhibition of the DPPH by the above antioxidants was calculated according to the formula reported by Yen and Duh[29].

Collection of blood

At the end of the experiment treatment, overnight fasted rats in each group were anaesthetized and blood drawn from cardiac puncture. The blood from each rat in a given group was collected in heparinised tubes, mixed gently by inversion 2-3 times and incubated at 4^{0}C for 2 h. Plasma was separated from blood by centrifugation at 2,500 rpm for 30 min, aliquoted and either stored at 4^{0}C or frozen at –20^{0}C for use in other experiments.

Preparation of liver, lung and kidney homogenate and post-mitochondrial supernatant

At the end of the experiment, liver, lung and kidney from each rat were promptly excised and chilled in ice cold saline. After washing with saline, liver, lung and kidney were blotted and weighed. Each liver, lung and kidney were cut into pieces, mixed and 10 g of wet tissue was homogenized with 90 ml of chilled 0.1 M sodium phosphate buffer, pH 7.4, containing 1.17 % KCl in a waring blender. The volume of each homogenate was recorded and centrifuged at 1,000 rpm for 10 min at 4^{0}C. After centrifugation, a portion of each homogenate from liver, lung and kidney thus obtained was aliquoted and stored at –20^{0}C. The remaining portions of the liver, lung and kidney homogenates were centrifuged at12, 000 rpm for 20 min at 4^{0}C. The post-mitochondrial supernatant thus obtained was aliquoted and stored at –20^{0}C for future use.

Measurement on plasma lipids

Plasma lipids [Total lipids (TL), Triglycerides (TG), free fatty acids (FFA), Total cholesterol (TC)] were evaluated in normal and hypercholesterolemic rats. Triglycerides were determined by using enzymatic kit. The method uses a modified Trinder colour reaction to produce a fast, linear, end point reaction as described by Trinder [30]. Free fatty acid in plasma was estimated as described by Duncombe[31]. The procedure of Folch *et. al.*[32] was used for extracting free fatty acid from plasma lipids. The absorbance was recorded at 440 nm against a reagent blank in Beckman DU 640 spectrophotometer. Total cholesterol in plasma was determined by Annino and Giese[33] the absorbance was read at 550 nm in Beckman DU 640 spectrophotometer.

Measurement of plasma "total antioxidant power" (FRAP)

The method of Benzie and Strain[34] was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimate the "total antioxidant power", with minor modification. Ferric to ferrous ion reduction at low pH results in the formation of a collared ferrous-tripyridyl triazine complex. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of plasma in 0.1 ml and 900 μl of freshly prepared FRAP reagent, prepared by mixing 10.0 ml of 22.78 mM sodium acetate buffer, pH 3.6, 1.0 ml of 20 mM ferric chloride and 1.0 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine solution prepared in 40 mM HCl. Before starting the reaction, both FRAP reagent and plasma samples were pre incubated for 5 min at 30^{0}C. Incubation was done for 5 min at 30^{0}C and absorbance was recorded at 593 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Ferrous sulphate was used as a standard for calculating the "total antioxidant power".

Estimation of lipid peroxides in plasma, liver, lung and kidney homogenates

For the extraction of Lipid contents from plasma and tissues, the method of Folch *et al.*[35] was employed. One volume of plasma or tissue homogenate was mixed with 5.0 volume of chloroform: methanol (2:1), followed by centrifugation at 1,000 rpm for 5 min to separate the phases. Most of the upper layer was removed; and 3.0 ml of the lower chloroform layer was recovered. The chloroform layer was placed in a test tube and incubated at 45°C till dryness. For the determination of conjugated dienes in plasma, liver, lung or kidney, corresponding lipid residues were dissolved in 1.5 ml of cyclohexane and the absorbance was recorded at 234 nm against a cyclohexane blank in a Beckman DU 640 spectrophotometer. The concentration of conjugated dienes formation was calculated by using a molar extinction coefficient of 2.52 x l 0^4 M^{-l} cm^{-l}.

Lipid peroxide contents in plasma were assayed by the method of Yagi[36]. Plasma (25 μl) was mixed with 4.0 ml of 0.083 N H_2SO_4 followed by the addition of 0.5 ml of 10 % phosphotungstic acid. The samples were mixed and incubated for 5 min at room temperature and then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the sediment was mixed with 2.0 ml of 0.083 N H_2SO_4 and 0.3 ml of 10 % phosphotungstic acid. The mixture was centrifuged at 3,000 rpm for 10 min, the sediment was suspended in 4.0 ml of water and 1.0 ml of thiobarbituric acid (TBA) reagent (a mixture containing equal volumes of 0.67 % aqueous TBA solution and glacial acetic acid) was added. The reaction mixture was heated for 60 min at 95°C, cooled and the tubes were centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was determined at 532 nm against a reagent blank in a Beckman DU 640 spectrophotometer.

Determination of malondialdehyde in erythrocytes

The determination of MDA in erythrocytes was carried out according to the method of Stocks and Dormandy[37]. Briefly, 0.3 ml of packed erythrocytes in triplicate was made up to 1.0 ml in phosphate buffered saline, pH 7.4. To each tube, BHT in ethanol (0.75mM) was added, followed by the addition of 0.5 ml of 30 % TCA. Tubes were vortexes and allowed to stand in ice-bath for 2 h. Tubes were then centrifuged at 2,000 rpm for 15 min and 1.0 ml of the supernatant of each tube was transferred to another tube. To each tube, 75 μl of 100 mM EDT A and 250 μl of 1 % thiobarbituric acid in 50 mM NaOH was added, mixed and kept in a boiling water bath for 15 min. After cooling the tubes to room temperature the absorbance of each sample was read against a reagent blank at 532 nm in a Beckman DU 640 spectrophotometer. Malondialdehyde was used as a standard for the calculation of MDA concentration[38].

Measurement of malondialdehyde release from intact erythrocytes

The procedure of Cynamon[39] was employed for the determination of malondialdehyde (MDA) release from erythrocytes. Two aliquots of 0.22 ml of washed packed erythrocytes in duplicate were taken in two separate tubes. One series of aliquots were suspended in 4.18 ml of phosphate buffered saline, pH 7.4, and the second series of aliquots of erythrocytes were suspended in 4.18 ml of phosphate buffered saline, pH 7.4, containing 4 mM sodium azide. Both the suspensions were vortexes for 15 seconds. The samples were mixed for 10 seconds and incubated for 1 h at 37^{0}C. At the end of incubation, 1.0 ml of 28 % TCA containing 100 mM sodium arsenite was added to each tube and centrifuged at 3,000 rpm for 10 min. Two ml of the supernatant from each tube was taken in triplicate and mixed with 0.5 ml of 1 % thiobarbituric acid prepared in 50 mM NaOH. The samples were then boiled for 15 min at 95^{0}C, cooled to room temperature and the absorbance was recorded at 535 nm against a reagent blank in a Beckman DU 640 spectrophotometer.

GC-MS and HPLC of aqueous ethanolic extract of roots *Boerhaavia diffusa* (Bd) and Black Caraway Oil (BCO)

GC-MS of *Boerhaavia diffusa* (Bd) and Black Caraway Oil (BCO)

The analysis of Bd and BCO volatile components were carried out by gas chromatography and gas chromatography-mass spectrometry (GC-MS)[52,53].Gas-chromatography measurements were carried out with Bush 610 instrument (Darmstadt, Germany) Equipped with a capillary silicon column: 60m; diameter:0.25mm; film thickness: 0.25mm; stationary phase: RTX-5MS; column temperature: 40^{0}C; heated at 10^{0}C/min; 310^{0}C isotherm for 10min; carrier gas: helium at a linear velocity of 32 cm/s; injected solution: 2µl; Finningan MS detector: start: 9min after injection, SCAN mode by electron impact ionization; mass range: 40-650; scanning rate: 1analysis/s.

High Performance Liquid Chromatography (HPLC) of extract of *B.diffusa* (Bd) and

Black Caraway Oil (BCO)

The technical details have been described by Simpson, C.F.[54] Extraction of the constituents from Bd and BCO were carried out using C18 PrepSep mini columns followed by quantification of the recovered constituents by HPLC on a reversed-phase Bondapack C18 analytical column, using an isocratic mobile phase of water: methanol: 2-propanol (50: 45: 5% v/v) at a flow rate of 1ml/min, detection was 254nm.

RESULTS

Quantification of Analytical GC-MS of extract of *B. diffusa* (Bd) and Black Caraway Oil (BCO)

There are fourteen peaks of *B. diffusa* (Bd) and eight main peaks found in Black Caraway Oil (BCO) in GC-MS. The *B. diffusa* (Bd) contains a large number of compounds as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins and glycoprotein. The following are few important chemical constituents present in *B. diffusa*.

Alkaloid- Punarnavine ($C_{17}H_{22}N_2O$)

Rotenoid- Boeravinone A_1, B_1, C_2, D, E, F

Hypoxanthine- 9-L-arabinofuranoside and Punarnavoside, Ursolic acid, β-sitosterol

Lignens- Liirodendrin and Syringaresinol mono-β-D-glucose.

The plant contained large quantities of potassium nitrate, besides punarnavine. The immunosuppressive activity of the two glycoside (flavonoids glycoside) compounds identified as BdI (eupalitin-3-O-β-D-glactopyranoside) and BdII (eupalitin) has been isolated from the root portion of the plant using flash chromatography technique by employing the solvent system of $CHCl_3$ and methanol 19:1[44].

The Black Caraway Oil contains Eight fatty acids (99.5%) have been identified in the fixed oils. The main fatty acids of the fixed oil were linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid 12.5%). The major compounds of volatile oil were trans-anethol (38.3%), p-cymene (14.8%), limonene (4.3%) and carvone (4.0%).

High Performance Liquid Chromatography (HPLC) extracts of *B. diffusa* (Bd) and Black Caraway Oil (BCO)

The fraction was purified by HPLC have eleven peaks of *B. diffusa* (Bd) shows Boeravinone A1, B1, C2, D, E, F, Hypoxanthine, Punarnavoside and Urosolic acid as the major phytoconstituents and 4 main peaks found in Black Caraway Oil (BCO) in HPLC. Pharmacological active constituents of the Black Caraway Oil (BCO) as found as thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY).

Antioxidative Activities of *B. diffusa* (Bd) and Black Caraway Oil (BCO)

Antiradical activity or hydrogen donating ability of *B. diffusa* (Bd) and Black Caraway Oil (BCO) was measured by using DPPH (2,2-diphenyl,1-picrylhydrazyl), which reflects the antioxidative properties of these compounds. As shown in **Fig.1**, the half quenching concentrations (IC_{50}) were as follows: *B. diffusa* (Bd), 44.42 µM; Black Caraway Oil (BCO), 38.11 µM. These findings indicate that as compared to *B. diffusa* (Bd) 89% was found more efficient then Black Caraway Oil (BCO) 76% respectively. *B. diffusa* shows more free radical scavenging property then Black Caraway Oil.

Effect on plasma lipids

As seen in table 1, the entire plasma lipid parameters were significantly increased in DMBA-Induced control (I-C) rats, when compared to normal value (N-C). Total lipids (TL), triglycerides (TG), free fatty acids (FFA), and total cholesterol (TC) significantly increased from 390, 54, 134 and 89 mg/dl in normal control to 510, 115, 151 and 155 mg/dl respectively in infected group. After 16 weeks of *B. diffusa* and Black Caraway Oil treatment level of TL, TG, FFA and TC were significantly decreased by *B. diffusa* and Black Caraway Oil 5%, 42%, 9%, 28% and 5%, 45%, 11%, 34% respectively, in comparison to corresponding values in infected groups. These results demonstrated that DMBA-Induced infected rats with two equal does of 2 ml/Kg.b.w.o. *B. diffusa* and Black Caraway Oil mediated a similar and significant reduction in all the parameters.

Impact on plasma total antioxidants and lipid peroxidation products

The antioxidant impact of *B. diffusa* and Black Caraway Oil on plasma concentrations of total antioxidants, conjugated diene, lipid hydroperoxide and MDA in infected (I-C) rats. In I-C rats, plasma total antioxidants level was reduced from a control value of 48 to 37 (24 %) µmole/dl. Treatment of I-C rats with *B. diffusa* and Black Caraway Oil for 16 weeks resulted in a significant increase of total antioxidants levels by 25 % and 14 %, when compared to N-C value. The oxidative stress induced in I-C rats **(Table 2)** significantly enhanced plasma lipid peroxidation products, such as conjugated diene, lipid hydroperoxide and MDA. Formation of conjugated diene, lipid hydroperoxide and MDA in plasma was increased from 9.31, 2.51 and 3.42 in N-C to 14.36 (54 %), 3.81 (52 %) and 5.91 (73 %) µmole/dl, respectively, in I-C. After *B. diffusa* treatment, in I-BdT, a significant decrease of 13 %, 18 % and 18 % were found in the formation of conjugated diene, lipid hydroperoxide and MDA, respectively, compared to corresponding values in I-C rats. Similarly in I-BCOT, conjugated diene, lipid hydroperoxide and MDA in plasma were also significantly decreased by 8%, 16 % and 15 %, respectively, when compared to corresponding values in I-C rats. These results demonstrate that in I-C rats, due to increase in oxidative stress, total antioxidants level was decreased, whereas, concentration of plasma conjugated diene, lipid hydroperoxide and MDA were significantly increased. *B. diffusa* and Black Caraway Oil treatment significantly restored the total antioxidants level and blocked the increase in plasma conjugated diene, lipid hydroperoxide and MDA to a level close to corresponding normal value.

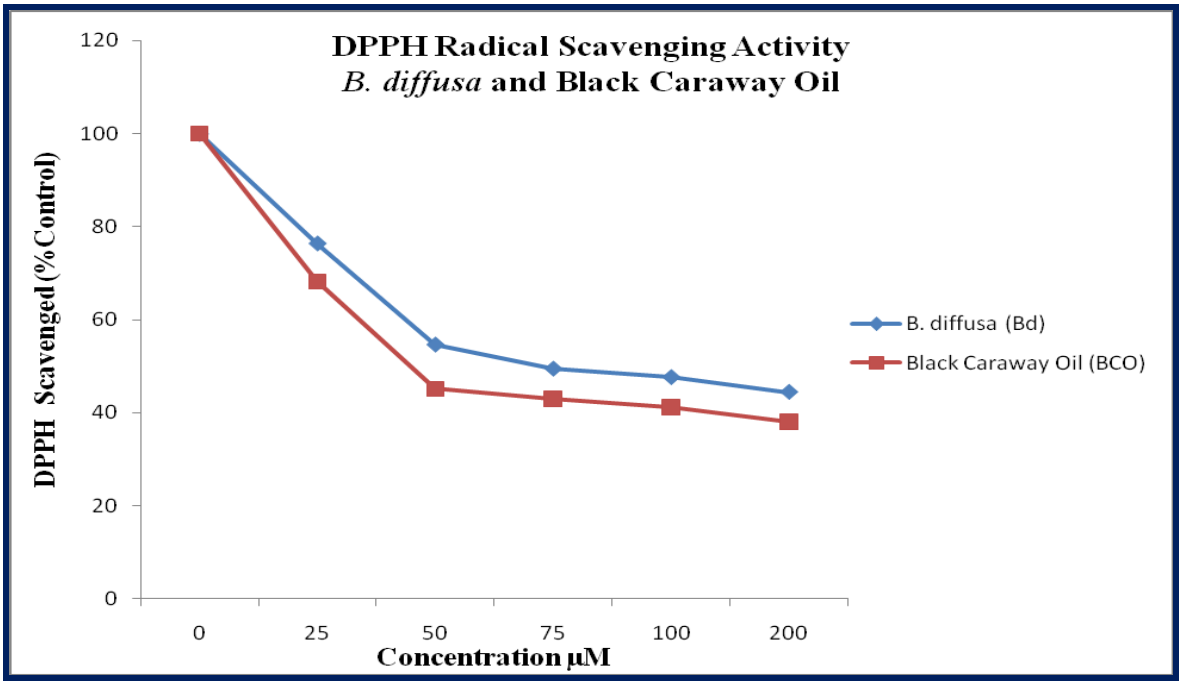

Fig. 1: Free radical scavenging activities of *B. diffusa* (Bd) and Black Caraway Oil (BCO).

The antioxidant activities of the above compounds at the indicated concentrations were carried out as described in methods. The assay is based on the reduction of 2, 2-diphenyl, 1-picrylhydrazyl (DPPH), which gives strong absorption maxima at 517nm. Values represent the mean of triplicate determinations. The average error in the data points in these assay were mean ± less than 3 %. The average absolute absorbance value of 100 % DPPH was 0.00786 ± 0.00029.

Table 1: Impact of *B. Diffusa* (I-BdT) and black caraway oil (I-BCOT) on TL, TGs, FFA and TC in DMBA-induced rats after 16 weeks of treatment

Group	Total lipid	Triglycerides	Free fatty acid	Total cholesterol
N-C	390.19±1.40*	54.22±1.17	134.15±0.412	88.66±2.46
I-C	510.36±2.23*	114.61±2.43	151.29±0.512	154.68±3.86
	(+30.79 %)[a]	(+111.38 %)[a]	(+12.78 %)[a]	(+74.46 %)[a]
I-BdT	483.22±1.56*	66.24±1.92	137.31±0.291	110.62±2.88
	(-5.32 %)[b]	(-42.20 %)[a]	(-9.05%)[b]	(-28.48 %)[a]
I-BCOT	486.31±1.12*	62.68±1.86	134.15±0.212	102.21±1.76
	(-4.71 %)[b]	(-45.3 %)[a]	(-11.33 %)[a]	(-33.92 %)[a]

*Values are mean (mg/dl) ± SD from pooled plasma of 15 rats in each group.

N-C, normal control; I-C, Infected control; I-BdT, through orally in two equal doses of 2ml/Kg b.w.orally and I-BCOT, given through orally in two equal doses of 2ml/Kg b.w.orally for 16 weeks.

Significantly different from N-C at [a]p < 0.001.

Significantly different from I-C at [a]p<0.001 and [b]p<0.05.

Table 2: Antioxidant Impact of *B. Diffusa* (I-BdT) and black caraway oil (I-BCOT) on Total antioxidants, Conjugated diene, Lipid hydroperoxide & Malondialdehyde in DMBA-induced rats after 16 weeks of treatment

Group	Total antioxidants	Conjugated diene	Lipid hydroperoxide	MDA
N-C	48.37±0.056*	9.31±0.011	2.51±0.032	3.42±0.081
I-C	36.89±0.121*	14.36±0.016	3.81±0.015	5.91±0.116
	(-23.73 %)[a]	(+54.24 %)[a]	(+51.79 %)[a]	(+72.80 %)[a]
I-BdT	46.24±0.034*	12.46±0.014	3.12±0.006	4.82±0.044
	(+25.34 %)[a]	(-13.23 %)[a]	(-18.11 %)[a]	(-18.44 %)[a]
I-BCOT	42.10±0.020*	13.21±0.022	3.20±0.024	4.99±0.062
	(+14.12 %)[b]	(-8.00 %)[b]	(-16.01 %)[a]	(-15.57 %)[a]

*Values are mean (µmole/dl) ± SD from pooled plasma of 15 rats in each group.

N-C, normal control; I-C, Infected control; I-BdT, through orally in two equal doses of 2ml/Kg b.w.orally and I-BCOT, given through orally in two equal doses of 2ml/Kg b.w.orally for 16 weeks.

Significantly different from N-C at [a]p<0.001.

Significantly different from I-C at [a]p<0.001 and [b]p<0.05.

Effect on membrane lipid peroxidation in erythrocytes

Erythrocytes from DMBA-Induced rats (I-C) group showed a greater susceptibility to hydrogen peroxide-induced lipid peroxidation than those from N-C group. A substantial increase of 146 % in the MDA content in I-C was observed, when compared to N-C value. Formation of MDA was markedly decreased by 51 % and 36 %, after the administration of *B. diffusa* and Black Caraway Oil, respectively, when compared to the corresponding values in I-C **(Table 3).** Similarly, release of MDA erythrocytes was increased from 9.82 in N-C to18.21 nmol/gHb (85 %) in I-C. A highly significantly decrease of 37 % and 21 % in MDA level was observed in DMBA-Induced rats treated with twice equal does of 2 ml/Kg.b.w.o. *B. diffusa* and Black Caraway Oil, respectively, when compared to corresponding values in I-C rats. These results demonstrate that DMBA-Induced to rats for 16 weeks was associated with a significant increase in both *ex vivo* and *in vivo* erythrocytes membrane lipid peroxidation product and MDA, which was significantly prevented by the administration of *B. diffusa* and Black Caraway Oil.

Table 3: Malondialdehyde content & *in vitro* MDA release in erythrocytes of in DMBA-induced rats after 16 weeks of treatment *B. Diffusa* (I-BdT) and black caraway oil (I-BCOT).

Group	MDA (nmole/g Hb)	Erythrocytes MDA release (percent)
N-C	26.88±0.106*	9.82±0.106
I-C	66.22±0.103* (+146.35 %)[a]	18.21±0.121 (+85.44 %)[a]
I-BdT	32.18±0.212* (-51.40 %)[a]	12.11±0.130 (-33.50 %)[a]
I-BCOT	42.26±0.262* (-36.18 %)[a]	14.37±0.046 (-21.09 %)[a]

*Values are mean ± SD from pooled packed erythrocytes of 15 rats in each group.

N-C, normal control; I-C, Infected control; I-BdT, through orally in two equal Doses of 2ml/Kg b.w.orally and I-BCOT, given through orally in two equal doses of 2ml/Kg b.w.orally for 16 weeks.

Significantly different from N-C at [a]p<0.001.

Significantly different from I-C at [a]p<0.001.

Impact on liver, lung and kidney lipid peroxidation products

The formation of conjugated diene, lipid hydroperoxide and MDA in liver of DMBA-Induced rats was significantly increased by 34 %, 48 % and 28 %, respectively, whereas in lung, these levels were significantly increased by 28 %, 43% and 32 %, respectively. Similarly, in kidney formation of conjugated diene, lipid hydroperoxide and MDA were significantly increased by 47 %, 57 % and 36 %, respectively, when compared to corresponding values in N-C. Feeding of *B. diffusa* and Black Caraway Oil to DMBA-Induced rats, was associated with a significant decline in the formation of liver conjugated diene, lipid hydroperoxide and MDA by 21 %, 11 % and 22 % respectively, in I-BdT rats whereas, in I-BCOT, these levels were reduced by 20 %, 10 % and 8 %, respectively, when compared to corresponding values in I-C group **(Table 4).** Similarly, in lung, conjugated diene, lipid hydroperoxide and MDA were significantly reduced by 13 %, 25 % and 20% respectively, in I-BdT and in Black Caraway Oil treated rats; these values were decreased by 15 %, 22 % and 20 %, respectively, when compared to corresponding values in I-C rats. In addition, formation of conjugated diene, lipid hydroperoxide and MDA in kidney was significantly reduced by 17 %, 31 % and 9 % respectively, in I-BdT. Similarly, the above three lipid peroxidation products in kidney were significantly decreased by 16 %, 27 % and 12 %, respectively, in I-BCOT group, when compared to corresponding values in I-C group. These results demonstrate that increased levels of conjugated diene, lipid hydroperoxide and MDA in liver, lung and kidney of DMBA-Induced rats were significantly reduced after treatment.

Table 4: Effect Of *B. Diffusa* (I-Bdt) & Black Caraway Oil (I-BCOT) On Liver, Lung & Kidney Conjugated Diene, Lipid Hydrperoxide & Malondialdehyde Content In DMBA-Induced Rats After Treatment.

Group	Liver			Lung			Kidney		
	Conjugated diene	Lipid hydroperoxide	MDA	Conjugated diene	Lipid hydroperoxide	MDA	Conjugated diene	Lipid hydroperoxide	MDA
N-C	6.12±0.021*	1.498±0.001	3.61±0.014	2.52±0.054	0.526±0.02	3.01±0.024	2.21±0.011	0.446±0.004	4.28±0.060
I-C	8.18±0.015* (+33.66%)[a]	2.212±0.001 (+47.66%)[a]	4.61±0.084 (+27.70%)[a]	3.24±0.034 (+28.54%)[a]	0.754±0.003 (+43.35%)[a]	3.99±0.016 (+32.56%)[a]	3.10±0.025 (+47.27%)[a]	0.702±0.012 (+57.39%)[a]	5.84±0.020 (+36.45%)[a]
I-BdT	6.42±0.054* (-21.51%)[a]	1.963±0.002 (-11.26%)[a]	3.58±0.066 (-22.34%)[a]	2.80±0.035 (-13.58%)[a]	0.565±0.003 (-25.07%)[a]	3.20±0.026 (-19.80%)[a]	2.58±0.024 (-16.77%)[a]	0.485±0.004 (-30.91%)[a]	5.32±0.031 (-8.90%)[a]
I-BCOT	6.50±0.011* (-20.54%)[a]	1.978±0.022 (-10.58%)[a]	4.24±0.011 (-8.03%)[d]	2.74±0.034 (-15.43%)[d]	0.585±0.002 (-22.41%)[a]	3.17±0.044 (-20.55%)[a]	2.59±0.020 (-16.45%)[a]	0.510±0.006 (-27.35%)[a]	5.16±0.010 (-11.64%)[c]

*Values are mean (nmole/mg protein)± SD from homogenate of pooled liver, pooled lung or pooled kidney 15 rats in each group.

N-C, normal control; I-C, Infected control; I-BdT, through orally in two equal doses of 2ml/Kg b.w.orally and I-BCOT, given through orally in two equal doses of 2ml/Kg b.w.orally for 16 weeks.

Significantly different from N-C at [a]p<0.001. Significantly different from I-C at [a]p<0.001 and [c]p<0.02.

Lipid Lowering Effect on liver, lung and kidney triglycerides, total cholesterol and free fatty acids

Hepatic levels of triglyceride (TG), total cholesterol (TC) and free fatty acids (FFA) were significantly increased in DMBA-Induced rats (I-C) by 27 % 82 % and 36 %, respectively, when compared to corresponding values in N-C. Similarly, in lung, TG and TC levels were significantly increased by 16 % and 28 % respectively, whereas, FFA level was not affected, while, TG, TC and FFA levels, in kidney of I-C rats were significantly increased by 25 %, 25 % and 51 %, respectively, when compared to respective values in N-C **(Table 5).** Feeding of *B. diffusa* and Black Caraway Oil to DMBA-Induced rats for 16 weeks was associated with a significant decline in liver TG, TC and FFA levels by 13 %, 26 % and 19 % respectively, in I-BdT, whereas, in I-BCOT group, TG, TC and FFA levels were reduced by 5 %, 34 % and 14 % respectively, when compared to corresponding values in I-C group. Similarly, in lung, TG, TC and FFA levels were reduced by 9 %, 21 % and 8 % respectively, in I-BdT. Whereas, in I-BCOT a decline of 12 %, 17 % and 8 % in TG, TC and FFA levels, respectively was seen. In kidney, *B. diffusa* mediated a decline of 19 %, 13 % and 23 % in TG, TC and FFA, whereas, these lipid parameters were reduced by 14 %, 15 % and 16 %, respectively, in I-BCOT, when compared to corresponding values in I-C group. These results demonstrate that similar to plasma TG, TC and FFA levels in liver, lung and kidney were significantly increased in DMBA-Induced rats. In addition, feeding of *B. diffusa* and Black Caraway Oil to DMBA-Induced rats resulted in a significant decline of TG, TC and FFA to a level similar to corresponding values in N-C. The combined results demonstrate that levels of TG, TC and FFA in plasma, liver, lung and kidney lipids were significantly increased in DMBA-Induced rats. Treatment of these stressed rats with *B. diffusa* and Black Caraway Oil mediated a significantly decline in the above lipid parameters, similar to corresponding values in N-C rats.

Table 5: Effect of *B. Diffusa* (I-Bdt) & Black Caraway Oil (I-BCOT) On Liver, Lung And Kidney Triglycerides, Total Cholesterol and Free Fatty Acid Content In DMBA-Induced Rats After 16 Weeks Of Treatment

Group	Liver			Lung			Kidney		
	Triglycerides (mg/100mg protein)	**Total cholesterol (mg /100mg protein)**	**Free fatty acid (mg /mg protein)**	**Triglycerides (mg /100mg protein)**	**Total cholesterol (mg /100mg protein)**	**Free fatty acid (mg /mg protein)**	**Triglycerides (mg /100mg protein)**	**Total cholesterol (mg /100mg protein)**	**Free fatty acid (mg /mg protein)**
N-C	0.682±0.001*	2.72±0.066	21.24±0.566	0.584±0.003	2.50±0.012	12.82±0.143	0.559±0.005	2.26±0.003	8.21±0.081
I-C	0.866±0.006* (+26.98%)[a]	4.96±0.025 (+82.35%)[a]	28.96±0.231 (+36.35%)[a]	0.679±0.005 (+16.27%)[a]	3.21±0.043 (+28.40%)[a]	13.02±0.04 (+1.56%)[e]	0.697±0.005 (+24.68%)[a]	2.82±0.005 (+24.78%)[a]	12.43±0.112 (+51.40%)[a]
I-BdT	0.758±0.004* (-12.47%)[a]	3.69±0.022 (-25.60%)[a]	23.50±0.112 (-18.85%)[a]	0.621±0.004 (-8.54%)[c]	2.53±0.045 (-21.18%)[a]	11.96±0.652 (-8.14%)[a]	0.565±0.003 (-18.94%)[a]	2.46±0.005 (-12.76%)[a]	9.58±0.065 (-22.93%)[a]
I-BCOT	0.824±0.004* (-4.85%)[a]	3.26±0.021 (-34.27%)[a]	24.86±0.093 (-14.16%)[b]	0.596±0.001 (-12.22%)[c]	2.66±0.056 (-17.13%)[a]	11.99±0.096 (-7.91%)[a]	0.596±0.003 (-14.49%)[a]	2.41±0.004 (-14.54%)[a]	10.48±0.142 (-15.69%)[a]

*Values are mean ± SD from homogenate of pooled liver, pooled lung or pooled kidney 15 rats in each group.

N-C, normal control; I-C, Infected control; I-BdT, through orally in two equal doses of 2ml/Kg b.w.orally and I-BCOT, given through orally in two equal doses of 2ml/Kg b.w.orally for 16 weeks. Significantly different from N-C at [a]p<0.001.

Significantly different from N-C at [a]p<0.001 and [e]p not significant. Significantly different from I-C at [a]p<0.001, [b]p<0.05 and [c]p<0.02.

DISSCUSSION

Our data show that due to sustained free radical load in hypercholesterolemia, oxidation of lipid/lipoprotein particles is considerably enhanced. *B. diffusa* (Bd) [42,44,45] and Black Caraway Oil[43,44,46] (BCO) were generally considered the having potent antioxidant activity[40]. They quench free radicals in cell membranes and protect them against lipid peroxidation. The higher antioxidant potency of Bd as compared to BCO is attributed to the combined effects of three properties: it's higher recycling efficiency from chromanoxyl radical, its more uniform distribution in membrane bilayer, and its stronger disordering of membrane lipids which makes interaction of chromanols with lipid radicals more efficient. Since, in the present study of *B. diffusa* and Black Caraway Oil have examined in the efficacy of individual Bd and BCO as a scavenger of peroxyl radical. Our result showed that, by using DPPH, the order of antiradical activity or hydrogen donating ability, expressed in terms of half quenching concentration (IC_{50}) was Bd > BCO. The reduction in the free radical quenching efficiency of *B. diffusa* (44.42 µM) in comparison to BCO (38.11 µM). *B. diffusa* was found more efficient scavenger of peroxyradical then Black Caraway Oil. All the plasma lipid parameters (TL, TG, FFA and TC) were significantly increased from normal control to infected control group, consistent with other reports [41-51,54-56]. After both treatment level of plasma lipids were significantly decreased in comparison to infected groups. Conjugated diene (which measure the initial phase of lipid peroxidation), lipid hydroperoxide (intermediate product of lipid peroxidation) and MDA (which measure the degradation phase of lipid peroxidation) in plasma are significantly increased in DMBA-Induced. The increase in plasma lipid peroxidation products is associated with a significant decline in plasma total antioxidants. The former suggests increased production of oxidants while later indicates diminished antioxidant defence. Both the changes indicate an existence of profound oxidative stress.

In response to oxidative stress, DMBA-induced hyperlipidemic rats, our data show a significant increase in lipid/lipoprotein peroxidation products. Conjugated diene, lipid hydroperoxide and MDA in plasma, liver, lung and kidney were significantly increased in I-C rats. A similar increase in hepatic conjugated diene, lipid hydroperoxide and MDA has been reported in rats induced to DMBA; however, protective effect of a hypolipidemic agent with antioxidant property on the formation of three lipid peroxidation products in plasma and above tissues as well as MDA content or its release in erythrocytes of I-C rats has not been reported. The increase in plasma lipid peroxidation products is closely associated with a significant decline in total antioxidants of plasma. The increase in lipid peroxidation products in plasma, liver, lung and kidney and decrease in plasma total antioxidants is consistent with the well known prooxidant effect of DMBA-Induced in rats. Treatment of I-C rats with either 2ml/Kg.b.w.o for 16 weeks was associated with a significant decline in lipid peroxidation products of plasma, liver, lung and kidney and a significant increase in plasma total antioxidants, indicating a potent antioxidant effect of *B. diffusa* and Black Caraway Oil. Similar to plasma and other tissues, MDA content of erythrocytes in DMBA-Induced rats were significantly increased

(8 %). The intact erythrocytes isolated from I-C rats exhibited a further increase (>2-fold) in susceptibility to hydrogen peroxide induced lipid peroxidation and MDA release, as compared to the basal MDA content in erythrocytes. *B. diffusa* and Black Caraway Oil significantly blocked the *in vivo* as well as *in vitro* susceptibility of erythrocytes to lipid peroxidation and MDA release. *B. diffusa* (I-BdT) mediated normalization of MDA was 51% of N-C value, while Black Caraway Oil treatment (I-BCOT) caused a lesser degree of MDA reversal (36%), indicating a more potent antioxidative property of *B. diffusa* (I-BdT). These results indicate that due to massive and sustained load of free radicals and elevated levels of lipid peroxidation products formed in hyperlipidemic I-C rats may further aggravate the tissue oxidant/antioxidant imbalance and damage the antioxidant defence systems, which may lead to disruption of cellular functions and oxidative damage to membranes of erythrocytes and other tissues. In addition, treatment of hyperlipidemic I-C rats with two potent hypolipidemic agents with strong antioxidant activity may mediate a reduction in both lipid levels as well as lipid peroxidation products by scavenging cellular free radicals, thus improving overall oxidant/antioxidant balance as well as possibly protecting the oxidative damage to membranes and tissues. It is interesting to note that the changes observed in plasma total antioxidants, conjugated diene, hydroperoxide and MDA as well as erythrocytes MDA content and its release during *in vitro* H_2O_2 induced peroxidation from I-C rats. However, MDA release in intact erythrocytes from I-C (Table 3) rats was substantially higher, that is, >3-fold, in comparison to their respective *ex vivo* basal MDA content in erythrocytes, indicating a massive oxidative stress in rats DMBA-Induced. Consistent with the increase in plasma TG, FFA and TC levels of I-C rats, significant increases in these lipids of liver, lung and kidney were also observed, consistent with other reports[41-51] While, *B. diffusa* (I-BdT) and Black Caraway Oil (I-BCOT) treatment of I-C rats for 16 weeks was associated with a significant decreases in TG, FFA and TC of each tissue. These results demonstrate that sustained DMBA-Induced in I-C rats is also able to induce hyperlipidemia in the above tissues with a maximum increase in hepatic TC, while both *B. diffusa* (I-BdT) and Black Caraway Oil (I-BCOT) effectively blocked these increases and restored the TG, FFA and TC levels of liver, lung and kidney close to corresponding normal control values.

In response to oxidative stress, hypercholesterolemic and carcinogenic DMBA-Induced hyperlipidemic rats, our data show a significant increase in lipid/lipoprotein peroxidation products. Conjugated diene, lipid hydroperoxide and MDA in plasma, liver, lung and kidney were significantly increased in I-C rats. The increase in plasma lipid peroxidation products is closely associated with a significant decline in total antioxidant capacity of plasma. The increase in lipid peroxidation products in plasma, liver, lung and kidney and decrease in plasma total antioxidants is consistent with the well known prooxidant effect of DMBA-Induced in rats. Treatment of I-C rats with either 2ml/Kg.b.w.o *B. diffusa* and Black Caraway Oil was associated with a significant decline in lipid peroxidation products of plasma, liver, lung and kidney and a significant increase in plasma total antioxidants, indicating a potent antioxidant effect of *B. diffusa* and Black Caraway Oil. It is interesting to note that the changes observed in plasma total antioxidants, conjugated diene, hydroperoxide and MDA as well as erythrocytes MDA content and its release during *in vitro* H_2O_2 induced peroxidation from I-C rats is qualitatively similar to the changes seen in the infected rats (I-C). However, MDA release in intact erythrocytes from I-C rats was substantially higher, that is, >2-fold in infected rats (I-C), in comparison to their respective *ex vivo* basal MDA content in erythrocytes, indicating a massive oxidative stress in DMBA-Induced rats. In addition, consistent with significantly higher free radical scavenging property of dietary *B. diffusa* than Black Caraway Oil, *B. diffusa* offered much better protection against both *in vivo* and *in vitro* H_2O_2 induced lipid peroxidation in erythrocytes from I-C rats, than Black Caraway Oil.

However, since, dietary *B. diffusa* (I-BdT) and Black Caraway Oil (I-BCOT), because of their potent hypolipidemic/antiatherogenic, antioxidant actions and anticarcinogenic, were able to substantially ameliorate/normalize all the altered parameters including atheroprotective function of HDL described in the thesis, we initially recommend daily supplementation of infected control (I-C) with dietary *B. diffusa* (I-BdT) and Black Caraway Oil (I-BCOT). In conclusion, based on *B. diffusa* mediated multiple therapeutic benefits, described in the present study, daily intake of Black Caraway Oil as a dietary supplement by hypolipidemic/antiatherogenic/antihypercholesterolemic, antioxidant actions and anticarcinogenic may be useful in the prevention and treatment of DMBA-Induced hyperlipidemia/ and atherosclerosis. In addition, daily use of dietary *B. diffusa* and Black Caraway Oil will be efficacious, cost effective, no side effects and a good source of hypolipidemic/antiatherogenic, antihypercholesterolemic, antioxidant actions and anticarcinogenic.

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