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Research Article

EFFECT OF *Bacopa monniera* LINN. IN ATTENUATING HEPATIC OXIDATIVE STRESS IN HYPERCHOLESTEROLEMIC INDUCED RATS

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ABSTRACT

Hypercholesterolemia is an important risk factor associated with atherosclerosis and coronary heart disease. The present study investigates the lipid lowering action of alcoholic extract of Bacopa monniera (AEBM) on body weight, liver weight, hepatic lipid levels, hepatic antioxidant status, hepatic- marker enzymes, membrane bound ATPases, glycoproteins and DNA fragmentation of liver against high cholesterol diet (HCD) induced rats. Hypercholesterolemia was induced by Hypercholesterolemic diet [HCD- normal rat chow, supplemented with 4% cholesterol (w/w) and 1% cholic acid (w/w)] for 45 days. Treatment with AEBM (40mg/kg b.w/day orally, for last 30days) has shown to possess a significant protective role on HCD induced alteration in body weight, liver weight, hepatic lipid levels, hepatic antioxidant status, hepatic- marker enzymes, membrane bound ATPases, glycoproteins and DNA fragmentation of liver. The results of the present study conclude that the AEBM can be used as hypocholesterolemic against HCD induced hypercholesterolemia.

Keywords: Bacopa monniera, hypocholesterolemic, antioxidant status, ATPases, glycoproteins.

INTRODUCTION

Hypercholesterolemia is one of the most important risk factors for atherosclerosis and subsequent cardiovascular disease [1]. Hypercholesterolemia and Hyperlipidemia has been ranked as one of the greatest risk factors contributing to the prevalence and severity of coronary heart diseases. In developing countries, the incidence of cardiovascular disease is increasing alarmingly especially; India is on the verge of a cardiovascular epidemic [2,3].

Feeding animals with cholesterol has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolemia-related metabolic disturbances [4]. Exogenous hypercholesterolemia causes fat deposition in the liver and depletion of the hepatocyte population; it can also cause malfunctioning of the liver, which apparently follows micro vesicular stenosis due to the intracellular accumulation of lipids [5]. In addition, feeding cholesterol-rich diets induces free radical production (ROS), followed by oxidative stress and hypercholesterolemia [6,7].

A large number of allopathic hypolipidemic drugs are currently available in the market but these lag behind the desired properties such as efficacy and safety on long term use, cost and simplicity of administration. These factors do not fulfill conditions for patient's compliance [8].

Hence plant products are increasingly recognized as having protective role in coronary artery disease through several mechanisms including antioxidant (scavenging free radical), anti-inflammatory and hypolipidemic properties and also they do not have any adverse effect too [9,10].

Bacopa monniera (Linn) wettst.(Syn. *Herpestis monniera* (Linn.H.B) is a small creeping herb known as Brahmi in Ayurvedic medicine and is widely used in India, especially for enhancing memory, analgesia (pain relief), and epilepsy [11]. *Bacopa monniera* has traditionally been used to treat asthma, hoarseness, mental disorders, improve mental performance, nervine tonic, cardiotonic and diuretic (increases urine flow) [12]. Preclinical and clinical studies have shown that B*acopa monniera* improves memory and mental function [13]. The plant has been shown to possess a potent free radical scavenging and antioxidant properties [14]. Besides it also exhibits cardio-protective [15], vasodilatory [16], anti-inflammatory [17], calcium antagonistic [18], mast cell stabilizing [19], antiulcer [20] and anti-addictive [21] properties.

Bacopa monniera has been reported to contain triterpenoid saponins, alkaloids, phytosterol and flavonoids [22,23] . As far as high cholesterol diet induced hypercholesterolemia is concern there are no report available on the hypocholesterolemic activity of *Bacopa monniera*. Hence we have attempted to investigate the effect of *Bacopa monniera* on diet induced hypercholesterolemia in rats. Preliminary work on dose dependent study with various doses (20, 40 and 80 mg/kg b.w) of *Bacopa monniera* on hypercholesterolemia induced rats (data not shown) were carried out and we found that the dosage at 40 mg/kg b.w was highly significant in lowering lipid profile level when compared to other doses. Hence we followed the above dose (40 mg/kg b.w) for the present study.

MATERIALS AND METHODS

Chemicals

Lipid profile kit and diagnostic enzymatic kits are purchased from Spin React (Spain). DNA isolation kit were purchased from Medox Biotech Pvt Ltd (India), Cholesterol, Cholic acid, Hydrogen peroxide (H_2O_2), Formalin, ATP, Isopropanol, Digitonin, Trichloro acetic acid (TCA), Xylene, 2,4-dinitrophenyl hydrazine (DNPH), $CHCL_3$ were obtained from SRL, Chennai India. Folin Ciocalteau reagent, EDTA was obtained from SISCO research laboratory, Chennai, India. All other chemicals and solvents used were of analytical grade and highest purity.

Collection of plant material and extraction procedure

The plant material was collected at Chennai, Tamil Nadu and was authenticated by Dr.P.Brindha, Botanist, Captain Srinivasa Murthi Drug Research Institute for Ayurveda, Arumbakkam, Chennai. The shade-dried and coarsely powdered whole plant material (1kg) was extracted with 90% ethanol in the cold (48hrs). The extract was filtered and distilled on a water bath to get a dark green syrupy mass. It was finally dried in vacuo (52gm). The extract was dissolved in water and given orally as a suspension [21].

Experimental animals

Healthy male albino rats (195-205g) of Wistar strain were used for the study. The animals were procured from Central Animal House Block, Dr. ALM PG IBMS, University Of Madras, Taramani Campus, Chennai-113. The animals were housed in a large spacious cage, bedded with husk and were given food and water. The animal house was ventilated with a 12hr light/dark cycle, throughout the experimental period. The animals were maintained on a commercial rat feed manufactured by M/s. Pranav Agro Industries Ltd., India, under the trade name 'Amrut rat feed'. The feed contains 5% fat, 21% protein, 55% nitrogen free, 4% fiber (wt/wt) with adequate vitamin and mineral content. Experimental animals were handled according to the University and institutional legislation, regulated

by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment & Forests (Animal Welfare Division), Government of India (IAEC No. 01/09/2010).

Experimental design

After the adaptation period (7 days), the animals were divided into four groups of six rats each.

Group I Control rats fed with normal diet for 45 days.

Group II Rats fed with hypercholesterolemic diet (HCD) for 45 days [rat chow supplemented with 4% cholesterol (w/w) and 1% cholic acid (w/w); HCD] [24].

Group III Rats fed with HCD for 45 days + administrated with AEBM (40mg/kg, body weight/day orally) for last 30 days.

Group IV Rats fed with normal diet for 45 days + administrated with AEBM (40mg/kg, body weight/day orally) for last 30 days.

On 46th days the animals were sacrificed by cervical decapitation with mild anesthesia. The liver tissues were excised immediately, washed with ice-cold saline. A 10% homogenate of liver tissue were prepared by using 0.1M Tris HCl buffer pH 7.4. Blood was collected for biochemical analysis.

Body weight

Body weight was measured on day 1 (before experiment) and 45th day (after experiment). To reduce the error originating from feeding, all the animals were fasted (with water) for 12 hrs before measurement.

Biochemical Analysis

Hepatic total lipids (TL) were extracted in chloroform: methanol (2:1) ratio [25]. Hepatic TC, TG, PL were also estimated by using spin react (made in Spain) lipid profile kit according to manufacture instruction. The hepatic antioxidant enzyme Viz., SOD [26], CAT [27], Vit C [28] and VitE [29] were estimated according to the standard protocols. Hepatic serum marker enzymes Viz., SGOT, SGPT, LDH, GGT and ALP by span diagnostic enzymatic kit according to manufacture instruction. Hepatic Na^+-K^+ ATPase was assayed according to the method of Bonting [30], Ca^{2+} ATPase was estimated according to the method of Hjerten and Pan [31] and Mg^{2+} ATPase were assayed by the method of Ohinishi et al. [32]. Inorganic phosphorus was estimated by the method of Fiske and Subbarow [33].

For the estimation of glycoproteins, the tissues were defatted by the method of Folch method and the defatted tissues were treated with 0.1 N H_2SO_4 and hydrolysed at 80^{0}C, and aliquot was used for sialic acid estimation. To the remaining solution, 0.1 N NaOH was added. The aliquots were used for hexose, hexosamine and sialic acid estimation. Glycoproteins of hepatic tissue were estimated- Hexose was estimated by the method of Niebes [34], Hexosamine was estimated by the method of Wagner [35] and Sialic acid was determined by the method of Warren [36] with modifications. Protein was estimated by the method of Lowry [37].

Molecular Analysis

DNA from liver was isolated according to the manufacturer's instruction (DNA isolation kit, Medox Biotech Pvt Ltd) and DNA fragmentation was detected by Agarose gel electrophoresis by the method of Iwasa [38].

Statistical analysis

Results were expressed as mean ± SD (n=6) for six animals in each group. Differences between each group were assessed by one way analysis of variance (ANOVA) using SPSS 17 version. Post hoc testing was performed for the inter-group comparisons using the least significance difference (LSD) test. A value of P<0.01 was considered as statistically significant.

RESULTS

Effect of AEBM on body weight and liver weight in HCD induced control and experimental rats

Table. 1 shows the change in body and liver weight of control and experimental rats. The body weight and liver weight were found to be increased significantly (P<0.01) in hypercholesterolemic induced rats when compared to control rats. AEBM treatment significantly decreased the body and liver weight when compared to HCD rats. AEBM itself had no change in the body and liver weight, when compared with control rats.

Table 1: Effect of AEBM on body weight and liver weight in HCD induced control and experimental rats.

Group	Bodyweight (g)		Liver wet weight (g/100g b.w)
	Initial (g)	Final (g)	
Control	202.23 ± 10.44	243.34 ± 12.85	3.49 ± 0.25
HCD	201.43 ± 9.11	255.19 ± 9.55 a*	3.63 ± 0.15 a*
HCD+AEBM	199.12 ± 8.86	239.62 ± 12.44 b*	3.54 ± 0.19 b*
AEBM	203.49 ± 11.95	241.26 ± 11.88	3.50 ± 0.21

Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a- Compared with Control
b- Compared with HCD

Effect of AEBM on hepatic lipid status in HCD induced control and experimental rats

The levels of hepatic Total cholesterol (TC), Triglyceride (TG) and Phospholipids (PL) were depicted in figure 1. The levels of hepatic TC, TG, PL were significantly (P<0.01) increased in HCD induced rats when compared to normal rats. On supplementation with AEBM significantly reverted the HCD induced alterations in the levels of plasma TC, TG and PL (P<0.01) when compared to HCD group.

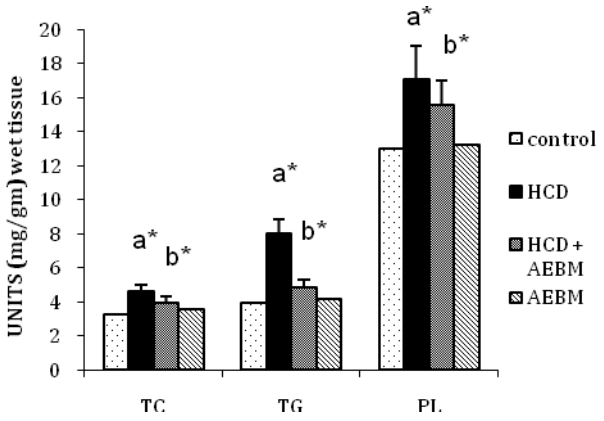

Figure 1: Effect of AEBM on hepatic lipid levels in HCD induced control and experimental rats
Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a- Compared with Control
b- Compared with HCD

Effect of AEBM on hepatic antioxidant status in HCD induced control and experimental rats

Table 2 shows the activity of hepatic antioxidant enzymes SOD, CAT, Vit-C and Vit-E in control and experimental rats. Significant decrease (P<0.01) in the activity of SOD, CAT, Vit-C and Vit-E were observed

in HCD induced groups when compared to the control rats. All the antioxidant activities were restored to normal level on AEBM treatment when compared to HCD induced rats.

Table 2: Effect of AEBM on hepatic antioxidant status in HCD induced control and experimental rats

Groups	SOD	CAT	VIT C	VIT E
Control	3.42 ± 0.71	68.34 ±6.77	2.49±0.30	2.08±0.25
HCD	2.24 ± 0.48 a*	48.71 ± 5.14 a*	1.75±0.14 a*	1.32±0.16 a*
HCD+AEBM	3.28 ± 0.68 b*	64.59 ± 5.68 b*	2.12±0.16 b*	1.86±0.14 b*
AEBM	3.19 ± 0.75	69.56 ± 7.23	2.28±0.32	2.04±0.18

Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a- Compared with Control
b- Compared with HCD
SOD : Units/mg protein, one unit is equal to amount of enzyme that inhibits the auto-oxidation reaction by 50%
CAT : µmoles of H_2O_2 consumed/min/mg protein.
Vit C and E : µg/mg protein

Effect of AEBM on hepatic marker enzymes in HCD induced control and experimental rats

Figure 2 portraits the effect of AEBM on hepatic markers- SGOT, SGPT, GGT and ALP on control and experimental animals. In HCD induced group there was a significant increase (P<0.01) in the activity of SGOT, SGPT, GGT and ALP when compared to control group. Treatment with AEBM significantly lowered (P<0.01) the activity of SGOT, SGPT, GGT and ALP when compared to HCD group.

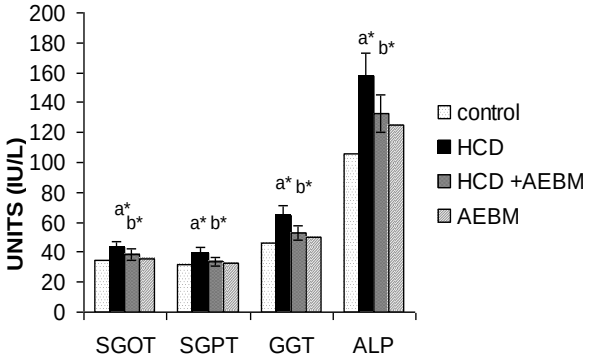

Figure 2: Effect of AEBM on hepatic marker enzymes in HCD induced control and experimental rats
Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a- Compared with Control
b- Compared with HCD

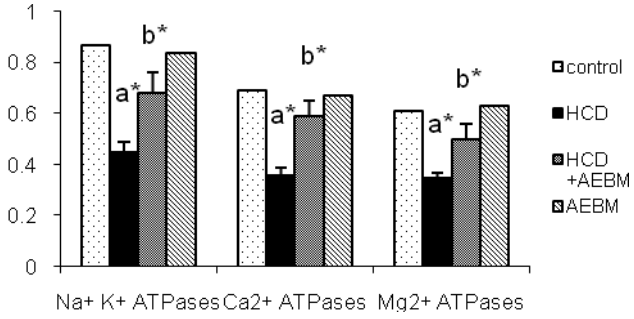

Figure 3: Effect of AEBM on hepatic ATPases in HCD induced control and experimental rats
Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a - Compared with Control
b - Compared with HCD

Effect of AEBM on hepatic ATPases in HCD induced control and experimental rats

Fig 3 represents the activities of hepatic Na^+-K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase on control and experimental animals. The activities of Na^+-K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase enzymes were found to be significantly decreased (P< 0.01) in HCD fed rats (Group II). Upon treatment with AEBM there was a significant increase (P< 0.01) in the activities of ATPases, when compared to HCD induced rats

Effect of AEBM on the levels of hepatic Glycoprotein components in HCD induced control and experimental rats

Table 3 shows the levels of glycoprotein components such as hexose, hexosamine, and sialic acid in hepatic tissue of normal and experimental rats. Hypercholesterolemic rats (group II) showed a significant increment (P< 0.01) in the levels of these glycoprotein components when compared with normo-cholesterolemic rats. Treatment with AEBM normalized (P< 0.01) the levels of these glycoprotein components when compared to HCD rats.

Table 3: Effect of AEBM on the levels of hepatic Glycoprotein components in HCD induced control and experimental rats

Group	Hexose (mg/g of defatted tissue)	Hexosamine (mg/g of defatted tissue)	Sialic acid (mg/g of defatted tissue)
Control	3.70±0.21	2.62±0.22	2.38±2.26
HCD	4.52±0.30 a*	3.86±0.30 a*	3.51±2.69 a*
HCD+AEBM	4.11±0.24 b*	2.98±0.26 b*	2.84±2.12 b*
AEBM	3.84±0.23	2.64±0.23	2.54±2.23

Values were expressed as mean ± S.D for 6 rats in each group.
Statistical Significance (P value): *P<0.01
a- Compared with Control
b- Compared with HCD

Effect of AEBM on Agarose gel electrophoretic pattern of hepatic DNA- FRAGMENTATION in HCD induced control and experimental rats

Fig 4 shows the agarose gel electrophoretic pattern of DNA fragmentation in control and experimental rats. DNA ladder (100-1000bp) where shown in lane 1. It was found that that there was a marked increase in the levels of DNA fragmentation in HCD induced rats (lane 3) when compared to control rats (lane 2). On treatment with *AEBM* which restores the HCD induced DNA fragmentation to near control (lane 4).

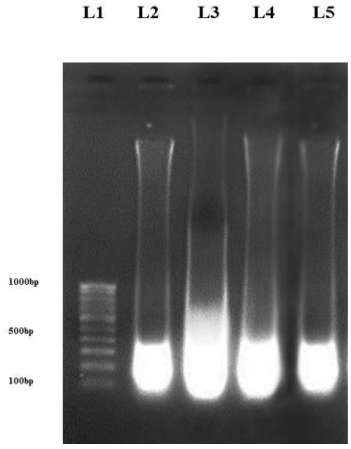

Figure 2: Effect of AEBM on Agarose gel electrophoretic pattern of hepatic DNA- FRAGMENTATION in HCD induced control and experimental rats
Lane 1 : DNA Marker (100-1000 bp)
Lane 2 : Control
Lane 3 : HCD
Lane 4 : HCD + AEBM
Lane 5 : AEBM

AEBM alone treated group didn't show any significant change in body weight, lipid levels, antioxidant status, hepatic marker enzymes, ATPases, glycoprotein and DNA fragmentation levels when compared with control rats.

DISCUSSION

Hypercholesterolemia/Hyperlipidemia is known to enhance the risk of coronary heart disease- atherosclerosis, myocardial ischemia, fatty liver disease, hepatic stenosis and carcinogenesis, which is associated with reactive oxygen species formation [39,40]. Liver being the major organ responsible for cholesterol transport, metabolism and excretion, it is reasonable to study hepatic oxidative disturbances in hypercholesterolemia [41]. The present study was therefore undertaken to investigate the anti-hypercholesterolemic action of alcoholic extract of *B. monniera (AERBM)* with respect to, hepatic damage produced by HCD via free radical/oxidative stress.

There is the concomitant increase in the body and liver weight in hypercholesterolemic induced rats when compared to control it may be due to increased absorption of cholesterol thus ends in increased body weight as well as liver weight. Similar observations were reported in several studies [42-44]. AEBM treatment markedly decreases the body weight and liver by decreasing the absorption of exogenous cholesterol due to the presence of flavonoid and saponins (Bacoside A and B) it could have precipitate cholesterol from micelles formation and interfere with enterohepatic circulation of bile acids making it unavailable for intestinal absorption.

In the current study, the HCD fed rats showed increased levels of hepatic cholesterol (TC), triglycerides (TG) and phospholipids (PL) levels compared to normal control rats. Treatment with AEBM significantly decreases the levels of hepatic TC, TG, PL when compared to HCD induced rats. Saponins are reported to precipitate cholesterol from micelles and interfere with enterohepatic circulation of bile acids making it unavailable for intestinal absorption, this forces liver to produce more bile from cholesterol (plasma) and hence the reduction in plasma cholesterol level (data not shown) which leads to reduction of hepatic lipid levels. Due to the presence of both flavanoids and saponins in *AEBM* [22,23] could have been contributed in reducing the levels of lipid status (TC, TG, PL) in hepatocytes and this might be the reason for also reduction of both body as well as liver weight.

Antioxidants are substances that either directly or indirectly protect cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions. The SOD decomposes superoxide radicals ($O2\bullet^-$) and produce H_2O_2. H_2O_2 is subsequently removed to water by CAT in peroxisomes, or by GPx oxidizing GSH in the cytosol [45,46]. Therefore, activities of these enzymes have been used to assess oxidative stress in cells. Ascorbic acid is the most widely cited form of water soluble antioxidant; it prevents oxidative damage to the cell membrane induced by aqueous radicals. Tocopherol, a known biological antioxidant, is concentrated in the hydrophobic interior of cell membranes. It has the potential to quench lipid peroxidation and protects cellular structure from the attack of free radicals [46]. Many studies have indicated that HCD can also alter antioxidant activities by inhibiting functional SH groups in several enzymes such as SOD, CAT because of its high affinity for sulfhydryl (SH) groups in these enzymes [43].

In the present study there is a concomitant decrease in the activity of SOD, CAT, Vit-C, Vit-E were observed in HCD induced groups when compared to the control rats this may be due to the increased amount of ROS generation. Many reports also supported our result that due to high fat induction there will be increase production of free radical (Peroxide) which contributes to the reduction of antioxidant level in HCD group [10,39,43].

All the antioxidant activities were restored to normal level on AEBM treatment when compared to HCD induced rats. Earlier it has been reported that AEBM has an antioxidant activity owing to the presence of its saponins, flavanoids and phytosterol [14].

The relationship between LPO and hypercholesterolemia is well recognized, a cholesterol rich diet results in increased LPO by the induction of free radical production [48]. Our results show a significant elevation in the levels of diagnostic hepatic serum marker enzymes such as SGOT, SGPT, GGT and ALP in HCD induced rats is due to Peroxide formation induced by hypercholesterolemia [24] result in increased cellular membrane permeability, intracellular fluid transfers into intercellular space, resulting in hepatocytes damage which leads to the leakage or release of hepatic marker enzymes from hepatocytes to serum and hence the level of marker enzymes are raised in HCD fed rats. Treatment with AEBM significantly reduced the activity of SGOT, SGPT, GGT and ALP to near normal levels due to its free radical scavenging property.

Membrane components of the cell play an important role in the regulation of cell function. It has been shown that the lipid composition of membranes can modify by dietary fats [49,50]. It is in agreement with the fact that the activity of many membrane bound enzymes and transport system is dependent on the physical state of the membrane lipid microenvironment [51].

It has been observed that an increase in the cholesterol content of plasma membranes results in decreased activities of ATPases [52]. Free radical produced by HCD suggested exerting their cytotoxic effects by causing peroxidation of proteins and membrane phospholipids (PL), which can result in an elevation in membrane fluidity, which increases permeability and loss of membrane integrity [53,54].

In the present work the activities of hepatic Na^+-K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase enzymes were significantly decreased in HCD fed rats due to increased peroxidation and lipid accumulation in hepatocytes. Upon treatment with AEBM significantly restores the activity of hepatic Na^+-K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase to normal group due to its antioxidant and hypolipidemic properties.

Glycoproteins are carbohydrate linked protein macromolecules found in the cell surface, which form the principle components of animal cells. Hexoses, hexosamine, and sialic acid are the basic components of the glycosaminoglycans and glycoproteins. They play an important role in membrane transport, cell differentiation and recognition, the adhesion of macromolecules to cell surface and the secretion and absorption of macromolecules [55].

The levels of glycoproteins are reported to be significantly increased in cardiovascular disease [56]. An increase in glycoprotein components has been reported to relate to the duration, severity and existence of degenerative Cardio vascular diseases [57]. Our study clearly shows an increase in the glycoprotein components namely hexoses, hexosamine, and sialic acid in HCD induced rats.

One possible explanation could be secretion (or) shedding of glycoproteins from cell membrane into the circulation due to peroxidative damage of membrane proteins for repairing the damage cause by free radical by HCD [58]. Treatment with AEBM significantly normalizes the activity of hepatic glycoproteins by repairing the damaged membrane structure caused by peroxidation, and scavenging the free radical and does not allow hepatocytes to undergo necrosis [59].

In our present study we found that that there was a marked increase in the levels of DNA fragmentation in HCD induced rats (lane 3) when compared to control rats (lane 2) due to increased ROS production which alters the macromolecules such as lipid, protein and especially nucleic acid –DNA [60]. *AEBM* treatment restores the HCD induced DNA fragmentation to near control (lane 4) due to its free radical scavenging activity. *AEBM* itself had no change in the level of DNA fragmentation when compared with control rats.

The result obtained in this study suggests that the AEBM has beneficial effects in preventing hypercholesterolemic induced hepatic oxidative stress and also a potent hypocholesterolemic agent. In future the active compounds of AEBM can be used to explore the mechanism behind the anti-hypercholesterolemic effect.

Declaration of interest

The authors declare that there is no declaration of interest to disclose.

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