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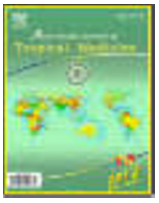

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Antihypercholesterolemic effect of *Bacopa monniera* linn. on high cholesterol diet induced hypercholesterolemia in rats

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ABSTRACT

Objective: To explore the effect of alcoholic extract of *Bacopa monniera* (AEBM) on high cholesterol diet-induced rats. **Methods:** The shade-dried and coarsely powdered whole plant material (*Bacopa monniera*) was extracted with 90% ethanol, finally filtered and dried in vacuum pump. The experimental rats were divided into 4 groups: control (group-I), Rats fed with hypercholesterolemic diet (HCD) for 45 days [4% cholesterol (w/w) and 1% cholic acid], Rats fed with HCD for 45 days+AEBM (40mg/kg, body weight/day orally) for last 30 days (group-III) and AEBM alone (group-IV). Blood and tissues (Aorta) were removed to ice cold containers for various biochemical and histological analysis. **Results:** AEBM treatment significantly decreased the levels of TC, TG, PL, LDL, VLDL, atherogenic index, LDL/HDL ratio, and TC/HDL ratio but significantly increased the level of HDL when compared to HCD induced rats. Activities on liver antioxidant status (SOD, CAT, GPx, GR, GST) were significantly raised with concomitant reduction in the level of LPO were obtained in AEBM treated rats when compared to HCD rats. Treatment with AEBM significantly lowered the activity of SGOT, LDH and CPK. Histopathology of aorta of cholesterol fed rat showed intimal thickening and foam cell deposition were noted. **Conclusions:** These results suggests that AEBM extended protection against various biochemical changes and aortic pathology in hypercholesterolemic rats. Thus the plant may therefore be useful for therapeutic treatment of clinical conditions associated hypercholesterolemia.

1. Introduction

High cholesterol diet is regarded as a crucial factor in the development of hypercholesterolemia, atherosclerosis and ischemic heart disease. Cardiovascular disease is the primary cause of mortality in the United States, Europe and most parts of Asia[1]. In hypercholesterolemia, the cholesterol content was elevated in serum as well as in erythrocytes, platelets and endothelial cells. This increased cholesterol is reported to activate these cells and cause the enhanced production of oxygen free radicals[2,3]. The elevation of serum total cholesterol and low-density lipoprotein (LDL) cholesterol as well as alteration of other lipid parameters has been implicated as a primary risk factor for cardiovascular disease[4-6].

Raised serum lipid levels, particularly of cholesterol along with generation of reactive oxygen species (ROS) play a key role in the development of coronary artery disease and atherosclerosis[7]. The body has evolved a complex defense strategy to minimize the damaging effects of various oxidants, central to this defense are the antioxidant enzymes. They include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) which act in concert to protect the organism from oxidative damage[8].

Varieties of plants have been used traditionally in the treatment of various cardiovascular diseases. A number of plants with potent therapeutic components such as fibers, phytosterols, saponins, polyphenols, flavanoids, etc., have been investigated for their antihyperlipidemic, antioxidant and anti atherosclerotic properties[9].

Bacopa monniera (Linn) wettst. (Syn. Herpestis monniera (Linn.H.B) (*B. monniera*) 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[10]. *B. monniera* has traditionally been used to treat asthma, hoarseness, mental disorders,

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improve mental performance, nervine tonic, cardiotonic and diuretic (increases urine flow)[11]. Preclinical and clinical studies have shown that *B. monniera* improves memory and mental function[12]. The plant has been shown to possess a potent free radical scavenging and antioxidant properties[13]. Besides it also exhibits cardio-protective[14], vasodilatory [15], anti-inflammatory[16], calcium antagonistic[17], mast cell stabilizing[18], antiulcer[19] and anti-addictive[20] properties.

B. monniera has been reported to contain triterpenoid saponins, alkaloids and flavonoids[21,22]. Despite the availability of literature on the medicinal properties of *B. monniera* and its chemical constituents, no report exits on its hypocholesterolmic properties. We therefore have attempted to investigate the effect of *B. monniera* on diet induced hypercholesterolemia in rats by monitoring lipid and lipoprotein status, antioxidant status, cardiac marker enzyme and histological changes of aorta.

2. Materials and methods

2.1. 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, India. The shade-dried and coarsely powdered whole plant material (1kg) was extracted with 90% ethanol in the cold (48 h). The extract was filtered and distilled on a water bath to get a dark green syrupy mass. It was finally dried in vacuo (52 g). The alcoholic extract of *B. monniera* (AEBM) was dissolved in water and given orally as a suspension.

2.2. Experimental animals

Healthy male albino rats of Wistar strain weighing (200± 250) g were used for the present study. The animals were purchased from Central Animal House Block, Dr. ALM PG IBMS, University Of Madras, Taramani Campus, Chennai-113. The animals were housed in large spacious cages. Food and water were given *ad libitum*. The animal house was ventilated with a 12 h light/dark cycle, throughout the experimental period. Rats were allowed to adapt to their environment condition for at least 10 days before the initiation of experiment. All experiment and protocols described in this study were approved by the Institutional Animal Ethics Committee (IAEC No: 01/09/10) of Dr.ALM PGIBMS, University of Madras, Taramani Campus, Chennai-113.

The rats were randomly divided into four groups of six rats each.

Group I Control rats fed with normal diet.

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)].

Group III Rats fed with HCD for 45 days[23] + 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 (40 mg/kg, body weight/day orally)[20] for last 30 days.

At the end of the experimental period (45 days), the animals were fasted overnight and sacrificed by cervical decapitation. The aorta and liver tissues were excised immediately, washed with ice-cold saline and then dried with filter paper. A 10% homogenate of liver were prepared by using 0.1M Tris HCl buffer pH 7.4. The slice of aorta tissue was fixed with 10% formalin amd stained with Haematoxylin and eosin stain for histopathological studies. Blood was collected in two different tubes, i.e., one with anticoagulant-EDTA for the separation of plasma and another without anticoagulant for the serum. The above samples were used for biochemical analysis.

2.3. Chemicals

Lipid profile kit purchased from Spin React (Spain). Nitro Blue Tetrazolium (NBT), L- α -phosphatidyl choline was obtained from Sigma chemicals (St. Louis, USA). Hydrogen peroxide (H2O2), Chromatrophic acid, 5,5′ Dithiobis-(2-nitrobenzoic acid) (DTNB), Sodium dodecyl sulphate (SDS), Ethylene-diaminetetra acetic acid (EDTA), dextran sulphate, Trichloro acetic acid (TCA) and Formalin were obtained from SRL, Mumbai, India. Folin Ciocalteau reagent, Thiobarbituric acid (TBA) Sialic acid was obtained from CDH, New Delhi, India.

2.4. Biochemical analysis

Plasma total cholesterol (TC), triglyceride (TG), phospholipids (PL), LDL-C, VLDL-C and HDL-C levels were measured using commercial kits from Spin react (Girona, Spain) according to the manufacturer's specifications. LDL/HDL ratio, TC/HDL ratio, and atherogenic index (AI) were calculated by the method[24]. Activity staining of SOD 8% and CAT 5% on gel were performed by using a native gel activity stain[25]. The antioxidant enzyme *viz*., GPx [26], GR[27] and GST[28] were estimated according to the reported methods. The GSH content in liver were measured by the method[29]. The lipid peroxidation product (LPO) in liver was measured by the method [30]. Serum cardiac marker enzymes Viz., SGOT[31], LDH[32] and CPK[33] were also evaluated according to the reported method. Protein was estimated by the method [34].

2.5. Histopathological studies

A midline thoracotomy was performed and the portion of thoracic aorta was quickly removed with heart from control and experimental animal and were fixed in 10% formalin, then dehydrated in the descending grades of isoproponol and xylene. The aorta was then embedded in molten paraffin wax and sectioned at 5 μm thickness and were stained with hematoxylin and eosin (H&E), viewed under light microscope for histological changes.

2.6. Statistical analysis

The values 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 and least significant difference (LSD) was determined using Post hoc test at the level of P<0.05.

3. Results

3.1. Effect of AEBM on plasma lipid status

The levels of plasma total cholesterol (TC), triglyceride (TG) and Phospholipids (PL) were given in Figure 1. The levels of plasma TC, TG, PL were significantly (P<0.01) increased in HCD induced rats when compared to normal rats. Treatment with AEBM significantly reverted the HCD induced alterations in the levels of plasma TC, TG (P<0.01) and PL (P<0.05) when compared to HCD group.

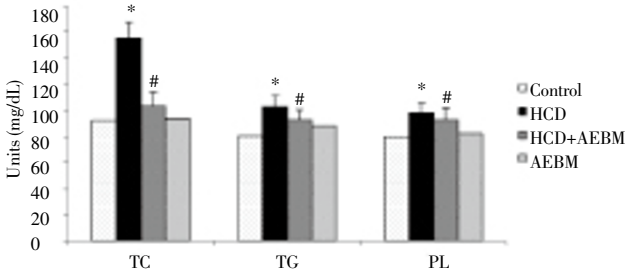

Figure 1. Effect of AEBM on plasma lipid status (TC, TG, PL) in control and experimental rats.
Values were expressed as mean ± S.D for 6 rats in each group.
*P<0.05 compared with control, **P<0.01 compared with control.
#P<0.05 compared with HCD, ##P<0.01 compared with HCD

3.2. Effect of AEBM on the level of plasma lipoproteins and ratios

Table 1 shows the effect of AEBM on the levels of plasma lipoproteins (HDL, LDL, VLDL) and the ratio of LDL/HDL, TC/HDL and atherogenic index of control and experimental rats. A significant (P<0.01) increase in the levels of LDL, VLDL, LDL/HDL ratio, TC/HDL ratio and atherogenic index with a significant decrease (P<0.01) in the level of HDL were observed in HCD induced rats when compared to control rats. Treatment with AEBM, reverted back all the changes in terms of lipoprotein and its ratios to normal level when compared to HCD induced rats.

3.3. Effect of AEBM on activity staining of hepatic SOD and CAT

Figure 2 and 3 shows the activity staining of hepatic superoxide dismutase (SOD) and catalase (CAT) in control and experimental rats by Native-PAGE. A marked decrease in the activity staining of the SOD and CAT, were observed in HCD induced rats (lane 2) when compared to control rats. AEBM treatment (lane 3) restores the HCD induced alterations in the activity staining of the SOD and CAT to near control. AEBM alone treated rats (lane 4) did not show any changes in the activity staining of the SOD and CAT and resembled very much similar to that of control rats (lane 1).

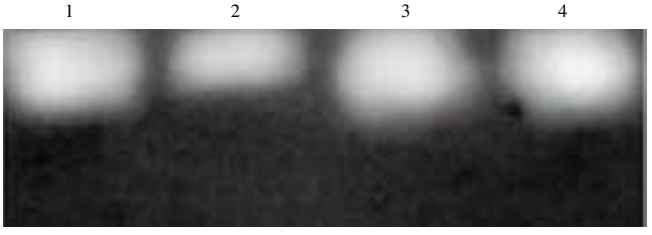

Figure 2. Activity staining of hepatic SOD in control and experimental rats by Native-PAGE.

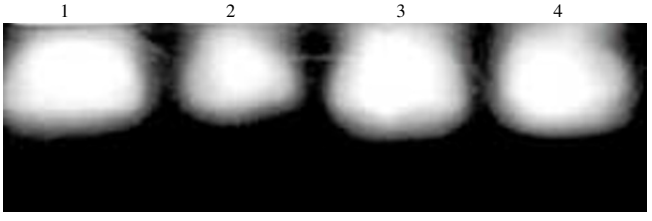

Figure 3. Activity staining of hepatic CAT in control and experimental rats by Native-PAGE.

Table 1
Effect of AEBM on the level of plasma lipoproteins and ratios in control and experimental rats.

Group	Plasma lipoproteins (mg/dL)			Ratios		
	HDL	LDL	VLDL	Atherogenic index	LDL/HDL	TC/HDL
Control	32.60±2.14	41.37±3.17	18.45±1.27	1.83±0.14	1.26±0.11	2.83±0.25
HCD	27.92±1.05 **	105.74±4.11 **	21.46±1.50 **	4.55±0.38 **	3.78±0.33 **	5.55±0.52 **
HCD+AEBM	30.05±1.82 ##	54.05±2.19 ##	19.70±.92.00 #	2.45±0.19 ##	1.79±0.17 ##	3.45±0.37 ##
AEBM	33.16±3.35	44.35±1.88	17.09±0.82	1.79±0.14	1.33±0.12	2.85±0.31

Values were expressed as mean ± S.D. for 6 rats in each group, *P<0.05 compared with control, **P<0.01 compared with control, #P<0.05 compared with HCD, ##P<0.01 compared with HCD.

Table 2
Effect of AEBM on the activities of liver antioxidant enzymes (GPx, GR, GST) in control and experimental rats.

Group	GPx (U/mg protein[1])	GR (U/mg protein[2])	GST (U/mg protein[3])
Control	13.49±1.30	0.80±0.05	0.53±0.04
HCD	10.75±1.10 **	0.52±0.06 **	0.38±0.02 **
HCD+AEBM	12.12±1.16 ##	0.68±0.10 ##	0.45±0.03 ##
AEBM	13.28±1.34	0.74±0.04	0.46±0.07

Values were expressed as mean ± S.D for 6 rats in each group, **P<0.01 compared with control, ##P<0.01 compared with HCD, 1 μg of GSH oxidized/min, 2 μmol NADPH oxidized/min, 3 nmol CDNB-GSH conjugate/min.

3.4. Effect of AEBM on the activities of hepatic antioxidant enzymes

Activities of liver antioxidant enzymes GPx, GR and GST were shown in Table 2. The activities of these antioxidant enzymes were found to be significantly (P<0.01) reduced in HCD induced rats when compared to normal rats. In the case of AEBM treated rats, there was a pronounced increase (P<0.01) in the activities of these enzymes.

3.5. Effect of AEBM on the levels of hepatic LPO and GSH content

Figure 4 and 5 portraits the levels of hepatic LPO and GSH content in control and experimental rats. Significant increase (P<0.01) in the level of LPO with the concomitant decrease (P<0.01) in glutathione content were observed in HCD induced groups when compared to the control rats. Supplementation with AEBM caused significant (P<0.05) restoration in the levels of glutathione content and LPO products, when compared to HCD induced rats.

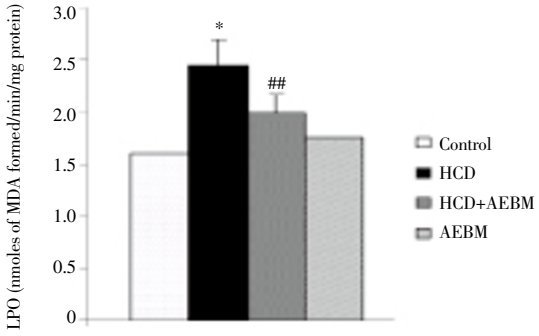

Figure 4. Effect of AEBM on the levels of hepatic LPO in control and experimental rats.
Values were expressed as mean ± S.D. for 6 rats in each group.
**P<0.01 compared with control, ##P<0.01 compared with HCD

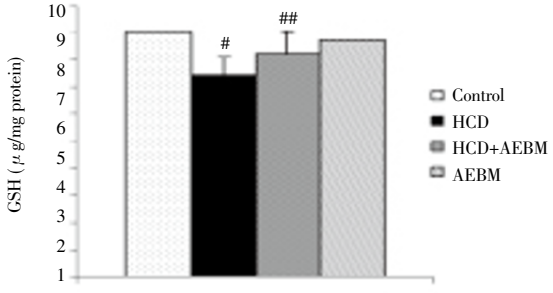

Figure. 5. Effect of AEBM on the levels of hepatic GSH in control and experimental rats.
Values were expressed as mean ± S.D. for 6 rats in each group.
**P<0.01 compared with control, ##P<0.01 compared with HCD

3.6. Effect of AEBM on the activity of serum cardiac markers enzymes

The activity of serum cardiac marker enzymes where shown in Figure 6. In HCD induced group there is a significant increase (P<0.01) in the activity of serum cardiac marker enzymes SGOT, LDH and CPK when compared to control group. Treatment with AEBM significantly lowered (P<0.01) the activity of SGOT, LDH and CPK when compared to HCD group (Figure 6). AEBM alone treated group doesn't show any significant change in lipid levels, antioxidant status and marker enzyme when compared with control rats.

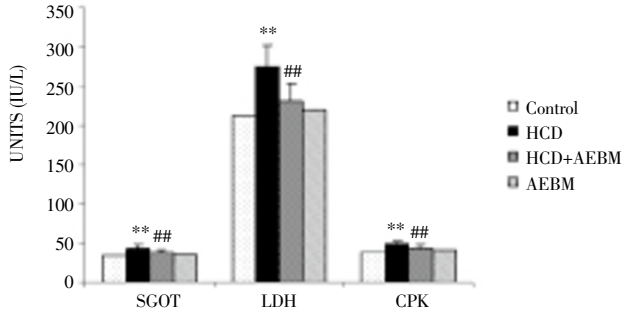

Figure 6. Effect of AEBM on the activity of serum cardiac markers enzymes (SGOT, LDH, CPK) of control and experimental rats.
Values were expressed as mean ± S.D. for 6 rats in each group.
**P<0.01 compared with control, ##P<0.01 compared with HCD

3.7. Effect of AEBM on Histological changes

Figure 7A showed no pathological changes (normal intima) in rats of control group (100X). Figure 7B rats fed with an HCD developed typical plaques characterized by thickening of the intima, migration of smooth muscle cells to the intima (shown by arrow mark), adhesion and infiltration of macrophages, appearance of foam cells and loss of normal arrangement of elastic lamellae under the endothelium are seen (100X). Figure 7C pathological changes of thoracic aorta in HCD+AEBM groups were less visible than that in HCD alone group, but shown less thickening of the intima and foam cells count (100X). Figure 7D AEBM alone treated rats showed normal intimal-medial thickening without inflammatory cellular infiltration, and was similar with that in control group (100X).

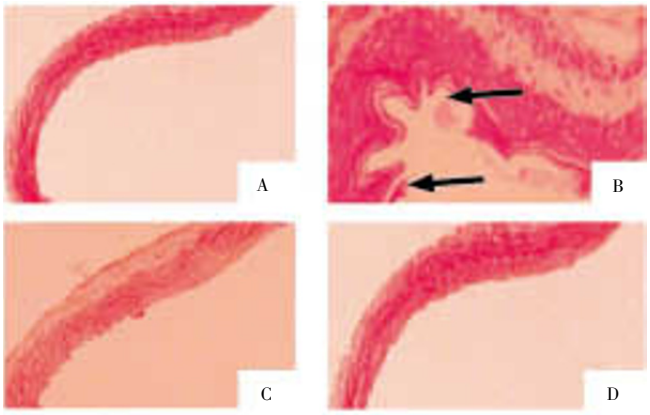

Figure 7. Effect of histopathological changes of aorta in control and experimental rats.
Figure 7A showed no pathological changes (H&E) in rats of control group (100X). Figure7B rats fed with an HCD, showed development of typical plaque and appearance of foam cells and loss of normal arrangement of elastic lamellae under the endothelium (100X). Figure 7C pathological changes of thoracic aorta of HCD+AEBM rats showed mild intimal thickening (100X). Figure 7D AEBM alone treated rats showed normal intimal-medial thickening and resembles control group (100X).

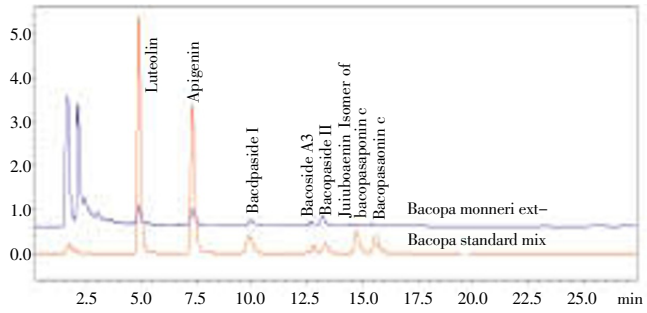

Figure 8. Hplc-chromatogram of *B. monniera* extract.
Fiugre 8 shows the overlaid chromatogram of *B. monniera* extract by HPLC along with bacosides and other flavonoids (luteolin and apigenin).

4. Discussion

Hypercholesterolemia and the resulting atherosclerosis have been implicated in the pathophysiology of coronary heart diseases and myocardial ischemia. Lowering cholesterol level may decrease the risk of CVD, and therefore enormous efforts have been extended to achieve this aim[7]. The hypocholesterolemic activity of alcoholic extract of *B. monniera* (AEBM) against hypercholesterolemia was monitored on the Lipid profile status, antioxidant status, activities of serum cardiac marker enzyme, level of LPO and histological changes of aorta.

In this study the high cholesterol diet (HCD) is used which consists of normal rat chow supplemented with 4% cholesterol (w/w) and 1% cholic acid (w/w) because cholic acid improves dietary absorption of cholesterol and hence diet supplemented with both cholesterol and cholic acid has been used in many experimental hypercholesterolemia[23]. The diet-induced hypercholesterolmia animal model has long been used for the assessment of agents with beneficial effects on cholesterol[35].

In the current study, the HCD fed rats showed increased levels of plasma cholesterol (TC), triglycerides (TG) and phospholipids (PL) levels compared to normal control rats. Treatment with AEBM significantly decrease the levels of plasma TC, TG, PL when compared to HCD induced rats. Saponins are also 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. Saponins are also reported to lower triglycerides by inhibiting pancreatic lipoprotein lipase[36]. Similarly in our study also, the presence of both flavanoids and saponins in AEBM [21,22] could have been contributed in reducing the levels of lipid status (TC, TG, PL).

Elevated levels of plasma low density lipoprotein cholesterol (LDL) and very low density lipoprotein cholesterol (VLDL) are often accompanied by premature atherosclerosis and other CVD. A low level of high-density lipoprotein cholesterol (HDL) is also an important risk factor for cardiovascular disease[37]. The cardioprotective effects of HDL have been attributed to its role in reverse cholesterol transport, its effects on endothelial cells, and its antioxidant activity. Flavanoids can increase HDL-C and also decreases oxidation of LDL-cholesterol[38]. High cholesterol diet increases plasma LDL levels and oxidative stress which results in the production of increased oxidized LDL and thereby increases atherosclerotic plaque formation[39]. From the present study it is evident that HCD induced rats showed increased plasma LDL and VLDL levels with the concomitant reduction in plasma HDL level, when compared to normal rats. Supplementation with AEBM reduced the plasma LDL and VLDL levels and increased the plasma HDL level which could be due to reduction in plasma total cholesterol and increasing LDL receptor activity by the flavanoids and phytosterol present in the plant extract. Also it could be presumed that the reduction of total cholesterol by AEBM could have been associated with a reduction of its LDL fraction, which is the target of several hypolipidemic drugs.

LDL/HDL and TC/HDL ratio had direct correlation with the cardiovascular disease (CVD) risk. An increase in the LDL/HDL and TC/HDL ratio is directly proportional to increased risk of CVD. From our study, we observed that, the HCD fed rats showed increased levels of Atherogenic index (AI), LDL/HDL and TC/HDL ratio compared to normal control rats. Supplementation with AEBM markedly decreases the Atherogenic index (AI), LDL/HDL and TC/HDL ratio due to its action on reducing the plasma total cholesterol level.

Oxidative stress is believed to contribute to the pathogenesis of hypercholesterolemic atherosclerosis hence, various antioxidant compounds are being evaluated for potential anti-hypercholesterolemic effects[9]. A high fat diet brings about remarkable modifications in the antioxidant defence mechanisms of rat tissues by the process of lipid peroxidation. Several reports have shown that hypercholesterolemia diminishes the antioxidant defence systems[40] by producing free radicals and thereby elevating the lipid peroxide products, resulting in the production of toxic intermediates.

SOD is the first enzyme in antioxidant defense that protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical (O^{2-}), which damages the membrane and biological structures[41]. CAT has been shown to be responsible for the detoxification of significant amounts of H_2O_2[42]. SOD and CAT are the two major scavenging enzymes that remove the toxic free radicals. From our study we observed that there was a reduction in the activity staining of hepatic SOD and CAT in HCD induced rats (lane 2) when compared to control rats (lane 1), this may be due to the enhanced production of Reactive Oxygen Species (ROS) by HCD. This free radical affects the antioxidant activity and hence resulted in the decreased activity staining of SOD and CAT. Treatment with AEBM (lane 3) restores the HCD induced alteration in the activity of staining of the SOD and CAT to near control due to its free radical scavenging activity.

The glutathione-S-transferase (GST) family of enzymes comprises a long list of cytosolic, mitochondrial, and microsomal proteins that catalyze the conjugation of reduced glutathione via the sulfhydryl group, to electrophilic centers on a wide variety of substrates[43]. This activity is useful in the detoxification of endogenous compounds such as deoxidized lipids[44]. GPx has been shown to be responsible for the detoxification of H2O2. Glutathione reductase is

responsible for the reduction of oxidised glutathione to glutathione (reduced). The increased oxidant stress in hypercholesterolemic conditions exhausts the GSH pools[35].

Activities of hepatic antioxidant enzymes *viz*., Glutathione peroxidase (GPx) and Glutathione Reductase (GR) enzymes and Glutathione contents were significantly decreased in HCD induced rats. On oral administration with AEBM, the activities of these antioxidant enzymes in liver were reverted back to normal level. Earlier it has been reported that AEBM has an antioxidant activity owing to the presence of its saponins, flavanoids and phytosterol[13].

LPO is regarded as one of the basic mechanisms of cellular damage caused by free radicals[45]. The relationship between LPO and hypercholesterolemia is well recognized, a cholesterol rich diet results in increased LPO by the induction of free radical production[46]. Hypercholesterolemia and lipid peroxidation are believed to be critically involved in development of Atherosclerosis[47]. In our study we found that a significant increase in LPO levels were observed in HCD fed groups when compared to the control group. AEBM supplementation brought down the level of LPO to near normal. It has been already reported that AEBM has an antioxidant activity owing to the presence of its saponins, flavanoids and phytosterol[13], thus it decrease the concentration of free radicals, which might terminate the initiation and propagation of LPO.

Several reports showed that high cholesterol level can cause cardiac damage[48-50]. Elevation in the levels of diagnostic cardiac marker enzymes such as SGOT, LDH and CPK in serum of HCD induced rats is due to Peroxide formation induced by hypercholesterolemia in the form of ROS[23]. This ROS production increases cellular membrane permeability, intracellular fluid transfers onto intercellular space, resulting in muscle and cardiac damage which leads to the leakage or release of cardiac marker enzymes from cardiac tissue to serum and hence the level of marker enzymes are raised in HCD fed rats. There was a significant elevation in the levels of cardiac marker enzymes such as SGOT, LDH and CPK were observed in HCD induced rats when compared with control rats. Treatment with AEBM significantly reduced the activity of SGOT, LDH and CPK to near normal levels.

Macroscopic observation of thoracic aorta showed that tenacity of vessel in control group was better than that of hypercholesterolemic rats. Furthermore, surface of intima in rats fed with normal rat chow was smooth and glossy, and there is no thickening of the intima or migration of smooth muscle cells to the intima, whereas surface of intima of HCD induced rats showed a typical plaques characterized by thickening of the intima, migration of smooth muscle cells to the intima, infiltration of macrophages, appearance of foam and lamellar calcification under the endothelium.

From our result we observed that there was an increased foam cell formation which leads to intimal thickening in HCD induced group. These results were accordance with the reports of [51,52]. These foam cell counts were reduced in AEBM treated group which could be due to its inhibition activity on cholesteryl ester and thereby reduces the specific binding sites of acetyl LDL hence can reduce the foam cell formation and leads to less thickening of intima.

The result obtained in this study suggests that the alcoholic extract of *B. monniera* has beneficial effects in preventing hypercholesterolemia by lowering lipid status, improving antioxidant status as well as protecting the aortic morphology.

The present experimental data therefore suggest that alcoholic extract of *B. monniera* has an atheroprotective potential. In our laboratory we have started to work on major active component namely Bacoside-A from the plant extract for its hypocholesterolemic activity. Further studies are needed to check the indepth mechanism of hypocholesterolemic activity of AEBM at molecular level.

Conflict of interest statement

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

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