Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2014, Volume 2, Issue 7
ISSN:2321–3647(online)

Anti-Atherosclerotic and Lipolytic Property of Polyherbal Formulation

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ABSTRACT

The incidence of coronary heart disease has markedly increased in India over the past few years. Ischemic heart disease, the largest cause of morbidity and mortality in the developed and developing countries today is overwhelmingly contributed by atherosclerosis. The main objective of the study is to evaluate the phytochemical screening of the plants and find out the potency of polyherbal formulation in high fat diet induced atherosclerosis in rats. *Terminalia arjuna, Nardostachys jatamansi, Garcinia combogia, Tribulus terrestris, Inula racemosa* are ayurvedic medicinal plants used in India to treat several ailments. The present study was aimed at performing pre-clinical studies of a polyherbal formulation prepared from the above mentioned plants in high cholesterol diet induced atherosclerosis in rats. The study highlights the activity of the polyherbal formulation in the treatment of atherosclerosis. 6 groups of male rats containing 5 animals per each group were taken and given 200mg/kg/day, 300mg/kg/day and 400mg/kg/day of hydroalcoholic extract of polyherbal formulation orally for 28 days along with the high cholesterol diet. The experiment showed promising results by significantly decreasing cholesterol, triglycerides, and LDL levels. Significant reduction in serum total cholesterol and increased HDL cholesterol was seen. The extract also showed hepato protective activity. MDA levels were reduced considerably. Our studies indicate that hydro alcoholic extract of plants used in polyherbal formulation exerts potent hypolipidemic effects in atherosclerotic rats which are evident from the histopathological results.

Keywords: Atherosclerosis, Plaque, Cholesterol, Atherogenic index, Cardiovascular diseases.

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Received 21 June 2014, Accepted 27 June 2014

Please cite this article in press as: Vedasri R et al., Anti-Atherosclerotic and Lipolytic Property of Polyherbal Formulation. American Journal of Pharmacy & Health Research 2014.

INTRODUCTION

Cardiovascular diseases are the largest cause of mortality, accounting for around half of all deaths resulting from Non Communicable Diseases (NCDs). Overall, CVDs accounted for around one-fourth of all deaths in India in 2008. CVDs are expected to be the fastest growing chronic illnesses between 2005 and 2015, growing at 9.2% annually, and accounting for the second largest number of NCD patients after mental illnesses. A more worrying fact is that the incidences of CVDs have gone up significantly for people between the ages 25 and 69 to 24.8%, which means we are losing more productive people to these diseases[1].

Coronary artery disease (CAD) starts with the formation of atherosclerotic plaques in the coronary arteries. Abrupt occlusion of these atherosclerotic arteries due mainly to thrombosis leads to coronary heart diseases: unstable angina, acute myocardial infarction and sudden death[2].

According to World Health Organization (WHO), approximately 80% of the world's population currently uses herbal medicines in healing different ailments. Among the estimated 400,000 plant species, only 6% have been studies for biological activity and about 15% have been investigated phytochemically[3].

Synthetic antilipidemic drugs clearly reduce cardiovascular mortality but are expensive and sometimes highly prone to side effects. However they are not covered by most health care insurers when used primarily for prophylactic purpose. The plant kingdom represents a rich storehouse of organic compounds, many of which have been used for medicinal and other purpose. There exists a plethora of knowledge, information and benefits of herbal drugs in our ancient literature of Ayurvedic and Unani medicines[4].

Present investigation is an attempt for pharmacological evaluation of atherogenic properties of a polyherbal preparation.

MATERIALS AND METHODS

Source of Plant Material:

Plant materials were obtained from forests in and around Warangal, AP and from Mithali herbals, Vijayawada, Andhra Pradesh. Taxonomic identification and authentication was given by Botanist Dr. Sunitha, Head of the department, St. Marys College, Yosufguda, Hyderabad.

Preparation of extract:

The required parts of the five plants used in the preparation of polyherbal formulation were taken, shade dried and coarsely powdered by using grinder mixer. The powdered material was macerated in sufficient quantity of ethanol and water (1:1 ratio) with small quantity of

chloroform to prevent fungal growth and kept for 3 days. During maceration it was shaken twice daily. On 3rd day it was filtered and dried at 60 °C on water bath. The extract was then preserved in the desiccator and then used for phytochemical and pharmacological studies. All the drugs used in this study were of pharmaceutical grade supplied by Sigma Aldrich.

Instruments Used:

Hot air oven (everflow scientific instruments)**,** Lyophiliser (delta sceintific equipments)**,** Centrifuge (perkin elmer)**,** Semi Auto analyzer (Hitachi 7070)**,** Soxhelet (borosilicate glass equipment)

Animals:

Male albino rats (180-200 g) were used for study. Animals were maintained at a temperature of 25±1°C and relative humidity of 45 to 55 % under 12 hr light and 12 hr dark cycle. The animals had free access to food pellets and water ad libitum. Ethical clearance was obtained from Institutional Animal Ethical Committee (IAEC), Hyderabad (CPCSEA Reg No: 1675/RO/c/12/CPCSEA, Project No: DLL/13/66, IAEC Approval No: 0012/13) After the duration of treatment blood was collected from the retro-orbital sinus and tests were done for biochemical parameters.

Acute Toxicity Studies:

Acute oral toxicity was carried out according to the OECD guidelines following acute oral toxicity-acute toxic class method (423 guidelines). The methanolic extract of polyherbal preparation showed no mortality in Albino Wistar rats upto the dose of 2000 mg/kg for duration of 24 hours. Hence, as per OECD guidelines 1/10th and 1/5th of 2000mg/kg i.e. 200mg/kg and 400mg/kg were selected as doses for the study. Also a median dose 300mg/kg was considered.

Induction of atherosclerosis:

In rats hypercholesterolemia was induced by daily administration by gavage a cocktail containing 20% Cholesterol, 10% Lard, 1% Protein mixture, 3.5% Mineral mixture, 0.2% Choline bitartarate, 0.3% l-methionine, 15% Corn starch per 50gm diet weight over a period of 28 days. The test compound was administered simultaneously for 28days. Before 24 hrs of sacrificing, the animals were kept for fasting and had free access to water. Blood was collected by retro orbital route for biochemical estimation. Then rats were sacrificed and aorta was collected and was kept in 15% V/V formalin solution for histopathological examination. All the drugs were given orally.

Experimental design

Rats were randomly divided into six groups with five animals per group.

Group 1: Control group treated with saline solution

Group 2: Disease Control (DC)

Group 3: DC + Atorvastatin at (10 mg/kg)

Group 4: DC+ PHF (400 mg/kg bw)

Group 5: DC+ PHF (300 mg/kg bw)

Group 6: DC+ PHF (200 mg/kg bw)

PHF: Polyherbal formulation

Phytochemical screening of the extract:

The extract prepared from the five plants taken were subjected to qualitative analysis for the various phytoconstituents like alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids and flavonoids[5].

Measurement of various Parameters:

Physical Parameters**:** The body weight was recorded on the first day and then last day of the study period in each group.

Biochemical Estimations:

Lipid parameters were determined in blood serum. At the end of 28 days, animals were fasted overnight and blood was collected from retro orbital plexus under light ether anesthesia, centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept at 4^{o}C until used.

After the completion of duration of study (28 days), the animals were fasted overnight and sacrificed by cervical decapitation. Their livers were dissected out on a petri dish kept in an ice-bath to keep the sample fresh. Sufficient care was taken to complete the entire procedure within 5-7 min to prevent loss of the enzyme activity in liver samples. The aorta was excised immediately, washed with cold saline. The aorta was fixed by 10% formalin for histopathological studies[4]. The quantitative estimation of lipid profile was carried out using Cholesterol kit, triglyceride kit. Estimation of VLDL-C and LDL-C was done by using the Friedward's formula[4].

VLDL-C = Triglycerides/5

LDL-C = Total cholesterol – (HDL-C + VLDL-C)

Measurement of Coronary Disease Risk Factor

Atherogenic Index (AI), which is a measure of the atherogenic potential of an agent, was calculated using the following formula and the results were tabulated[6].

Atherogenic Index = log [TG/HDL]

Assay for Thiobarbituric acid reactive substance (Maleic dialdehyde)

Lipid peroxidation was estimated colorimetrically in the liver by quantifying TBARS according to the method of Ohkawa and Ohishi. For the estimation, 0.5 ml of supernatant was treated with 0.2 ml of of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid adjusted to pH 3.5 with NaOH and 1.5 ml of 0.8% solution of thiobarbituric acid. The volume of the mixture was adjusted to 4.0 ml with distilled water and heated in a water bath at 85˚C for 60 min. Light pink color was developed. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of mixture of n-butenol and pyridine (15:1, V/V) was added. The mixture was shaken vigorously and then centrifuged at 4000 rpm for 5 min. After centrifugation, the organic layer was separated and its absorbance was read at 532 nm using a UV-visible spectrophotometer (UV-1700, Schimadzu, Japan) against a reagent blank. The amount of TBARS was calculated by using 1.56×105 $M^{-1}cm^{-1}$ as molar extinction co-efficient and the level of lipid peroxidation was expressed as n mole of maleic dialdehyde/mg of protein (MDA)[4].

Determination of Aspartate aminotransferase (AST or SGOT), Alanine aminotransferase (ALT or SGPT) levels

The blood sample was centrifuged at 5000 rpm for 10 min and the plasma was collected in micro-centrifuge tubes. Plasma samples were stored at −20°C for the determination of ALT and AST levels. These marker enzymes were measured using commercially available kits (ALT kit, AST kit, Merck, Mumbai, India) according to manufacturer's instructions and the absorbances were recorded at 340 nm. All histopathological evaluations were done by a pathologist blinded to the experimental design.

Statistical analysis:

The values were expressed as mean ± SEM (n=5). The statistical significance was assessed using student t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$, $P<0.01$, $p<0.0001$ were considered to be statistically significant. Data were processed with graph pad prism version 6 software.

RESULTS AND DISCUSSION

The preliminary phytochemical investigations of the hydroalcoholic extract of polyherbal formulation showed the presence of phytoconstituents like glycosides, alkaloids, carbohydrates, phenolic compounds and tannins. The acute toxicity studies of the PHF showed no symptoms of toxicity or behavioral changes at the maximum dose (2000 mg/kg). Effect of HCD, PHF and atorvastatin on animal body weight were analyzed on 28th day of the experiment. The HCD fed animals showed significant increases in body weight compared to normal diet fed animals). The

results obtained where shown as in Table 1. There was increase in body weight of animals from the beginning to the end of the experiment in all six groups, but at the end, increase in body weight was low in PHF treated groups as compared to high cholesterol diet treated group.

Table 1: Effect of PHF on body weight (gm)

Groups	Before treatment	After treatment
Control	184.6±1.71	192.2±1.15
CD	185±1.57	235±2.23
CD + ATR (10mg/kg)	186.4±2.72	196±1.86
CD + PHF (400mg/kg)	184±1.86	212±5.14
CD + PHF (300mg/kg)	182±2.54	221±5.99
CD + PHF (200mg/kg)	183±2.54	235±2.23

The effect of PHF on lipid profile (total cholesterol, triglyceride, HDL, VLDL and LDL levels, and AI) over a period of 28 days treatment, in the treatment and control groups were analysed on 28th day of treatment and the values were depicted in the Figure 1-6. In HCD fed rats showed significant increase in lipid profile level.

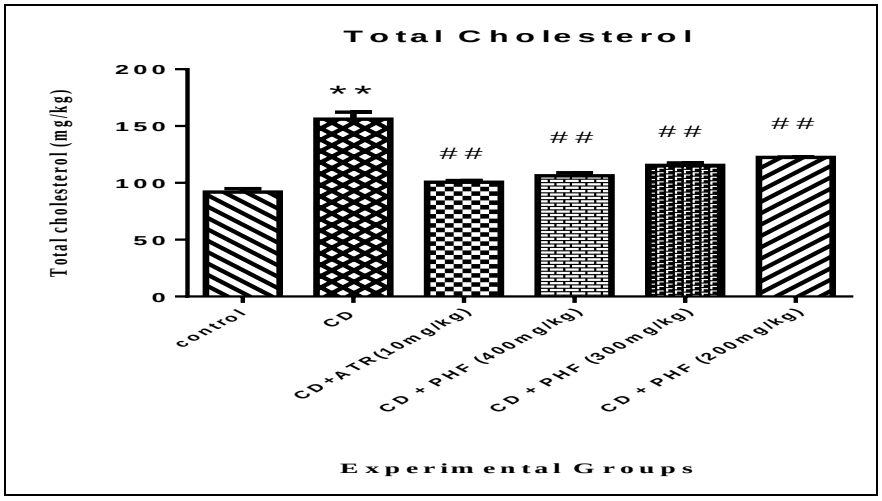

Figure 1: Effect of PHF on serum cholesterol levels

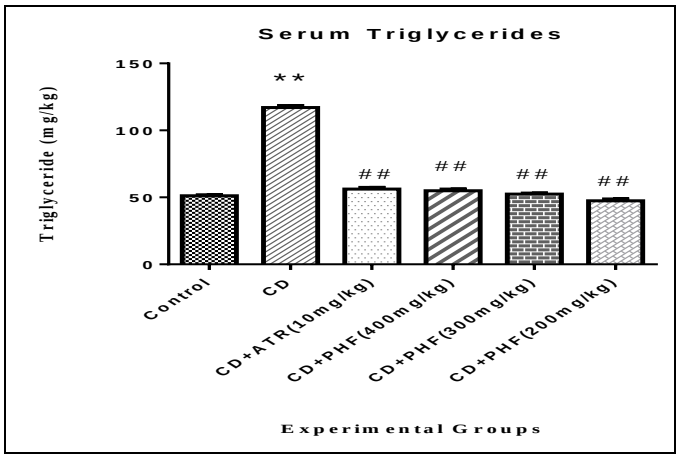

Figure 2: Effect of PHF on Triglycerides

Data was analysed using one way ANOVA and Tukey's t test. **p<0.0001 represent significant difference when compared with control group; ## p<0.0001, represent significant difference when compared with disease control (High cholesterol diet).

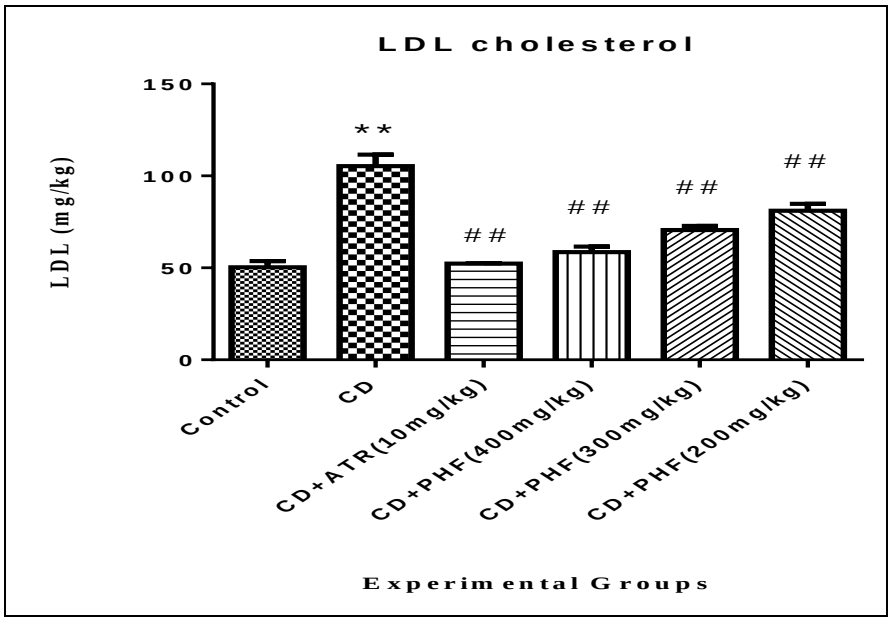

Figure 4: Effect of PHF on LDL-cholesterol

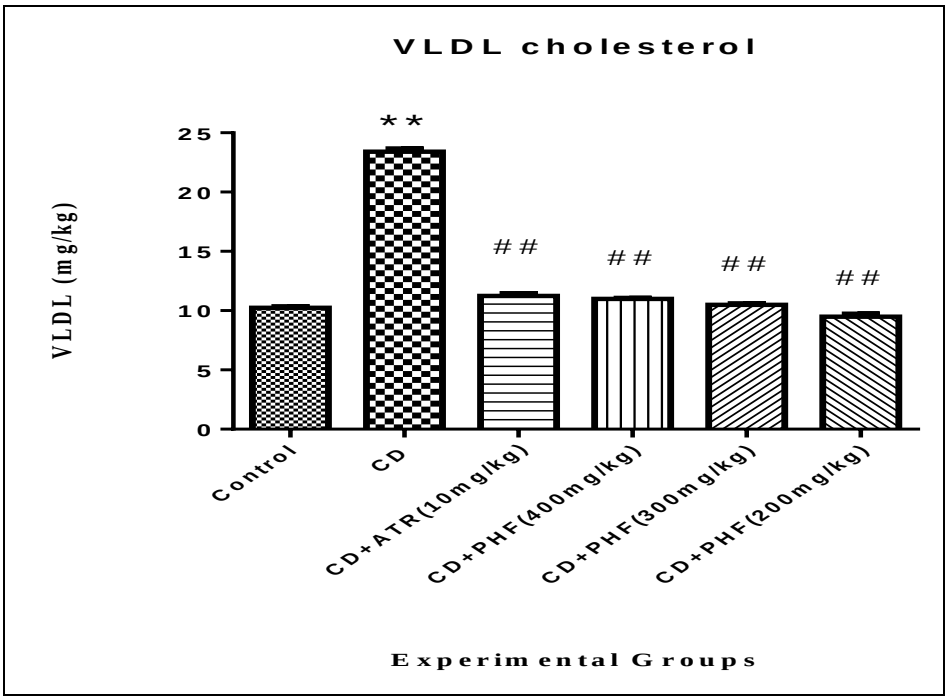

Figure 5: Effect of PHF on VLDL-cholesterol

Data was analyzed using one way ANOVA and Tukey's t test. **p<0.0001 represent significant difference when compared with control group; ## p<0.0001, represent significant difference when compared with disease control (High cholesterol diet).

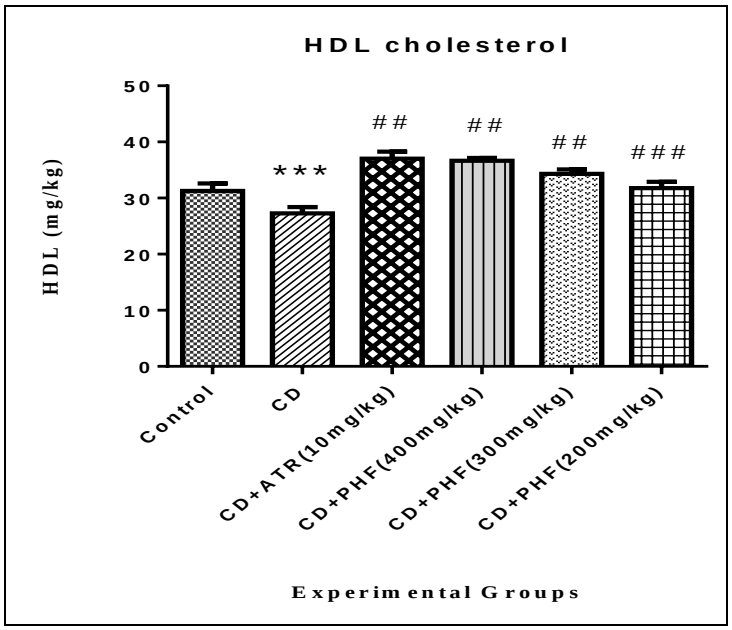

Figure 3: Effect of PHF on HDL-cholesterol

Data was analysed using one way ANOVA and Tukey's t test. ***p=0.001 represent significant difference when compared with control group; ##p<0.0001, represent significant difference when compared with disease control (High cholesterol diet).

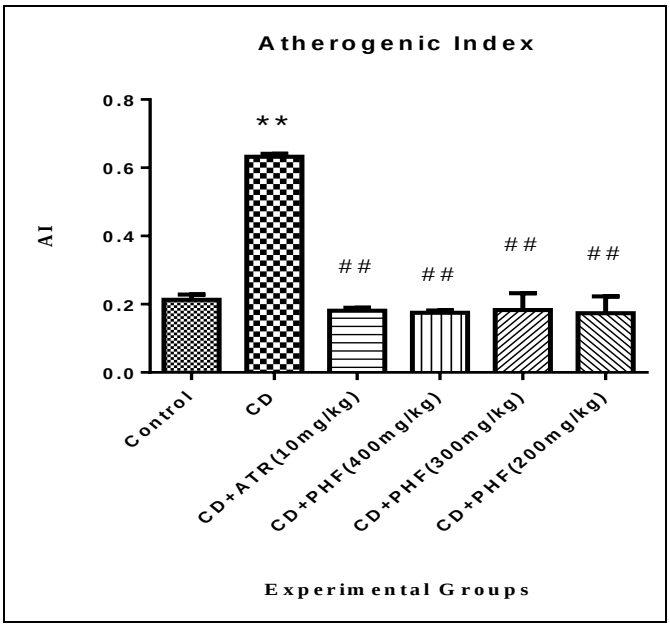

Figure 6: Effect of PHF on Atherogenic Index

Effect of PHF and atorvastatin on lever function (ALT, AST) was assessed using serum sample and depicted in table 2.

In this study, plasma MDA (as depicted in table 3) decreased in the group which received high cholesterol diet plus polyherbal formulation compared to the group which received high cholesterol diet.

Table 2: Effect of PHF on AST (Aspartate transaminase) and ALT (Alanine transaminase) (IU/L):

Groups	Mean±SEM AST	Mean±SEM ALT
Control	55.64±0.35	29.71±0.6
CD	162.45±1.69**	76.4 ±0.5**
CD + ATR (10mg/kg)	107.75±0.61##	48.62 ±0.44##
CD + PHF (400mg/kg)	125.93±1.94##	52.38±0.55##
CD + PHF (300mg/kg)	134.75±2.71##	67.66±0.9##
CD + PHF (200mg/kg)	144.34±1.64##	74.66±1.08

Table 3: Effect of PHF on Malondialdehyde and Glutathione:

Groups	MDA(nmol/ mg of protein)	Glutathione (permoles/mg protein)	Mean±SEM serum creatinine
Control	0.154±0.005	296.7 ± 2.7	29.71±0.6
CD	0.55±0.039**	187.4 ± 1.8**	76.4 ±0.5**
CD + ATR (10mg/kg)	0.172± 0.008##	268.7 ±0.9##	48.62 ±0.44##
CD + PHF (400mg/kg)	0.196±0.005##	281.7 ± 1.4##	52.38±0.55##
CD + PHF (300mg/kg)	0.23±0.007##	271.7±0.3##	67.66±0.9##
CD + PHF (200mg/kg)	0.27 ± 0.14	264.8 ± 1.8##	74.66±1.08

Data (Mean±SEM**)** was analysed using one way ANOVA and Tukey's t test. **p<0.0001 represent significant difference when compared with control group; ##p<0.0001, represent significant difference when compared with disease control (High cholesterol diet).

A significant decrease (p<0.0001, p<0.05) in the TC, TG and LDL-C levels were observed in the treatment groups with an increase in HDL-C. The serum AST, ALT and ALP levels were also decreased in PHF treated groups.

Histopathology of Aorta:

In histopathology study, normal group showed no pathological changes in endothelial lining. Control group (High cholesterol diet) showed severe damage to endothelial lining. Hydroalcoholic extract of polyherbal preparation treated groups (200 mg/kg, 300mg/kg, and 400 mg/kg) showed less damage to the endothelial lining as compared to control group as shown in Figures 7-11. Atorvastatin treated group showed mild damage to the endothelial lining.

Data was analyzed using one way ANOVA and Tukey's t test. **p<0.0001 represent significant difference when compared with control group; ##p<0.0001, represent significant difference when compared with disease control (High cholesterol diet).

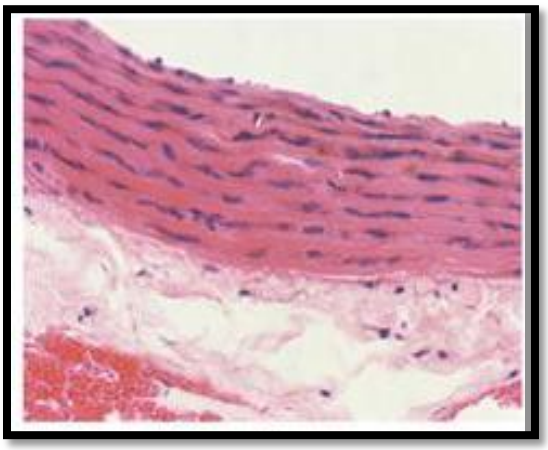

Figure 7. Arterial section in control group

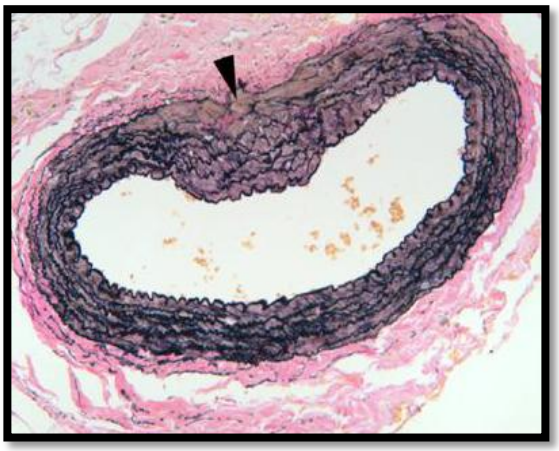

Figure 8. Arterial section in disease control

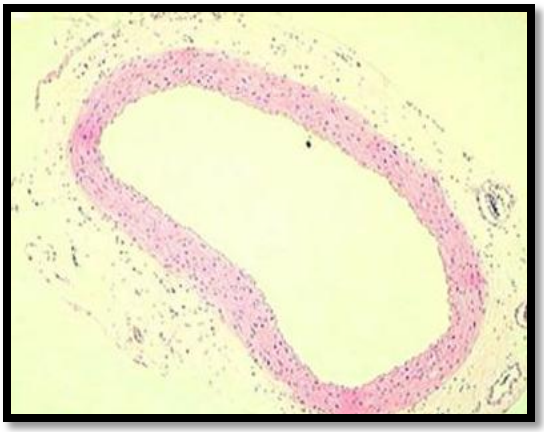

Figure 9. Arterial section in CD+Atorvastatin(10mg/kg)

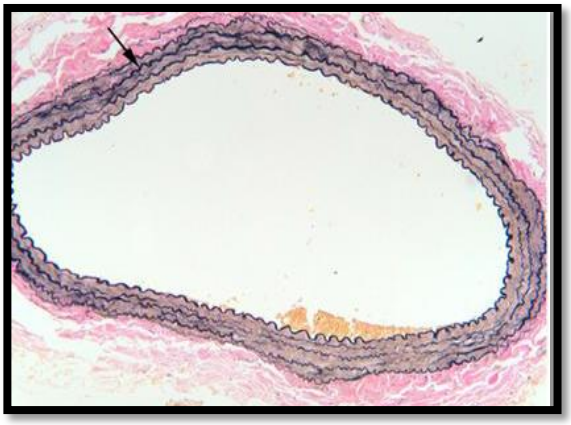

Figure 10. Arterial section in CD+PHF (400mg/kg)

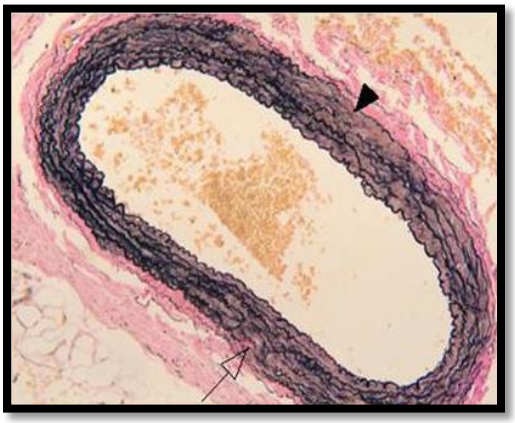

Figure 11. Arterial section in CD+PHF (200mg/kg)

Plant derived drugs offer many drug molecules for the treatment of serious diseases like cardiac disorders. They show fewer side effects when compared to the allopathic medicines. In present study, the ant atherosclerotic activity of hydroalcoholic extract of polyherbal preparation was evaluated in rats receiving high cholesterol diet (HCD). Determination of body weight in experimentally induced atherosclerosis is considered to be a positive factor to find out the

prognosis of disease. Results indicated increase in body weight of animals from the beginning to the end of the experiment in all six groups, but at the end, increase in body weight is low in hydroalcoholic extract of polyherbal preparation treated groups as compared to high cholesterol diet treated group.

Cholesterol is an essential structural element of the biological membranes. In addition, it is the precursor of many compounds such as synthesis of bile acids, steroid hormones and vitamin D. Despite this, high concentration of serum cholesterol increases the risk of coronary heart dieses (CHD) [4].

Our study demonstrated that rats fed with a high cholesterol diet (disease control group) exhibited a higher level of total cholesterol in serum as compared to rats fed with a standard laboratory diet (Normal control group); while oral administration of hydroalcoholic extract of polyherbal preparation dose (200 mg/kg, 300 mg/kg, 400mg/kg p.o) reduced the raised level of total cholesterol in serum. In our study, the administration of hydroalcoholic extract of polyherbal preparation dose (200 mg/kg, 300 mg/kg, and 400mg/kg p.o) significantly lowered triglyceride and VLDL level in serum. It is widely accepted that the elevation of plasma LDL-C level is major risk factor for CHD.

Direct correlation between LDL-C level and atherosclerosis as well as the reversibility of the related pathological events by lowering the serum level of LDL-C has already been reported.[7]

Beyond the role of LDL-C in the development of atherosclerosis, growing evidence suggests that high density lipoprotein cholesterol (HDL-C) is a powerful predictor of cardiovascular disease (CVD). Indeed, epidemiological, mechanistic and intervention studies suggest that low HDL-C is a major CVD risk factor and that increasing HDL-C plasma levels may be beneficial, particularly in patients with low HDL-C levels. The results clearly indicated that hydroalcoholic extract of polyherbal preparation was capable of increasing level of HDL-C in serum[8].

Further, it was observed that the PHF decreased triglyceride levels.

Atherogenic index (AI) is used as a marker to assess the susceptibility of atherogenesis. Significant reduction of AI was found in PHF treated group and the study results indicating protective role of PHF against aterogenesis[9].

Oxidative stress caused by atherogenic diet is associated with peroxidation of cellular lipids, which is determined by measurement of thiobarbituric acid reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results in increase of oxygen free radicals, which attacks the polyunsaturated fatty acids in cell membranes and cause LPO. The malondialdehyde (MDA) content, a measure of LPO was

assayed in the form of TBARS. Treatment with polyherbal extract and Atorvastatin significantly ($p<0.0001$) prevented the peroxidation by reducing the concentration of TBARS.

Elevated serum creatinine levels could be associated with atherosclerotic renovascular disease which corerelates with early development of atherosclerosis. With elevated serum creatinine levels, urinary excretion of creatinine is less in atherosclerotic condition. Hence, in the present study, serum creatinine level was estimated. A significant increase was observed in the levels of serum creatinine in disease control group (CD). There was decrease in the serum creatinine levels in atorvastatin treated group (CD+ATR) and in the group treated with polyherbal formulation (CD+PHF) [10].

Hyperlipidemia induces liver damage and changes in serum AST, ALT levels are markers of hepatotoxicity. A significant increase of serum AST, ALT levels was detected in CD group compared to normal control group. However, significant decrease in the levels of hepatic enzymes was observed in treated groups which indicate the hepatoprotective potential of the polyherbal extract[11].

CONCLUSION

In conclusion, our results have shown that treatment with polyherbal formulation in rats have anti atherosclerotic activity due to a number of factors like preventing the initiation of early endothelial dysfunction by its antioxidant action, decreasing LDL cholesterol. Increase in HDL enhances the reverse transport of circulating cholesterol. By a combination of these effects, the extract can effectively prevent atheromatous plaque. The antiatherosclerotic activity of the polyherbal formulation may be due to its phytochemical constituents like glycosides, alkaloids, terpenoids. Hence further studies on isolated chemical constituents are needed to find out potential antiatherosclerotic agent.

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