Original Research Article

Anti-atherosclerotic activity of root bark of *Premna integrifolia* Linn. in high fat diet induced atherosclerosis model rats

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**ABSTRACT**

*Premna integrifolia* Linn. is a medicinal plant used in “Dhasamula” drug preparation of Ayurvedic systems of medicine in the treatment of various ailments like bronchitis, dyspepsia, liver disorders, piles, constipation, hyperlipidemia and fever. The anti-atherosclerotic activity of hydroalcoholic extract (HAE) of root bark of *P. integrifolia* was evaluated in high fat diet induced atherosclerosis rats. Sixty Wistar rats were divided into six groups: the first group served as control, the second group was fed with high fat diet and the other three groups were fed with high fat diet along with various concentrations of HAE and the last group was treated with atorvastatin for 30 days. Lipid and lipoprotein profile, atherogenic index, and cardiac markers and histopathological evaluation of aorta were determined in high fat diet induced atherosclerosis rats. HAE of *P. integrifolia* produced a significant and dose-dependent anti-atherosclerotic activity in terms of reduction in lipids and lipoprotein profile, atherogenic index, HMG-CoA reductase activity, marker enzymes such as lactate dehydrogenase (LDH), creatine phosphokinase (CPK), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), alteration in collagen and calcium contents, mild mineralization and focal rupture of intima and media of aorta was noticed in treated groups as compared to the control. The results suggested that anti-atherosclerotic activity of HAE of *P. integrifolia* Linn. was due to its modulatory activity on metabolic pathway of lipid. The results contribute to the validation of the traditional use of Agnimantha in high fat diet induced atherosclerosis rats.

1. Introduction

Atherosclerosis is one of the major risk factors for coronary artery disease. It is a complex, multifactorial inflammatory disease, characterized by the presence of lesions due to the accumulation of lipids in the walls of arteries. There are a number of genetic, metabolic, and environmental factors involved in the initiation and evolution of the atherosclerotic plaque. A well-known risk factor in humans is hypercholesterolemia, i.e., elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDLc) [1], and other important contributors to this disease include inflammation, oxidative stress and insulin resistance [2,3]. Foods rich in saturated fat and cholesterol have been linked to elevations in circulating cholesterol levels [4]. Lipid-enriched diets are often used to induce or accelerate the rate of atherosclerotic lesion in murine models of atherosclerosis [5]. Therefore, using high fat diet for promoting atherosclerosis is a valuable tool for understanding the disease and treatment effect. Lipoprotein oxidation and oxidative processes in general play an important role in the pathogenesis of atherosclerosis. Disorders of lipid metabolism are manifested by elevation of plasma lipids and lipoprotein fractions, which in turn results in cardiovascular diseases.

The alternative systems of medicines like Ayurveda, Siddha, Unani and other tribal folklore medicines which are herbal based have significantly contributed to the health care of the population in India. Today, these systems are not only complementary but also competitive in the treatment of various diseases. Herbs and herb-based compounds...
have always been an important source of medicines for various diseases and have been paid considerable attention to in recent years due to their diverse pharmacological actions. All over the world, the genus Premna contains 200 species under the family Verbenaceae, out of which approximately 30 species are present in India. The genus Premna can be used traditionally in treating various ailments like rheumatism, asthma, dropsy, cough, fever, and boils. The different parts of this plant have been used for the treatment of various diseases. Hypolipidemic [6], anti-diabetic [7,8], anti-inflammatory [9,10], immuno-modulatory [11], anti-obesity [12], anti-bacterial and analgesic [13], anti-arthritic [10], antioxidant [14,15], antiparasitic [16], hepatoprotective and in vitro cytotoxic [17] activities and beneficial effect of *P. integrifolia* root extract on human leucocytes and erythrocytes against H₂O₂ induced oxidative damage [18] were reported. In Ayurveda, the roots are used in the treatment of chyluria, bronchitis, dyspepsia, liver disorders, piles, constipation and fever [19]. *P. integrifolia* is a medicinal herb and is popularly known as “Agamitha” in Ayurvedic system of medicine. Their roots are important ingredient of well-known Ayurvedic preparations like *Dasamula kwatha*, *Chyanprashavaleh*, *Haritakiavaleh*, *Ayushavardha tel*, and *Narayan tel*, valued for the treatment of variety of ailments [20]. Hence the present study was aimed to demonstrate the anti-atherosclerotic activity of hydroalcoholic extract (HAE) of *P. integrifolia* in high fat diet induced atherosclerosis rats.  

2. Materials and methods

2.1. Preparation of plant extract from root bark

The root bark of *P. integrifolia* was procured from Indian Medical Practitioners Co-operative Pharmacy and Stores (IMPCOPS), Chennai, Tamil Nadu, India, and authenticated by Shri. C. Arunkachalam, Research Officer (Botany), Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute, Chennai and the voucher specimen (00641/2014) was deposited in the Botany Department. The coarse powder of root bark of 500 g was soaked in 5 L of hydro alcohol (60:40, v/v) for 72 h with intermittent shaking at room temperature. The extract was filtered through Whatmann No. 1 filter paper; the filtrate was evaporated to dryness and stored in an air-tight container. The percentage yield was higher (7.6%) at 60:40 (v/v) than that at 50:50 (v/v) and 70:30 (v/v). Therefore, the ratio of 60:40 was chose for the following study.

2.2. Animals

Wistar rats of 6–8 weeks of age were purchased from King’s Institute of Preventive Medicine, Guindy, Chennai-600032, India. Experiment was conducted in 60 (30 males+30 females) rats. The rats were maintained in an institutional animal house facility with 12 h light and dark cycles. Temperature was maintained at 25±3 °C and feeding schedule consisted of rat pellet diet and water ad libitum. Daily intake of food was quantitated precisely. The proposal was duly approved by Institutional Animal Ethical Committee as per CPCSEA guidelines (Registration No. IAEC/CSMDRIAS/10/2014) prior to initiation of experiments.

2.3. Establishment of atherosclerosis model

Atherosclerosis model was established by feeding atherogenic diet consisting of 2.0 g of cholesterol, 8.0 g of saturated fat (groundnut oil) and 0.1 g of calcium (BYCALVIT-500; Biochem Pharmaceutical Industries Ltd., Mumbai, India) and mixing thoroughly with 90 g of powdered standard commercial pellet diet. The rats were fed with high fat diet along with weekly challenge of oral vitamin D₃ (3,00,000 IU) through per oral route [21]. Feed was prepared daily and intake was recorded.

2.4. Grouping and dosing methods

Sixty rats were randomly divided into 6 groups with 10 animals (5M+5F) in each group. Group I served as control and was fed with standard pellet diet; Group II was fed with high fat diet; Group III, IV and V were fed with high fat diet and administered 100, 200, 500 mg/kg HAE orally, respectively; Group VI was fed with high fat diet and atorvastatin for 30 days. HAE and vehicle (0.5% SCMC) were administered for 30 days by intragastric route using appropriate graduated syringe.

2.5. Biochemical analysis

At the end of experimental period (i.e. 31st day) 4 mL of blood sample was collected from retro-orbital plexus under mild ether anesthesia. Serum was separated using centrifuge (Remi C-24) at 3000 rpm for 10 min at 4 °C. Cross sections of 5 μm thickness of paraffin embedded aorta were stained with hematoxylin and eosin. TC, triglycerides (TG), high density lipoprotein cholesterol (HDLc), apoprotein B (ApoB), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and calcium were estimated by clinical chemistry fully auto-analyser (XL-640; Transasia). Instrument was calibrated with XL-Multical (Erba XL SysPack-Level 1-14441) using kits from ERBA. Quality control (Biorad) was run before analysis.

HMG-CoA/Mevalonate ratio [22], collagen [23] and total protein [24] were estimated in the liver by standard procedures. Free fatty acids (FFA) were measured in serum [25] with the color reagent of Itaya [26] using UV–Visible Spectrophotometer (Perkin-Elmer EZ-201). LDLc and very low density lipoprotein cholesterol (VLDLc) were calculated by standard method and atherogenic index was calculated using the following equation [27]:

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\text{Atherogenic Index} = \left[ \log_{10}(\text{TG/HDLC}) \right]
\]

2.6. Statistical analysis

Statistical analysis was carried out using graph pad prism software, version 5. All the values were expressed as mean ± SD (n=10). Analysis of variance (ANOVA) was used for multiple comparison of treatment groups with the vehicle control and disease control followed by Dunnett’s test. *p < 0.05* was considered to be statistically significant.

3. Results and discussion

Hypercholesterolemia and atherogenic dyslipidemia have been closely implicated in the pathogenesis of coronary artery disease. Changes in body weight are used to access the course of disease and response to therapy, and also indicate the adverse effects of drugs. Changes in body weight during the experiment are shown in Table 1. Results indicated that body weight of rats was increased from the beginning to the end of experiment in all six groups. On day 30, Group II showed an increase of 44.76% in body weight when compared to day 1 of the same group and an increase of 1.21 fold when compared to Group I. On treatment with HAE the body weight was maintained to near normal values and there was a decrease in body weight of 1.51 fold in Group III, 1.55 fold in Group IV, 1.62 fold in Group V and 1.32 fold in Group VI when compared to Group II (Table 1). There were no significant changes in the feed consumption and organ weight such as liver, heart and kidney between the control and experimental groups (Fig. 1 and Table 2). Reduction in body weights of experimental rats treated with HAE might be due to the anti-obesity potential of HAE in a dose-dependent manner. The result of the present study was in agreement with that of aqueous extract of root of *P. integrifolia* reported [12,28].
3.1 Lipid profile

The levels of TC, TG and FFA in serum of control and experimental groups are shown in Table 3. The levels of TC and TG, FFA were significantly increased in group II when compared to group I. The levels of TC, TG and FFA were significantly decreased in groups III, IV, V and VI, respectively, when compared to group II. No significant changes in lipid profile were noticed in groups III, IV, V and VI when compared to group I. Lipid metabolism normally maintains the synthesis and degradation of fat in biological tissues. Cholesterol is an essential structural element of the biological membranes and the precursor for synthesis of many compounds such as bile acids, steroidal hormones and vitamin D. High concentration of serum cholesterol increases the risk of coronary heart diseases [29]. There was a significant increase in the levels of plasma TC, TG and FFA in high fat diet fed rats. Cholesterol-enriched diet resulted in significant increase of TC and TG levels accompanied by increased serum LDLc and decreased circulating HDLc levels [29].

3.2 Lipoprotein status

The levels of HDLc, LDLc and VLDLc in serum of control and experimental groups are shown in Table 4. The level of HDLc was significantly decreased in group II when compared to group I and significantly increased in groups IV and V when compared to group II. The level of LDLc was significantly increased in group II when compared to group I and significantly decreased in groups IV, V and VI when compared to group II. No significant change in HDLc was noticed in group VI treated with

| Table 1 | Body weight (g) changes in experimental rats. |
|---------|---------------------------------------------|
| Group I | Day 1  | Day 7  | Day 14 | Day 21 | Day 30 |
| Group II| 107.20±8.31 | 112.90±9.05 | 127.40±5.46 | 132.90±5.76 | 136.80±8.27 |
| Group III| 105.00±9.23 | 116.10±8.91 | 129.70±7.59 | 140.00±6.73 | 152.00±6.69 |
| Group IV| 104.70±8.89 | 113.30±9.97 | 121.90±13.55 | 129.40±17.08 | 138.70±21.57 |
| Group V | 106.00±8.31 | 115.70±9.89 | 123.90±19.57 | 140.00±6.73 | 152.00±6.69 |
| Group VI| 103.90±8.89 | 113.30±9.97 | 121.90±13.55 | 129.40±17.08 | 138.70±21.57 |

Values are expressed as mean ± SD of respective group of rats. (n=10).

| Table 2 | Organ weights (g) of experimental rats. |
|---------|----------------------------------------|
| Group     | Liver       | Heart      | Kidney     |
| Group I   | 7.31±0.744 | 0.79±0.019 | 2.13±0.118 |
| Group II  | 8.09±0.336 | 0.76±0.030 | 2.12±0.089 |
| Group III | 7.71±0.289 | 0.75±0.024 | 2.09±0.056 |
| Group IV  | 7.41±0.453 | 0.77±0.042 | 2.13±0.117 |
| Group V   | 7.17±0.499 | 0.79±0.077 | 2.24±0.145 |
| Group VI  | 7.30±0.567 | 0.77±0.044 | 2.14±0.088 |

Values are expressed as mean ± SD of 10 rats; ANOVA was used for multiple comparisons.

| Table 3 | Estimation of TC, TG and FFA in serum. |
|---------|----------------------------------------|
| Group     | TC (mg/L)   | TG (mg/L)  | FFA (mg/L)  |
| Group I   | 8.23±1.52  | 6.48±1.64  | 6.48±1.64  |
| Group II  | 12.69±1.16 | 11.41±1.90 | 11.41±1.90 |
| Group III | 8.87±1.41  | 7.99±1.60  | 7.99±1.60  |
| Group IV  | 8.19±2.18  | 7.14±1.29  | 7.14±1.29  |
| Group VI  | 9.68±2.71  | 7.16±1.79  | 7.16±1.79  |

Values are expressed as mean ± SD of 10 rats; ANOVA was used for multiple comparison; compared with group I, **p < 0.01; compared with group II, *p < 0.05, **p < 0.01.

| Table 4 | Determination of LDLc, VLDLc and HDLc in serum. |
|---------|----------------------------------------|
| Group     | LDLc (mg/L) | VLDLc (mg/L) | HDLc (mg/L) |
| Group I   | 3.22±0.89  | 1.29±0.33  | 5.55±1.12  |
| Group II  | 4.82±0.51  | 2.28±0.38  | 3.75±0.55  |
| Group III | 3.91±0.75  | 1.64±0.36  | 4.69±0.99  |
| Group IV  | 3.65±0.88  | 1.59±0.32  | 5.33±1.22  |
| Group V   | 3.49±0.56  | 1.43±0.26  | 5.46±0.75  |
| Group VI  | 3.63±0.54  | 1.43±0.36  | 5.04±1.28  |

Values are expressed as mean ± SD of 10 rats; ANOVA was used for multiple comparison; compared with group I, *p < 0.01; compared with group II, *p < 0.05, **p < 0.01.
Fat diet-induced atherogenesis has a relation with vascular damage and oxidative stress and in turn leads to pathogenesis of various diseases [41]. TC/HDLc and LDLc/HDLc ratios were significantly high in high fat diet fed rats when compared to the control. These atherogenic factors are the better indexes reflecting the abnormality of lipid metabolism [42]. Increase in antioxidants and decrease in pro-oxidants upon HAE treatment were observed in the present study.

### 3.3. Status of atherosclerotic markers

The activities of serum ALT, AST and ALP in control and experimental groups are shown in Table 6. The activity of ALT was significantly increased in group II compared with group I and significantly decreased in groups IV, V and VI and were not changed in group III compared with group II. The activities of AST and ALP were significantly increased in group II compared with group I and significantly decreased in groups V and IV and no significant changes were noticed in other groups when compared to group II. No significant changes in ALT, AST and ALP were noticed in groups III, IV, V and VI compared with group I. The activities of LDH and CPK in serum of control and experimental groups are depicted in Table 7. The activity of LDH was significantly increased in groups II, III and IV compared with group I and significantly decreased in groups III, IV, V and VI compared with group I. Treatment with 200 and 500 mg/kg HAE showed a drastic reduction in LDH activity when compared to group III (100 mg/kg). The results showed that the activity was inversely proportional to the concentration of HAE on treatment. Also atorvastatin treated group showed a major decline in LDH activity. The activity of CPK was significantly increased in group II compared with group I and significantly decreased in groups IV, V and VI compared with group II. No significant increase in the activities of LDH and CPK was noticed in groups IV, V and VI compared with group I. Measurement of LDH activity in blood is considered to be a diagnostic marker for certain cardiovascular diseases and it is a sensitive marker of myocardial infarction [43]. AST level is used as a diagnostic marker for myocardial infarction. In pathological conditions, the enzymes such as CPK, LDH, AST and ALP leak from the necrotic heart cells to blood, which are important measuring markers of cardiac injury. These enzymes are not specific for myocardial injury individually; however, evaluation of these enzymes together may be an indicator of myocardial

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### Table 6

**Activities of ALT, AST and ALP in serum.**

| Group | ALT (U/L) | AST (U/L) | ALP (U/L) |
|-------|-----------|-----------|-----------|
| Group I | 101.07±23.00 | 118.50±31.15 | 470.10±73.80 |
| Group II | 143.34±10.60** | 162.44±9.14** | 619.11±55.70** |
| Group III | 120.11±25.90 | 132.28±21.80 | 514.60±62.60 |
| Group IV | 109.18±20.40* | 130.89±23.05 | 504.22±90.40* |
| Group V | 107.18±16.80** | 127.32±22.71** | 495.22±64.00** |
| Group VI | 110.74±19.70* | 134.37±27.09 | 531.33±120.20 |

Values are expresses as mean ± SD of 10 rats; ANOVA was used for multiple comparison; compared with group I, *p < 0.01; compared with group II, **p < 0.05, ***p < 0.01.
damage [44,45]. CPK, another important enzyme in energy metabolism in the body, maintains the high concentration of intracellular ATP through phosphorylation and is considered as another important index for atherosclerosis [46]. The results of the present study showed that serum AST, ALT, LDH and CPK levels were significantly increased in high fat diet fed rats when compared to the control. Higher activities of these enzymes in serum have been found in response to oxidative stress induced by high fat diet [30]. There was a significant restoration of these enzymes on administration with different doses of HAE. Markable decrease in AST, ALT, CKP and LDH enzyme activities demonstrated the cardio-protective effect of the drug. Also atorvastatin treated rats showed its protective potential on atherosclerosis.

Levels of HMG CoA/mevalonate ratio, collagen, calcium and total protein in liver of control and experimental groups are depicted in Table 8. HMG CoA/mevalonate ratio was increased in group II compared with group I. A significant improvement was observed in groups IV, V and VI compared with group II. The collagen content was significantly increased in groups II and III compared with group I, and significantly decreased in groups IV, V, and VI compared with group II. The calcium level in the liver was significantly increased in group II compared with group I, and significantly decreased in groups III, IV and V compared with group II. The level of total protein was significantly increased in group II when compared to group I and significantly decreased in groups IV, V, and VI compared with group II. No significant change in the level of total protein was observed in groups III, IV, V and VI compared with group I. HMG-CoA reductase activity was indirectly measured in terms of HMG CoA/mevalonate ratio in the liver tissue. Inhibitors of HMG-CoA reductase are used to lower the serum cholesterol levels and improve the survival of individuals at the risk of atherosclerotic vascular disease [47]. Their primary site of action is in the liver, where they inhibit HMG-CoA reductase, the metabolic pathway that produces cholesterol and isoprenoids [48]. Group V rats showed a significant reduction in the ratio of HMG-CoA/mevalonate, which might be due to the inhibition of HMG-CoA reductase activity by HAE; hence it is evident that the drug possesses the hypolipidimic activity. The liver is an important organ in which all metabolic reactions take place. One of the most effective and widely used drugs for the treatment of hyperlipidemia is the atorvastatin which competitively inhibits HMG-CoA reductase enzyme in the liver that converts HMG-CoA to mevalonate, an early precursor for cholesterol biosynthesis. Hence, this enzyme might be competitively inhibited by HAE, which was evident from the lipid and lipoprotein status of the present study. The unique distribution of hydroxy proline within the collagen can be easily studied [49]. It is a family of proteins which consists of several genetically distinct molecular species and is closely involved in tissue organization, function, differentiation and development. HAE treated group showed the reversibility of the changes when compared to high fat diet fed rats. Histopathology study revealed that alteration in collagen and calcium content, mild mineralization and focal rupture of intima and media of aorta were noticed in treated groups as compared to the control (Fig. 2). These parameters were significantly reverted to near normalcy on treatment with HAE of *P. integrifolia* in a dose-dependent manner.

### Table 8

| Group | HMG CoA/ Mevalonate ratio (µg/mg protein) | Collagen (mg/g tissue) | Calcium (µg/g tissue) | Total protein (g/mg tissue) |
|-------|------------------------------------------|------------------------|-----------------------|----------------------------|
| Group I | 1.16±0.110** | 38.83±16.25 | 12.02±2.28 | 198.6±32.2 |
| Group II | 1.40±0.144** | 72.52±24.67** | 14.6±1.64 | 254.7±39.9 |
| Group III | 1.20±0.105 | 82.07±26.17 | 12.34±1.34 | 208.3±35.3 |
| Group IV | 1.158±0.150** | 76.52±41.40 | 11.87±1.64 | 200.2±51.1 |
| Group V | 1.05±0.139** | 38.95±22.94 | 11.02±1.83 | 197.3±17.6 |
| Group VI | 1.10±0.130** | 40.07±29.66 | 13.30±1.94 | 203.5±23.6 |

Values are expresses as mean ± SD of 10 rats; ANOVA was used for multiple comparison; compared with group I, *p < 0.05, **p < 0.01; compared with group II, *p < 0.05, **p < 0.01.

### Table 7

| Group | LDH (IU/L) | CPK (IU/L) |
|-------|-----------|-----------|
| Group I | 249.90±42.46 | 166.50±44.40 |
| Group II | 539.78±89.82** | 282.43±35.50** |
| Group III | 432.70±78.02** | 231.28±58.70 |
| Group IV | 374.20±65.34** | 188.48±85.70** |
| Group V | 273.56±58.79** | 165.49±35.50** |
| Group VI | 309.22±61.24** | 195.09±57.00* |

Values are expresses as mean ± SD of 10 rats; ANOVA was used for multiple comparison; compared with group I, *p < 0.01; compared with group II, *p < 0.05, **p < 0.01.

### 4. Conclusion

The data of present study showed significant increases in serum TC, TG, LDLc, VLDLc, athereogenic index (TC/HDLc and LDLc/HDLc ratio), HMG-CoA reductase activity and a significant decrease in HDLc in rats fed with high fat diet. The present study revealed that this plant individually maintains the lipid and lipoprotein status through a metabolic key enzyme HMG-CoA reductase along with the maintenance of collagen and calcium contents, which plays an integral role in atherosclerosis. The efficacy of the drug was further proved through histopathological study. No data is available on the efficacy of root bark of *P. integrifolia* in high fat diet induced atherosclerosis model. Further study is required on the activity guided fractionation and exact molecular mechanisms involved in the anti-atherosclerotic activity of *P. integrifolia*. 

![Fig. 2. Histopathology of experimental rats. (A) No changes (Group I). (B) Multifocal severe fragmentation of elastic and collagen fibres in media with marked mineralization (Group II). (C) Moderate multifocal splitting of media with marked peripheral mineralization (Group III). (D) Moderate multifocal marked splitting of tunica media with vacuolation and mineralization (Group IV). (E) Mild diffuse thickening and fragmentation of media with mild mineralization; focal rupture of intima and media (Group V). (F) Mild multifocal fragmentation of elastic and collagen fibres in media with mineralization; focal rupture of intima and media (Group VI).](image-url)
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