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Premna integrifolia L. on Enzymatic Biomarkers in Atherosclerosis

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Authors' contributions

This work was carried out in collaboration between all authors. Author SC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author RA managed the analysis of the study. Author SG monitored the study. Author RI designed the experiment. Author KSDV provided the financial support. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Premna integrifolia L. belongs to family Verbenaceae and it is believed to prevent cardiovascular disease as per Ayurveda. In order to evaluate the cardiac biomarkers and treatment efficacy of hydroalcoholic extract (HAE) of root bark in atherosclerosis. Sixty Wistar rats were divided into six groups and ten animals in each group. Rats fed with high fat diet and various concentrations of HAE treatment were studied and standard drug, atorvastatin was compared with HAE. This extract showed a significant action against atherosclerosis in a dose dependent manner which was studied in terms of lactate dehydrogenase activity, HMG-CoA/Mevalonate ratio, collagen content in aorta, levels of transaminases, calcium and other marker enzymes indirectly involved in the cardio protection. Alteration in collagen deposition in aorta of histopathological evaluation was noticed in treated animals. Anti-atherosclerotic activity of HAE of *P. integrifolia* was due to its modulatory activity on cholesterol metabolism and our results contribute towards validation of the traditional use of *P. integrifolia* in high fat diet induced atherosclerosis.

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1. INTRODUCTION

Hyperlipidemia is one of the important factors associated with atherosclerosis, hypertension, smoking, diabetes mellitus etc. Atherosclerosis is a multi-factorial disease induced by various factors on suitable genetic backgrounds. It is characterized by the vascular areas containing mononuclear proliferation of smooth muscle cells resulting in thickening and hardening of the arterial walls [1]. The HMG-CoA reductase inhibitors play an important role in the reduction of lipid and lipoprotein levels. The most effective and widely used commercially available drugs for the treatment of hyperlipidemia are statins. Their primary site of action is in liver where they inhibit HMG-CoA reductase, the metabolic pathway that produces cholesterol and isoprenoids [2]. Impaired perfusion from decreased cardiac output may be associated with marked elevations in serum aminotransferases. Bridging fibrosis or cardiac cirrhosis can result from prolonged hemodynamic abnormalities, resulting in an impaired hepatic function with impaired coagulation, and alteration in the metabolism of several cardiovascular drugs, which can lead to unwanted toxicity [3]. Today, the Indian Systems of Medicine are not only complementary but also competitive in the treatment of various diseases. Herbs and herbal based compounds have always been an important source of medicines for various diseases and have received considerable attention in recent years due to their diverse pharmacological actions. Plants have a significant role in maintaining human health and improving the quality of life. *Premna integrifolia* is a medicinal plant and popularly known as "*Agnimantha*" in Ayurvedic systems of medicine and this plant root is commonly used in Dasamula drug preparation of Ayurveda. Hence the present study is focused on identification of liver abnormalities and complex relationship between cardiac and hepatic function in atherosclerosis. The hydroalcoholic extract of root bark of *P. integrifolia* may gain knowledge on hepatic biomarkers which further improve our understanding on disease process with less or no side effects in rats fed with high fat diet.

2. MATERIALS AND METHODS

2.1 Preparation of the Plant Extract

The root bark was procured, authenticated and voucher specimen (00641/2014) was deposited in the Botany Department, Captain Srinivasa Murthi Regional Ayurveda Drug Development Institute, Chennai, India. The bark was shade dried and coarsely powdered. The powder was defatted with petroleum ether (60-80℃) and then extracted with hydro alcohol (60:40 *v/v*) in a Soxhlet extractor and dried under vacuum. The percentage yield of HAE was 7.61% *w/w* and this extract was suspended in 0.5% sodium carboxyl methyl cellulose (SCMC) for the pharmacological screening.

2.2 Induction of Atherosclerosis

Experiment was conducted on 60 (30M+30F) Wistar rats of 6-8 weeks of age and purchased from King's Institute of Preventive Medicine, Guindy, India. Rats were randomly divided in to 6 groups and 10 animals (5M+5F) in each group, maintained in individual cage under controlled temperature, humidity and illumination conditions with water and diet *ad libitum*. High fat diet consisted of 2.0 g of cholesterol, 8.0 g of saturated fat and 0.1 g of calcium (BYCALVIT-500 (Biochem Pharmaceutical Industries Ltd., Mumbai, India) mixed thoroughly with 90 g of powdered standard commercial pellet diet along with weekly challenge of oral Vitamin D_3 (3,00,000 IU) through per oral route [4]. Extract, vehicle and standard drug were administered daily through intra gastric route for 30 days.

2.3 Experimental Protocol for Atherosclerotic Study

Rats fed with

Group-I : Standard pellet diet (n=10)
Group-II : High fat diet (n=10)
Group-III : High fat diet+100 mg/kg.b.wt. of hydroalcoholic extract (n=10)
Group-IV : High fat diet+200 mg/kg.b.wt. of hydroalcoholic extract (n=10)
Group-V : High fat diet +500 mg/kg.b.wt. of hydroalcoholic extract (n=10)
Group-VI : High fat diet +10 mg/kg.b.wt. of Atorvastatin (n=10)

2.4 Biochemical Analysis

At the end of experimental period, 4 mL of blood was collected in a vacutainer from retro-orbital plexus under mild ether anesthesia. Serum was

separated by centrifugation (Remi C-24) at 4℃ for 10 minutes at 3000 rpm. Euthanasia was performed with carbon dioxide and tissues of liver and aorta were removed. Liver was used for biochemical assays and aorta was preserved in 10% formalin for histopathology study of collagen deposition in aorta with picrosirius red staining [5]. The serum biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatine phosphokinase (CPK), bilirubin total and calcium (arsenazo, III-colorimetric) were assayed by clinical chemistry fully autoanalyser (XL-640; Transasia) using kits of system pack from ERBA. Instrument was calibrated with XL-multical (Erba XL SysPack-Level 1-14441) and quality control (Biorad) was run before started the analysis. Liver biochemical parameters AST & ALT [6], acid phosphatase (ACP) & ALP [7], LDH [8], HMG-CoA/mevalonate ratio [9], collagen [10] and total protein [11] were estimated by UV-visible spectrophotometer (Perkin-Elmer XE2-201) by standard procedures.

2.5 Data Analysis

Statistical analysis was carried out using Graph pad prism 5.0. All the values were expressed as mean ± SD (n=10). Groups of data were compared with the analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison. Values were considered statistically significant when $P<0.05$.

3. RESULTS

The serum ALT activity was increased in Group-II ($p<0.001$) when compared to Group-I. The decreased activity was observed in Group-V ($p<0.01$) when compared to Group-II. The activity of AST was increased in Group-II and in Group-III when compared to Group-I. The AST activity was decreased in Group-IV ($p<0.05$) and in Group-V ($p<0.001$) compared to Group-II. The activity of ALP was increase in Group-II ($p<0.05$) when compared to Group-I (Table 1). The serum LDH activity was increased in Group-II ($p<0.0001$), Group-III ($p<0.0001$) and Group-IV ($p<0.01$) compared to Group-I. The activity was decreased in Group-III ($p<0.05$), Group-IV ($p<0.0001$), Group-V ($p<0.0001$) and Group-VI ($p<0.0001$) when compared to Group II. The LDH activity was decreased in Group-V ($p<0.0001$) and in Group-VI ($p<0.01$) when compared to Group-III. The CPK activity was increased in Group-II ($p<0.001$) compared to Group-I and decreased in Group-IV ($p<0.01$), Group-V ($p<0.001$) and Group-VI ($p<0.05$) when compared to Group-II. The collagen content in liver was significantly increased in Group-II ($p<0.0001$) and III ($p<0.001$) when compared to Group-I. The level was significantly decreased in Group-IV ($p<0.05$), V ($p<0.001$) and VI ($p<0.01$) when compared to Group-II. A significant reduction was noticed in Group-V ($p<0.01$) and VI ($p<0.05$) when compared to Group-III. A significant reduction in calcium content of Group-III ($p<0.05$), IV & V ($p<0.01$) when compared to Group-II (Table 2).

The level of bilirubin total was elevated by 35% in Group-II compared to Group-I. 22.7% decreased in Group-III, 25.58% in Group-IV, 28.57% in Group-V and 31.71% in Group-VI when compared to Group-II (Fig. 1). The level of calcium was significantly increased in Group-II ($p<0.0001$) and III ($p<0.05$) when compared to Group-I. The level of calcium was significantly decreased in Group-IV ($p<0.05$) and V ($p<0.01$) when compared to Group-II (Fig. 2). The activity of liver ALT was significantly increased in Group-II ($p<0.001$) compared to Group-I. Group-V

Table 1. Assay of serum Alanine transaminase, Aspartate transaminase and Alkaline phosphatase

Group	Alanine transaminase (mg pyruvate liberated/ mg protein at 37℃ for 30 minutes)	Aspartate transaminase (mg pyruvate liberated/ mg protein at 37℃ for 60 minutes)	Alkaline phosphatase (µg phenol liberated/ mg protein at 37℃ for 15 minutes)
Group-I	0.584±0.086	0.389±0.117	0.482±0.082
Group-II	0.944±0.260[a***]	0.524±0.046[a*]	0.687±0.147[a*]
Group-III	0.798±0.167	0.415±0.072[a*]	0.581±0.185
Group-IV	0.728±0.166 [a,b]	0.398±0.088[b*]	0.522±0.097
Group-V	0.649±0.134[b**]	0.353±0.057[b***]	0.515±0.129
Group-VI	0.719±0.162	0.421±0.089	0.544±0.091

*[Values are mean ± SD; n=10; * p<0.05; ** p<0.01; *** p<0.001]*
[a]Results were compared with Group-I; [b]Compared with Group-II

Table 2. Assay of serum lactate dehydrogenase and creatine phosphokinase

Group	Lactate dehydrogenase (IU/L)	Creatine phosphokinase (IU/L)	Collagen (µg/mg protein)
Group-I	249.90±42.46	166.50±44.4	38.83±16.25
Group-II	539.78±89.82^{a****}	282.43±35.5^{a***}	92.51±24.67^{a****}
Group-III	432.70±78.02$^{a****; b*}$	231.28±58.7	82.07±26.17^{a****}
Group-IV	374.20±63.54$^{a**; b****;}$	188.48±85.7^{b**}	76.52±41.40 ^{b*}
Group-V	273.56±58.79$^{b****; c****}$	165.49±35.5^{b***}	38.95±22.94$^{b***; c**}$
Group-VI	309.22±61.24$^{b****; c**}$	195.09±57.0^{b*}	40.07±29.66$^{b**; c*}$

[Values are mean ± SD; n=10; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; p<0.0001]
^{a}Results were compared with Group-I; ^{b}Compared with Group-II; ^{c}Compared with Group-III

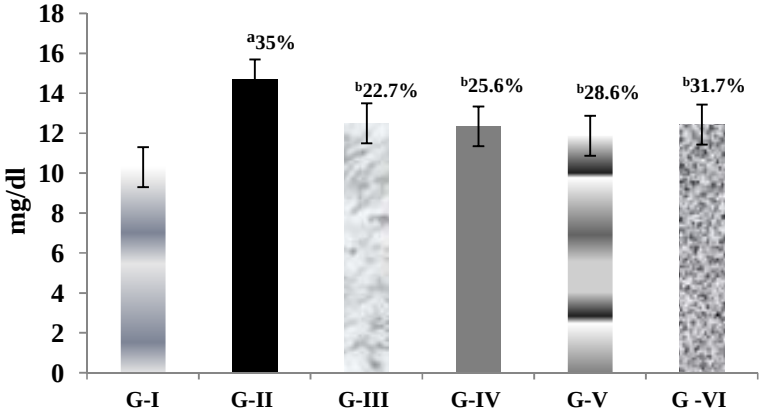


Fig. 1. Estimation of bilirubin total in serum
[Values are mean ± SD; n=10; ^{a}Results were compared with Group-I; ^{b}Compared with Group-II]

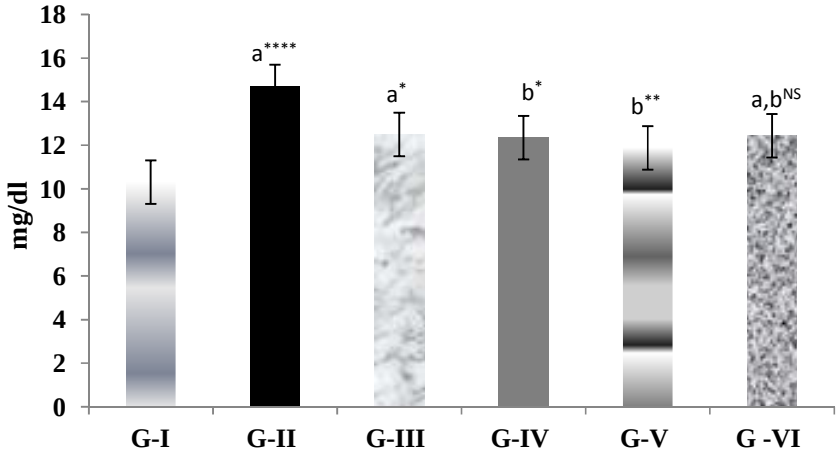


Fig. 2. Estimation of calcium in serum
[Values are mean ± SD; n=10; $^{*}p<0.05$; $^{**}p<0.01$; $^{****}p<0.0001$]
^{a}Results were compared with Group-I; ^{b}Compared with Group-II

showed a significant decrease (p<0.01) in activity than Group-II. The activity of liver AST was significantly increased in Group-II and III (p<0.05) when compared to Group-I. Group-IV (p<0.05) and V (p<0.001) showed a significant decrease in AST activity when compared to Group-II. The activity of ALP was significantly high in Group-II (p<0.05) when compared to

Group-I. The activity of ALP in liver was decreased but no significant difference was found in all other groups when compared to Group-II (Table 3). The activity of ACP in liver was significantly increased in Group-II ($p<0.01$), III ($p<0.05$) when compared to Group-I and significant decrease was observed in Group-V ($p<0.05$) when compared to Group-II. The activity of LDH in liver was significantly increased in Group-II, III, IV ($p<0.0001$) and VI ($p<0.05$) when compared to Group-I and significant decrease was noticed in Group-III, IV, V and VI ($p<0.0001$) when compared to Group-II. A significant decrease was observed in Group-IV and VI ($p<0.01$) and V ($p<0.0001$) when compared to Group-III. HMG CoA/mevalonate ratio was decreased in Group-II ($p<0.01$) compared to Group-I. A significant improvement was noticed in Group-IV, VI ($p<0.01$), V ($p<0.001$) when compared with Group-II (Table 4).

4. DISCUSSION

Measurement of LDH activity in blood is considered to be a diagnostic marker for certain cardiovascular diseases and it is a sensitive measure of myocardial infarction [12]. When inadequate end-organ perfusion and tissue hypoxia is persistent or when acute shock develops this protecting mechanism against hypoxic liver damage is overwhelmed. Hepatocellular injury ensues, accompanied by sharp elevation of the serum aminotransferases, LDH and occasionally functional renal impairment. Ischemic hepatitis is usually benign and self-limited. Aspartate transaminase level is used as a diagnostic marker for myocardial infarction. In pathological conditions, the enzymes such as CPK, LDH, AST and ALT leaks from the necrotic heart cells to blood, which are important measure of cardiac injury. These enzymes are not specific for myocardial injury individually; however, evaluation of these enzymes together may be an indicator of myocardial damage [13,14]. CPK is another important enzyme in energy metabolism in body which maintains the high concentration of intracellular ATP through phosphorylation and it is considered as important index for atherosclerosis [15]. Higher activities of these enzymes in serum have been found in response to oxidative stress induced by high

Table 3. Assay of liver Alanine transaminase, Aspartate transminase, Alkaline phosphatase

Group	Alanine transaminase (mg pyruvate liberated/ mg protein at 37℃ for 30 minutes)	Aspartate transaminase (mg pyruvate liberated/ mg protein at 37℃ for 60 minutes)	Alkaline phosphatase (µg phenol liberated/ mg protein at37℃ for 15 minutes)
Group-I	0.584±0.086	0.389±0.117	0.482±0.082
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Group-IV	0.728±0.166	0.398±0.088^{b*}	0.522±0.097
Group-V	0.649±0.134 ^{b**}	0.353±0.057 ^{b***}	0.515±0.129
Group-VI	0.719±0.162	0.421±0.089	0.544±0.091

[Values are mean ± SD; n=10; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$]
^{a}Results were compared with Group-I; ^{b}Compared with Group-II

Table 4. Assay of liver Acid phosphatase, Lactate dehydrogenase and HMG CoA/ mevalonate ratio

Group	Acid phosphatase (µg phenol liberated/ mg protein at 37℃ for 60 minutes)	Lactate dehydrogenase (µmoles/mg protein)	HMG CoA/ mevalonate ratio
Group-I	0.209±0.052	85.36±12.91	1.161±0.110
Group-II	0.408±0.076^{a**}	808.89±103.89^{a****}	1.404±0.144^{a**}
Group-III	0.366±0.138^{a*}	440.39±123.91^{a,b****}	1.204±0.105
Group-IV	0.336±0.152	300.15±56.01$^{a,b****; c**}$	1.158±0.150^{b**}
Group-V	0.241±0.123^{b*}	143.42±70.97 $^{b****; c****}$	1.056±0.139^{b***}
Group-VI	0.263±0.112	195.66±66.72$^{a*;b****; c**}$	1.108±0.130^{b**}

[Values are mean ± SD; n=10; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $p<0.0001$]]
^{a}Results were compared with Group-I; ^{b}Compared with Group-II; ^{c}Compared with Group-III

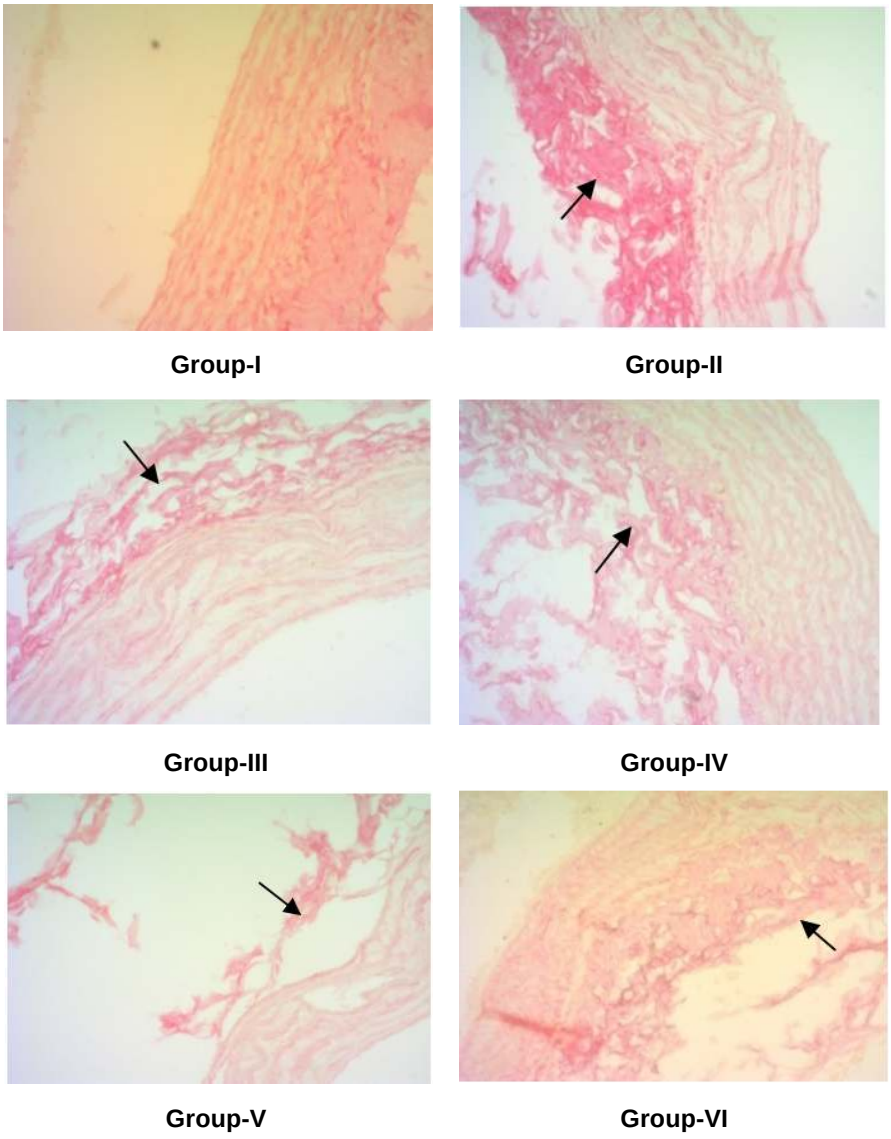


Fig. 3. Histopathology of collagen deposition in aorta

Legend:

Group-I	:	*No collagen accumulation*
Group-II	:	*Well defined collagen accumulation with mineralization*
Group-III	:	*Moderate accumulation of collagen with less demineralization*
Group-IV	:	*Mild collagen accumulation with marked demineralization*
Group-V	:	*Mild collagen accumulation with severe demineralization*
Group-VI	:	*Moderate collagen accumulation with less demineralization*

fat diet [16]. Impaired perfusion from low cardiac output results in cardiogenic ischemic hepatitis. The hall mark findings of ischemic hepatitis are severe jaundice with high bilirubin level and elevated liver function tests. Increase in LDH tends to be massive and ALT/LDH ratio may help distinguishing ischemic injury from other forms of acute hepatitis [17,18]. There was a significant restoration of these enzymes on administration with different concentrations of HAE of *P. integrifolia*. The activity of AST, ALT, CPK and LDH was inversely proportional to the concentration of HAE extract. Liver plays a central role in the absorption, distribution and elimination of majority of drugs. Collagen is a family of proteins consists of several genetically

different molecular species and is closely involved in tissue organization, function, differentiation and development [19]. The unique distribution of hydroxyl proline within the collagen can easily be studied [20]. Hepatic impairment can alter the pharmacokinetic profiles of cardiovascular drugs, which can lead to unwanted toxicity. On treatment with HAE, reversibility of changes was noticed when compared to high fat fed rats (Fig. 3).

Liver function tests abnormalities were common in patients with chronic heart failure. Elevated total bilirubin remained one of the strongest independent predictors of poor prognosis. An intracellular cation has significant role in the regulation of normal physiology and biochemistry of cardiac and smooth muscles. Flavonoids, have been found to suppress the intracellular high Ca^{2+} ion in a dose dependent manner, as well as the release of pro-inflammatory mediators such as tumor necrosis factor-α [21]. Studies revealed that tannins, triterpenoids and flavonoid are the major phytoconstituents of *P. integrifolia* [22,23]. HAE have an essential phytoconstituents including flavonoid might help in the reduction of intracellular calcium levels in the drug treated groups.

Elevated transaminases should be seriously considered in patients with cardiac disease and also elevation of each cholestatic liver function tests was significantly associated with the degree of tricuspid incompetence [24]. During hepatic impairment, the bioavailability is double and the total plasma clearance is halved. Marked decrease in liver transaminases, ACP and ALP levels demonstrate the cardio-protective effect of *P. integrifolia*. HAE has cadioprotective activity along with hepatoprotective activity which was evident in liver marker enzymes in extract treated groups. Due to the presence of flavonoid, a secondary metabolite in HAE could enhance the antioxidant defense mechanisms and prevents muscle cell damage. LDH is less specific than AST and ALT as a marker of hepatocyte injury, but LDH may disproportionately elevated after an ischemic liver injury. The activity of LDH was reported to be high in rats fed with high fat diet induced obesity [25]. The HAE might protect the cell by reducing the activities of these marker enzymes. HMG-CoA reductase activity was indirectly measured in terms of HMG CoA/Mevalonate ratio in liver tissue [9]. Inhibitors of HMG-CoA reductase are used to lower serum cholesterol levels and improve the survival of individuals at risk of atherosclerotic vascular disease [26]. Their primary site of action is in liver where they inhibit HMG-CoA reductase, the metabolic pathway that produces cholesterol and isoprenoids [27]. Atorvastatin treated 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. The most effective and widely used drugs for the treatment of hyperlipidemia are the atorvastatin and this drug competitively inhibit HMG-CoA reductase enzyme in liver that converts HMG-CoA to mevalonate an early precursor for cholesterol biosynthesis. Hence this enzyme might competitively inhibited by the HAE which was evident from the lipid and lipoprotein status.

5. CONCLUSION

In conclusion, all HMG-CoA reductase inhibitor undergo extensive hepatic metabolism. Active liver disease or persistent unexplained elevations in serum transaminases to above 3 times the upper limit of normal, the use of statins is contraindicated as they may worsen liver function [28]. Therefore the cardio tonic action of the HAE might attribute to the phytoconstituents present in it. From the above findings, HAE of *P. integrifolia* modulates enzyme activity in a dose-dependent manner and further research is required on which active principle is mainly involved in the anti-atherosclerotic activity of high fat diet-induced atherosclerosis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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