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Therapeutic Potential of Alcoholic Extract from *Lemna minor* Linn. in Attenuating Atherosclerosis Induced by High-Fat Diet in Male Wistar Rats

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Abstract

This study explored the cardioprotective effects of Alcoholic Extract of *Lemna minor Linn.* (AELM) against high-fat diet-induced atherosclerosis in male Wistar rats. AELM was administered at doses of 300 mg/kg and 800 mg/kg. Assessments included lipid profiles, oxidative stress markers, cardiac injury enzymes, and liver parameters associated with cholesterol synthesis. Results showed significant reductions in lipid levels, oxidative stress markers, and cardiac injury enzymes, particularly with the higher dose of 800 mg/kg. AELM also lowered the atherogenic index and improved HDLc levels, indicating the potential to mitigate atherosclerosis-related lipid imbalances. Furthermore, AELM exhibited a dose-dependent reduction in the HMG CoA/mevalonate ratio, suggesting inhibition of HMG-CoA reductase activity, crucial in cholesterol synthesis. Molecular docking studies supported AELM's antiatherosclerotic potential, with leucine demonstrating favourable binding energies with atherosclerosis-associated enzymes. Histopathological analysis revealed structural improvements in rat aortas with AELM treatment. In conclusion, AELM presents promise as a therapeutic agent against atherosclerosis through modulation of lipid metabolism, attenuation of oxidative stress, and inhibition of HMG-CoA reductase activity.

Keywords: Atherosclerosis, Cardioprotective, HMG-CoA Reductase, Lemna minor Linn., Oxidative Stress

1. Introduction

Heart diseases are a primary cause that contributes to mortality, frequently arising from atherosclerosis, a persistent inflammatory condition affecting medium to large arteries, primarily at sites of disturbed hemodynamic flow[1]. Recent surveys indicate that in individuals aged 30-79 in 2020, the global prevalence of increased carotid intima-media thickness, carotid plaque, and carotid stenosis consistently rose with age, with higher rates observed in men compared to women. Estrogen's beneficial effects on the cardiovascular system may protect women during their fertile years from atherosclerosis, as evidenced by lower age-specific risk before menopause[2,3]. Dietary factors, notably saturated fat intake, have been implicated in atherosclerosis pathogenesis, elevating atherogenic lipoproteins and promoting inflammation[4,5]. Notably, prolonged adherence to a low-protein diet has

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tended to reduce the occurrence of cardiovascular diseases according to protracted observational investigations[6,7].

Dietary regimens packed with fat and abundant in Saturated Fatty Acids (SFAs) may induce heightened lipoprotein concentrations besides a decrease in High-Density Lipoprotein Cholesterol (HDL-C), culminating in hepatic cellular oxidative stress and inflammatory cascades[8,9]. Choline, a constituent of Western high-fat diets, contributes to the development of a lipid triad characterized by elevated plasma Triglycerides (TG) and increased Low-Density Lipoproteins (LDL).

While statins remain a focal point in atherosclerosis management, they are accompanied by untoward effects, including myopathic complications, the onset of diabetes, and heightened susceptibility to haemorrhagic stroke[10]. Atherogenic processes within atherosclerosis intricately involve the mediation between the oxidative damage and conjointly advancement of active oxygen species, underscoring the quest for compounds capable of curtailing the responsive exchange of vascular reactive oxygen species and rehabilitation concerning endothelial integrity[11,12].

This study scrutinizes the potential of both natural and synthetic antioxidants to ameliorate oxidative modifications within lipoproteins and forestall the emergence of cardiovascular maladies[13]. Herbal agents, exemplified by flavonoid extracts, are deliberated upon for their antioxidative attributes, characterized by a diminished side-effect profile relative to synthetic analogs[14-16]. *Lemna minor* Linn., commonly known as duckweed, has demonstrated auspicious prospects in inhibiting lipid peroxidation and exerting immunomodulatory effects, based on antecedent investigations[17,18].

Lemna minor Linn. is distinguished as a bountiful reservoir of both amino acids either obligatory and nonetheless, amid those that are valine, leucine, and isoleucine assume significant roles[19]. Specifically, amino acids having extending chains, including leucine, have garnered attention in the realm of cardiovascular disorders and associated risk factors. Leucine has been correlated with the modulation of the level in the blood of the cholesterol-forming lipoprotein (HDL-C) and the display of antiatherosclerotic attributes. The plant-based origin of these bioactive constituents accentuates their bioavailability and underscores their potential in atheroprotection[20]. This inquiry is poised to further elucidate the antiatherosclerotic attributes of *Lemna minor* Linn. extract, capitalizing on its leucine-rich composition and the prospective advantages inherent to plant-derived therapeutics.

2. Materials and Methods

2.1 Accumulation and Extraction of Plant Product

The entire *Lemna minor* Linn. plant from the Araceae family, sourced from Hunsur Lake in Jigani, Bengaluru, India, was taxonomically verified by experts at the Central Ayurveda Research Institute, Bengaluru. Subsequently, 480 grams of the dried plant material were subjected to alcohol-based extraction using a Soxhlet apparatus at Green Chem, Bengaluru, Karnataka, resulting in a 90-gram alcoholic extract of *Lemna minor* Linn.

2.2 Preliminary Phytochemical Studies

Preliminary phytochemical analysis was undertaken as per definitive methods for the comparative assimilations of phytoconstituents[21,22].

2.3 GC-MS Evaluation

Methanol was the solvent employed to dissolve 10 mg of the *Lemna minor* Linn. extract. After that, the sample under scrutiny was subjected to analysis using Gas Chromatography-Mass Spectrometry (GC-MS) with a Perkin Elmer apparatus, namely the GC Model Clarus 680. The experimental parameters used were as follows: a 30-metre-long fused silica column filled with Elite-5MS (which is made up of 95% dimethylpolysiloxane and 5% biphenyl), having an inside diameter of 0.25 mm with an impermeable layer of 250µm. Helium constituted the vessel that carried gas, flowing at a steady 1 millilitre per minute. Throughout the chromatographic run, the injector's temperature was maintained at 260°C. After adding a 1µL volume of the extracted sample to the device, the oven temperature program was set to start at 60°C for two minutes, up to 300°C at a rate of 10°C per minute, and then hold that temperature for an additional six minutes. The temperatures of the ion source and transmission line have both been recorded at 240°C, and the electron impact ionization mode was seen at 70 eV. The scan interval was set at 0.1 seconds and the scan period was 0.2 seconds to identify mass. Pieces in the 40-600 Da range were examined by the mass spectrometer. Each component's spectrum was contrasted with the list of known component spectra kept within the GC-MS NIST (2008) library.

2.4 Experimental Animals

Male Wistar rats were acquired from the Central Animal House and weighed around 200-250 gms at Krupanidhi College of Pharmacy. They were maintained in a standard environmental condition with a 12-hour light/dark cycle at approximately 24ºC and 50% humidity. The rats had *ad libitum* accessibility to water from the faucet and a standard diet comprising pellets. The Institutional Animal Ethics Committee granted ethical approval for the study procedure, which complied with the rules stipulated by the Committee for the Purpose of Control and Supervision (Ref. No.378/PO/ReBi/S/01/CPCSEA 2001) on Experimental Animals (CPCSEA).

2.5 Acute Toxicity Study in Rats (LD_{50} Determination)

Male Wistar rats were chosen for the study of acute oral toxicity by OECD Directive No.425. For 14 days, the animals are monitored for symptoms and mortality by administering different dosages of alcoholic extract of *Lemna minor* Linn. at 100, 300, 750, 1000, and 2000 mg/kg *b.w*[23].

2.6 Induction of an Experimental Atherosclerosis Model

An atherogenic diet was formulated by combining saturated fat in the form of lard, bread, and a 0.1-gram supplement of calcium from Auspharma PVT Ltd, in conjunction with a 90-gram portion of a standard commercial pellet diet. The experimental rats were subjected to weekly oral administration of Vitamin D3 (Auspharma PVT Ltd) via the peroral route[24].

2.7 Grouping and Dosing Methods

Five groups, each with six animals, were assigned at random to the animals and were subjected to a 30-day treatment regimen as outlined in the following schedule. Group I functioned as the control and was subjected to vehicle treatment, Group II was administered a high-fat diet, Group III was exposed to a high-fat diet concurrently with a low dose of AELM (300 mg/kg, orally), Group IV was exposed to a high-fat diet in conjunction with a high dose of AELM (800 mg/kg, orally), and Group V, designated as the standard group, was treated with a high-fat diet along with Atorvastatin (20 mg/kg, orally)[25].

2.8 Biochemical Analysis

At the end of the experimental period (i.e., 31st day) under aseptic conditions two millilitres of blood were extracted from the retroorbital plexus whilst under a light ether anaesthesia. Extraction of the serum required centrifugation, which took 10 minutes at 3000 rpm and 4°C. Hematoxylin and eosin were used to stain aorta cross-sections that were 5 µm thick. Biochemical markers such as calcium, total cholesterol, triglyceride, high-density lipoprotein-cholesterol, aspartate aminotransferase, alanine transaminase, alkaline phosphatase, lactate dehydrogenase, and creatinine phosphokinase were analyzed using Transasia Bio-medicals Ltd's automated analyzer.

The hepatic ratio of Hydroxylmethylglutaryl-Coenzyme A to mevalonate[26], the amount of collagen[27], and the total protein content[28] were determined. Serum FFA levels[29] were quantified with a UV-visible spectrophotometer using Itaya's reagent[30]. Low-Density Lipoprotein-Cholesterol (LDL-C), Very Low-Density Lipoprotein-Cholesterol (VLDL-C), and the atherogenic index, calculated via established methodologies, were employed to assess atherosclerotic risk[31]:

$$AI = \left(Log10_{10} \left(\frac{TG}{HDLc} \right) \right)$$

2.9 Molecular Docking Analysis

Molecular docking utilized Auto-Dock 4.2.6 and Discovery Studio 4.5 for visualization, focusing on Nitric Oxide (NO) synthase and Caspase-3 in atherosclerosis. Compounds' structures were prepared in ChemDraw Ultra 8.0, and 3D protein structures (PDB ID: 3E7G and 2XYG) were retrieved from the Protein Data Bank. After preprocessing, modified structures were used in docking studies, employing Gasteiger charges for hydrogen addition and Lamarckian Genetic Algorithm (LGA). Results were assessed based on binding energy and hydrogen bonding. Reverse validation confirmed target compatibility.

2.10 Statistical Analysis

GraphPad Prism 10.0.2 software was used for statistical analysis, the data were analyzed by one-way ANOVA followed by Dunnet's test. The results were represented as Mean ± SEM. A value of $p < 0.05$ was considered significant.

3. Results

3.1 GC-MS Analysis of AELM

GC-MS analysis showed the presence of L-leucine being the primary compound. Additional components are listed in Table 1, and Figure 1 shows the gas chromatogram for each of these compounds. GC-MS analysis of methanol soluble compounds of alcoholic extract of *Lemna minor* Linn. has indicated the presence of branched-chain amino acid leucine which was not reported earlier. The L-leucine was found to be present in greater concentration, with a peak area of 26.675%.

Table 1. Specifications of the AELM from GC-MS

Serial numbers	Name of the compound	Molecular mass	Retention period	Peak region%
1	Silane, dimethyl (4- methoxyphenyl) heptadecyloxy-	436	23.23	22.42
2	L-Leucine, N- pentadecafluorooctanoyl-, hexadecyl ester	751	23.31	26.67
3	Benzo[1,2,3-cd:4,5,6-c'd] diperylene	500	23.33	7.62
4	Phosphoric acid, bis (3- methyl phenyl) 4-bromophenyl ester	432	23.42	7.46
5	Cyclodisilazane, 2,2,4,4- tetramethyl-1,3-bis (methyl diphenyl silyl)-	538	24.40	10.11
6	Tetra benzo[a,c,hi,qr]pentacene	452	25.11	3.03
7	Benzo[ghi]diindeno[1,2,3- cd:1',2',3'-lm]perylene	424	25.16	5.015

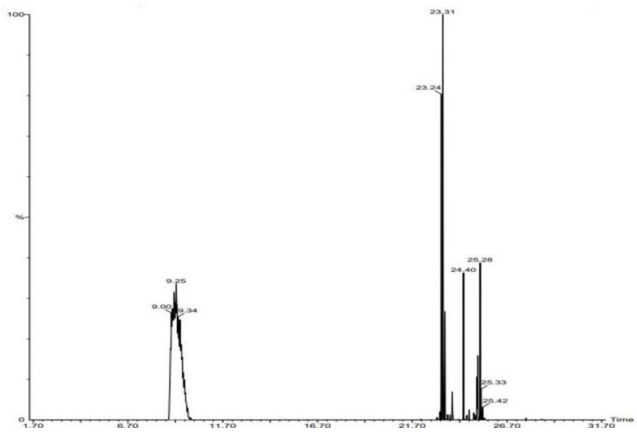


Figure 1. AELM GC-MS chromatogram.

No prior reports have been made on this compound's cardioprotective effect.

3.2 Impact Concerning AELM Affecting the Body Weight in Rats Employing an Atherosclerosis Model Developed Through a High-Fat Diet

A substantial elevation in body weight was noted in rats fed with a high-fat diet for 30 days. The high dose of AELM (800 mg/kg) treated group showed a significant reduction in body weight in contrast to the group under positive control (Table 2).

Table 2. Impact of AELM on body mass (g)

SI. No.	Experimental Group	0th Day	7th Day	14th Day	21st Day	28th Day
1.	Normal group	181.1 ± 0. 69	193.16 ± 0. 79	203.83 ± 1.51	214 ± 0.84	226.16 ± 1.2 2
2.	Positive Control group (High-fat diet 10mg/kg *b,w p.o.*)	333.6 ± 5. 27****	354 ± 5.05* ***	373.83 ± 4.89* ***	391.83 ± 5***	407 ± 4.95*** *
3.	A low dose of AELM (300 mg/kg *b,w p.o.*)	348.1 ± 2. 77*	338.33 ± 2. 80*	329.83 ± 2.65*	319.66 ± 2.52*	307.83 ± 2.6* *
4.	High dose of AELM (800 mg/kg *b,w p.o.*)	383.3 ± 0. 66*	363.33 ± 5. 62**	344.5 ± 1.33**	322.5 ± 2.18**	302.83 ± 0.8* **
5.	Standard drug (Atorvastatin 20 mg/kg *b,w p.o.*)	365.8 ± 5. 62**	353.33 ± 5. 62***	341.33 ± 5.80* **	333.83 ± 5***	314 ± 4.95***

Values are Mean±SEM(n=6), and numerous analogies were performed using ANOVA; groups I and II were compared assembling ****p<0.0001, respectively, *p<0.05, **p<0.01, ***p<0.001 on comparable days.

3.3 Impact of AELM Affecting the Weight of Organs in Rats Utilizing an Atherosclerosis Model Developed Through a High-Fat Diet

There have not been notable distinctive changes in weight disparities between the untreated and treatment groups for organs such as the liver, heart, and kidney (Table 3).

3.4 Impact Of AELM Affects Blood Level of Lipids in Rats Utilizing an Atherosclerosis Model Developed Through a High-Fat Diet

Serum TC, TG, as well as levels of FFA, were substantially elevated in rats given a diet that was high in fat for 30 days (as shown in Table 4), LDL (as indicated in Table 5), and the Atherogenic Index (as presented in Table 6). Conversely, HDL and VLDL levels (as displayed in Table 5) were notably decreased in this group.

However, in the group treated with considerable reductions in blood TC, TG, FFA, LDL, and the Atherogenic Index were noted at an elevated dosage of AELM (800 mg/kg) in contrast with the positive control group. Additionally, both the High dose of the AELM-treated group (800 mg/kg) and the group treated with the standard drug Atorvastatin (20 mg/kg) revealed a substantial decrease in VLDL levels and a substantial rise in HDL levels relative to the positive control group.

3.5 Evaluation of Atherosclerotic Markers in Serum

In the high dose of AELM group as in contrast to the group receiving a positive control, those given a daily dose of 800 mg/kg indicated that substantial reduction

Table 3. Impact of AELM on organ weight (g)

SI. No.	Experimental Group	Liver	Heart	Kidney
1.	Normal group	6.86 ± 0.06	0.92 ± 0.0	1.93 ± 0.04
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	8.08 ± 0.06	0.82 ± 0.0	1.88 ± 0.04
3.	A low dose of AELM (300 mg/kg b.w., p.o.)	7.25 ± 0.13	0.76 ± 0.01	1.93 ± 0.04
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	6.9 ± 0.13	0.70 ± 0.0	1.41 ± 0.10
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	7.18 ± 0.14	0.74 ± 0.0	1.85 ± 0.05

ANOVA was utilized for multiple comparisons, and values are displayed as Mean±SEM of six rats.

Table 4. Impact of AELM on TC, TG and FFA

SI. No.	Experimental Group	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	Free fatty acids (mmol/L)
1.	Normal group	84.98 ± 3.71	66.62 ± 0.09	64.54 ± 0.60
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	184.79 ± 0.62****	196.44 ± 1.21****	194.00 ± 1.32***
3.	A low dose of AELM (300 mg/kg *b.w.,* p.o.)	148.85 ± 0.74***	148.90 ± 0.74^{ns}	146.90 ± 0.84^{ns}
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	121.31 ± 0.74****	110.63 ± 2.93***	110.30 ± 2.16**
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	129.87 ± 0.24***	115.34 ± 2.01**	122.20 ± 0.58*

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were compared assembling ****p<0.0001, respectively, *p<0.05, **p<0.01, ***p<0.001, ns.

Table 5. Impact of AELM on LDLc, HDLc and VLDLc

SI. No.	Experimental Group	LDLc (mg/dL)	HDLc (mg/dL)	VLDLc (mg/dL)
1.	Normal group	22.79 ± 0.26	47.96 ± 1.74	13.32 ± 0.11
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	76.52 ± 1.12****	33.86 ± 0.42****	39.28 ± 0.24****
3.	Low dose of AELM (300mg/kg *b.w., p.o.*)	48.75 ± 0.86**	40.06 ± 0.56***	28.95 ± 0.86**
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	31.88 ± 1.53****	42.97 ± 0.52****	22.12 ± 0.58***
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	38.99 ± 1.06***	42.84 ± 0.44****	23.06 ± 0.40**

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were assessed alongside ****p<0.0001, respectively, **p<0.01, ***p<0.001.

Table 6. Impact of AELM on Atherogenic Index

SI. No.	Experimental Group	Atherogenic Index
1.	Normal group	1.39 ± 0.06
2.	Positive Control group (High-fat diet 10mg/kg *b.w.,* p.o.)	5.80 ± 0.07****
3.	Low dose of AELM (300mg/kg *b.w., p.o.*)	3.71 ± 0.03**
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	2.57 ± 0.06***
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	2.69 ± 0.06***

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were assessed alongside ****p<0.0001, respectively, **p<0.01, ***p<0.001.

in the levels of the enzymes ALT, AST, and ALP (Table 7), in addition to a reduction in LDH and CPK (Table 8).

3.6 Determination of HMG-CoA, Collagen, Calcium and Total Protein in The Liver

High-dose AELM treatment (800 mg/kg) and the standard drug Atorvastatin (20 mg/kg) exhibited a notable decrease in HMGCoA and collagen levels in comparison to the positive control group (Table 9). In addition, contrasted with the positive control group, the high-dose AELM treatment group (800 mg/kg) exhibited a substantial decrease in total protein as well as calcium levels (Table 9).

Table 7. Impact of AELM on ALT, AST and ALP

SI. No.	Experimental Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
1.	Normal group	99.04 ± 0.62	118.02 ± 1.05	410.39 ± 3.97
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	141.99 ± 0.67****	161.96 ± 0.76***	615.16 ± 0.91***
3.	Low dose of AELM (300mg/kg *b.w., p.o.*)	121.05 ± 0.87**	134.07 ± 0.47*	504.09 ± 0.91*
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	105.92 ± 0.49***	119.16 ± 1.72**	459.86 ± 4.30**
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	112.91 ± 1.15**	134.01 ± 0.39*	522.16 ± 1.31*

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were compared assembling ****p<0.0001, respectively, *p<0.05, **p<0.01, ***p<0.001.

Table 8. Impact of AELM on LDH and CPK

SI. No.	Experimental Group	LDH (IU/L)	CPK (IU/L)
1.	Normal group	237.15 ± 0.95	144.9 ± 0.32
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	447.34 ± 1.50****	249.5 ± 0.89***
3.	Low dose of AELM (300mg/kg *b.w., p.o.*)	371.92 ± 0.55**	163.73 ± 0.42**
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	262.41 ± 3.14****	136.96 ± 0.50****
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	302.55 ± 0.58***	184.50 ± 0.15***

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were assessed alongside ****p<0.0001, respectively, **p<0.01, ***p<0.001.

Table 9. Impact of AELM on HMG-CoA, Collagen, Calcium and Total protein in the liver

SI. No.	Experimental Group	HMG-CoA/ Mevalonate ratio	Collagen (µg/ mg protein)	Calcium (mg/ dL)	Total protein (g/ dL)
1.	Normal group	1.16 ± 0.00	37.68 ± 0.77	12.42 ± 0.17	172.62 ± 1. 13
2.	Positive Control group (High-fat diet 10mg/kg *b.w., p.o.*)	1.43 ± 0.00***	90.73 ± 0.46****	15.58 ± 0.21* ***	245.98 ± 2. 31***
3.	Low dose of AELM (300mg/kg *b.w., p.o.*)	1.19 ± 0.00*	75.48 ± 0.33**	11.42 ± 0.10* **	242.87 ± 0. 67ns
4.	High dose of AELM (800 mg/kg *b.w., p.o.*)	1.048 ± 0.00**	37.48 ± 0.61***	10.27 ± 0.06* ***	191.25 ± 2. 06****
5.	Standard drug (Atorvastatin 20 mg/kg *b.w., p.o.*)	1.091 ± 0.01 **	40.55 ± 0.33***	13.45 ± 0.09*	206.16 ± 0. 47***

The analysis is displayed as the Mean±SEM of six rats, and numerous analogies were performed using ANOVA; groups I and II were assessed alongside ****p<0.0001, respectively, *p<0.05, **p<0.01, ***p<0.001, ns.

3.7 Molecular Docking Results

The results obtained from docking of L-leucine, N-pentadecafluorooctanoyl hexadecyl ester with two atherosclerosis marker enzymes are given in Table 10. The enzyme with PDB ID 3E7G and 2XYG exhibited a binding energy of -7.5 and -8.6 kcal/mol appropriately. The intermolecular energy of atherosclerosis marker enzymes was found to be within a range of -9.86 (3E7G) and -6.03 (2XYG) kcal/mol. The inhibition constant kI for the atherosclerosis marker enzymes was found to be 68.16 (3E7G) and 43.85 (2XYG) μM respectively. All the selected atherosclerosis marker enzymes have better binding energy with L-leucine, N-pentadecafluorooctanoyl-, and hexadecyl ester as compared with the standard Atorvastatin (-5.7 kcal/mol). This data indicates that L-leucine, N-pentadecafluorooctanoyl-, and hexadecyl ester possess good antiatherosclerotic capacity and may be a better therapeutic entity to treat high-fat diet-induced atherosclerosis (Table 10).

Table 10. Docking results of L-leucine, N-pentadecafluorooctanoyl-, hexadecyl ester with Caspase-3 and NO synthase enzymes

Enzymes	PBD ID	Binding Energy (ΔG) (kcal/mol)	Inter molecular energy (kcal/mol)	Inhi bition constant kI (μM)
NO synthase	3E7G	-7.5	-9.86	68.16
Caspase-3	2XYG	-8.6	-6.03	43.85

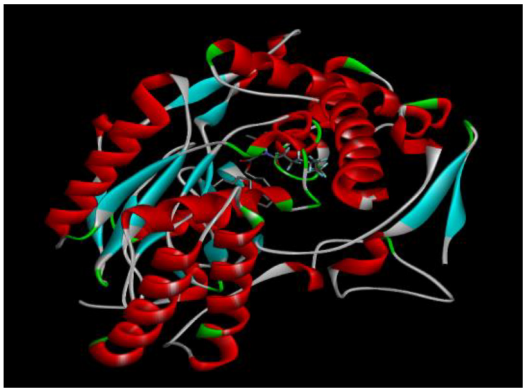


Figure 2. 3D Snapshot of Autodock model on the binding model on binding sites of L-leucine, N-pentadecafluorooctanoyl-, hexadecyl ester with NO synthase enzyme (PBI ID: 3E7G).

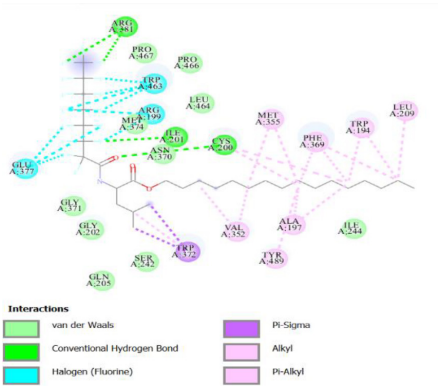


Figure 3. Snapshot of 2D drug-receptor interaction of the NO synthase enzyme with L-leucine, N-pentadecafluorooctanoyl-, hexadecyl ester revealing the binding sites and amino acid residues (PBI ID: 3E7G).

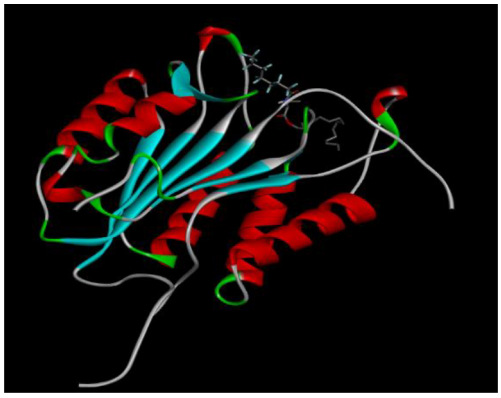


Figure 4. 3D Snapshot of Autodock model on the binding model on binding sites of L-leucine, N-pentadecafluorooctanoyl-, hexadecyl ester with caspase-3 enzyme (PBI ID: 2XYG).

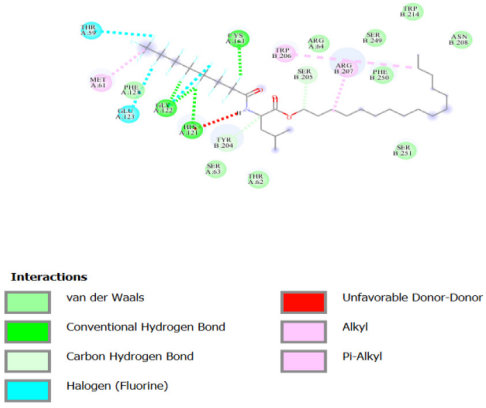


Figure 5. Snapshot of 2D drug-receptor interaction of the caspase-3 enzyme with L-leucine, N-pentadecafluorooctanoyl-, hexadecyl ester revealing the binding sites and aminoacid residues (PBI ID: 2XYG).

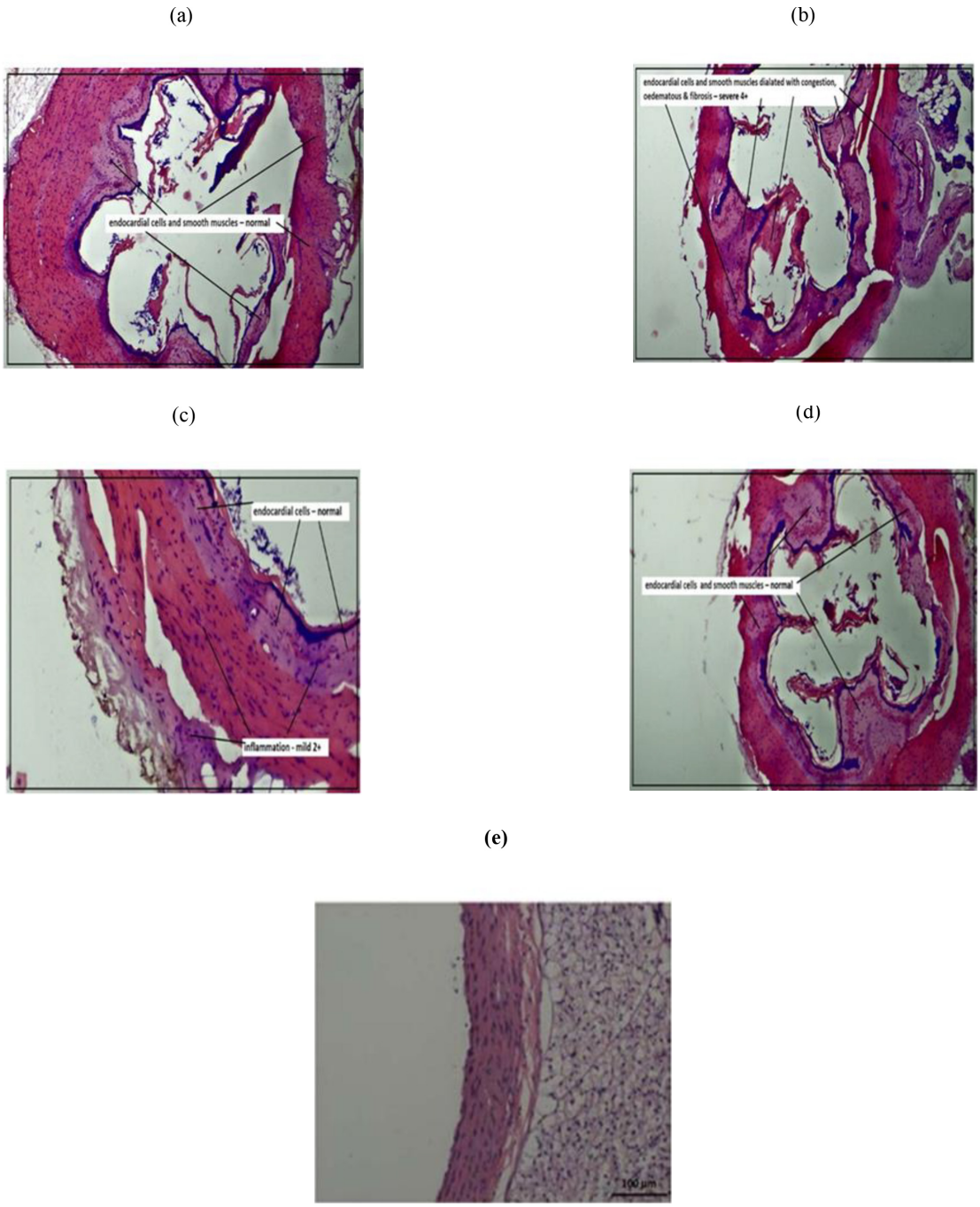


Figure 6. Illustrates the way AELM impacts the histological evaluation of aorta tissue in a rat model of atherosclerosis resulted due to a diet rich in fat. This figure presents photomicrographs of histopathological sections of rat aortic tissue from various experimental groups, captured at a magnification of 100X. **(a)** The control group exhibits the typical, unaltered histological architecture of rat aortic tissue. **(b)** In the positive control group, aortic tissue reveals pronounced dilation of endocardial cells and smooth muscle, along with marked congestion, oedema, and severe fibrotic changes, graded at 4+. **(c)** Rats who responded to a low dose of AELM (300 mg/kg) demonstrated mostly normal aortic tissue, characterized by intact endocardial cells with no apparent abnormality (NAD+), but displaying mild inflammation, graded at 2+. **(d)** In the group that responded to a high dose of AELM (800 mg/kg), aortic tissue maintains its normal appearance, with clear nuclear staining observed in both endocardial cells and smooth muscle. **(e)** Rats treated with Atorvastatin (20 mg/kg) exhibit normal aortic tissue structure, with nuclear staining apparent in a few endocardial cells and smooth muscle.

3.8 Aortic Histopathological Study

Microscopy photographs of the histopathological portions of rat aortas from different experimental groups are shown in Figure 6. These images were captured at a magnification of 100X. (a) Depicts the histologically normal structure of a rat aorta. (b) Illustrates aortic abnormalities in experimental rats subjected to a high-fat diet, characterized by dilated endocardial cells and smooth muscle, along with congestion, oedema, and severe fibrosis (graded as 4+). (c) Demonstrates the aortas of experimental rats responded to a low dose of AELM (300 mg/kg), revealing a generally normal aortic structure with the presence of mild inflammation (graded as 2+). (d) Depicts the aortas of experimental rats that responded to a high dose of AELM (800 mg/kg), showcasing normal aortic architecture with clear nuclear staining in both endocardial cells and smooth muscles. (e) Shows the aortas of experimental rats treated with Atorvastatin (20 mg/kg), displaying a normal aortic structure with nuclear staining observed in a few endocardial cells and smooth muscles.

4. Discussion

Cardiovascular Diseases (CVDs) are a major contributor to impairment and the world's leading cause of death. These conditions include stroke and Ischemic Heart Disease (IHD). Atherosclerosis, a complex condition, exhibits characteristics resembling a persistent inflammatory process. The consumption of a diet rich in fat results in severe hypercholesterolemia and characteristics of the metabolic syndrome, particularly obesity, elevated triglycerides, and heightened insulin levels. These characteristics disrupt normal immune regulation, making it a common experimental model known as the diet rich in fat-induced atherosclerosis model for evaluating the cardioprotective potential of interventions.

The study highlighted the growing concern over CVDs due to lifestyle changes and the side effects of available antihyperlipidemic medications, which can exacerbate the condition.

Numerous plant-derived compounds have demonstrated significant medicinal value in global healthcare systems. In an initial phytochemical analysis, AELM exhibited the presence of alkaloids, tannins, flavonoids, proteins, amino acids, and phenols. Further GC-MS analysis identified seven compounds, with Leucine being notably abundant as an essential amino acid. *Lemna minor* Linn., a monocotyledonous herbaceous plant from the Araceae family, is rich in Leucine. Leucine can influence macrophage cell activity, foam cell formation, and lipid metabolism, playing roles in both proatherogenic and antiatherogenic effects[20]. Acute oral toxicity studies indicated AELM's safety at 2000 mg/kg.

Based on these observations, two distinct doses, precisely 300 mg/kg and 800 mg/kg, were identified for further preclinical investigation of each extract.

In this study, monitoring body weight changes proved valuable for assessing disease progression, treatment effectiveness, and medication side effects. When Wistar rats were subjected to a diet rich in fat, the group under positive control consistently exhibited significant weight gain contrary to the normal group. However, the high-dose AELM treatment (800 mg/kg) led to a substantial reduction in body weight compared to all other experimental groups. Importantly, when the control group and the experiment group were contrasted, there was evident no notable variations in the weights of vital organs like the liver, heart, and kidneys. These results highlight AELM's potential to counteract high-fat diet-induced weight gain without adversely affecting organ weights.

Lipid metabolism in a healthy system involves continuous fat synthesis and breakdown. Cholesterol, crucial for building biological membranes and creating compounds like bile acids and hormones, is linked to an elevated risk of coronary heart diseases when present in excess[32]. In the serum, the high-fat diet group exhibited notably higher Triglycerides (TG), Free Fatty Acids (FFA), and Total Cholesterol (TC) values compared to the normal group. Remarkably, the high-dose AELM treatment (800 mg/kg) effectively lowered when compared with the group on a high-fat diet alone in terms of TC, TG, and FFA levels.

In dyslipidemia, oxidised Low-Density Lipoprotein (LDL) accumulates on artery walls, which is a vital indicator of risk for coronary artery conditions such as atherosclerosis. Managing or mitigating dyslipidemia requires addressing oxidative stress, as elevated development of atherogenesis is greatly impacted by oxidative stress[33]. Findings indicate unambiguously suggested a robust positive association between blood oxidant extents and atherogenic lipoprotein levels in patients with cardiovascular disorders[34].

The investigation assessed the amounts of lipoproteins, such as Very-Low-Density Lipoprotein cholesterol

(VLDLc), Low-Density Lipoprotein cholesterol (LDLc), and High-Density Lipoprotein cholesterol (HDLc), in the control and experimental groups. In the high-fat diet group, HDLc levels decreased, a known risk factor for atherosclerosis. However, both high-dose AELM and Atorvastatin treatment groups showed significant increases in HDLc levels, suggesting potential atherosclerosis prevention. Additionally, the high-fat diet group showed elevated VLDLc levels, but a high dose of AELM treatment (800 mg/kg) effectively reduced VLDLc levels, highlighting its ability to address atherosclerosis-related lipid imbalances.

Leucine, found in *Lemna minor* Linn., demonstrates an antiatherogenic effect by inhibiting macrophage lipid accumulation. This outcome is primarily achieved through a dose-dependent decrease in macrophage triglycerides and cholesterol levels, primarily attributed to reduced uptake of Very Low-Density Lipoprotein (VLDL), which is abundant in triglycerides[35]. Furthermore, leucine acts to suppress triglyceride synthesis by inhibiting diacylglycerol acyltransferase-1 (DGAT1), an essential enzyme involved in this metabolic pathway. By downregulating DGAT1, leucine interferes with the final step of triglyceride synthesis, ultimately mitigating lipid accumulation within macrophages and contributing to its antiatherogenic properties. Moreover, leucine curtails cholesterol synthesis while promoting macrophage cholesterol efflux. This action is mediated by the downregulation of ATP-binding cassette transporters G5 (ABCG5) and G8 (ABCG8), crucial for hepatic cholesterol export into bile[36]. Remarkably, *Lemna minor* Linn. contains leucine, and the augmented ATP synthesis and macrophage mitochondrial respiration elucidate this mechanism distinctly[37].

While the diet with a high-fat content group was contrasted to the normal group, the atherogenic index in the serum was significantly greater. However, bothv the high-dose AELM (800 mg/kg) and Atorvastatin treatment groups exhibited significantly lower atherogenic index values than the high-fat diet group. These findings underscore the potential of AELM in reducing atherogenic risk factors in the context of atherosclerosis.

The assessment of lactate dehydrogenase activity in the bloodstream serves as a sensitive diagnostic marker for specific cardiovascular conditions, particularly myocardial infarction. AST levels are diagnostic indicators for myocardial infarction, and during pathological states, catalysts such as creatine phosphokinase, lactate dehydrogenase, aspartate transaminase, and alanine transaminase are enzymes released from damaged cardiac cells into the bloodstream, collectively serving as significant markers for cardiac injury. CPK, a key enzyme in cellular energy metabolism, plays a vital role in maintaining phosphorylation increases intracellular ATP levels and assists in assessing atherosclerosis[38]. AST, ALT, CPK, and LDH activities have significantly decreased, indicating the beneficial role of *Lemna minor Linn.* Additionally, the leucine content in AELM mitigates atorvastatin's muscle damage side effects by maintaining the skeletal muscle's defence system against the development of Reactive Oxygen Species (ROS) and oxidative damage[39].

The levels of total protein, collagen, HMG CoA, and calcium in the livers of both experimental and control groups were assessed (Table 9). The high-fat diet group's parameters appeared prominently greater compared to those of the normal group, although they were also substantially lower than those of the high-dose AELM therapy group (800 mg/kg) in comparison with the high-fat diet group.

HMG-CoA reductase activity, a pivotal enzyme in cholesterol synthesis, was indirectly evaluated by assessing the HMG CoA to mevalonate ratio in liver tissue. Inhibition of this enzyme offers a strategy to lower cholesterol levels and prevent atherosclerotic vascular disease[40]. High-dose AELM (800 mg/kg) demonstrated a significant decrease in the HMG CoA/mevalonate ratio, highlighting its hypolipidemic properties. AELM may competitively inhibit HMG-CoA reductase by activating the mammalian Target of the Rapamycin (mTOR) pathway, a protein kinase that regulates various cellular processes including growth, proliferation, and metabolism, thereby contributing to reduced cholesterol synthesis. This underscores the critical role of HMG-CoA reductase in cholesterol homeostasis and its potential as a therapeutic target for managing dyslipidemia and atherosclerosis[41].

Molecular docking of Leucine with atherosclerosis-associated enzymes (PDB ID 3E7G and 2XYG, NO synthase and caspase-3) resulted in binding energies of -7.5 and -8.6 kcal/mol, signifying Leucine's promising antiatherosclerotic potential. High-fat diet-induced rat aorta histopathology revealed dilation, congestion, oedema, and fibrosis in the endocardial cells and smooth muscles. Treated groups exhibited normal aortas with nuclear staining, albeit some displayed endocardial cell inflammation. Further research is imperative to

unveil the mechanisms behind *Lemna minor* Linn.'s antiatherosclerotic effects and explore potential therapeutic strategies for atherosclerosis.

5. Conclusion

In conclusion, cardiovascular diseases, notably atherosclerosis, remain a global health concern, with lifestyle-related factors and medication side effects exacerbating the issue. The study highlights the potential of *Lemna minor* Linn., particularly its rich Leucine content, as an antiatherosclerotic agent. The research demonstrates that high-dose AELM treatment (800 mg/kg) effectively counteracts weight gain, dyslipidemia, atherogenic risk factors, and oxidative stress, indicating its cardioprotective properties. Moreover, molecular docking analyses suggest that Leucine can mitigate atherosclerosis. Overall, this study underscores the importance of further research into *Lemna minor* Linn. as a potential therapeutic intervention for atherosclerosis and related cardiovascular conditions, offering hope for improved treatment strategies. Further research is warranted to understand its mechanisms and validate clinical utility in managing atherosclerotic vascular disease.

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