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AMELIORATION OF INFLAMMATION AND CARDIOPROTECTIVE EFFECT OF *TERMINALIA ARJUNA* BARK EXTRACT IN HIGH-FAT DIET RAT MODEL

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Keywords:

ADIPOQ gene, High fat diet, MMP-9, Rats, *Terminalia arjuna*, TNF-α immunohistochemistry

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ABSTRACT: The objective of this study is to investigate the protective effect of aqueous bark extract of *Terminalia arjuna* (TA) on high-fat diet-induced cardiovascular changes in Wistar rats using histological, immunohistochemical, and gene expression analysis. Group 1- without treatment served as control, group 2- High fat diet (HFD) received corn oil orally at a dose of 10 mL/kg, group 3 - HFD+atorvastatin (ATV) - 10 mg/kg, group 4 - HFD+TA125 mg/kg , and group 5- HFD+TA250 mg/kg, 6 days a week for 5 weeks. The activity of MMP-9 was measured in blood, Hematoxylin and Eosin staining was analyzed with heart tissue and TNF-α immunohistochemistry and *ADIPOQ* gene expression analysis were conducted using heart tissue. When Compared to the control group, the HFD group showed higher levels of MMP-9 activity and morphological changes in heart histology. TNF-α expression was also significantly increased in HFD group and down-regulation of *ADIPOQ* gene expression levels. MMP-9 activity and pathological damage to cardiac tissue including *ADIPOQ* gene expression were significantly reverted when *T. arjuna* bark extract was administered along with a high-fat diet. The findings reveal that *T. arjuna* bark has protective against HFD-induced cardiovascular alterations.

INTRODUCTION: Heart disease remains the predominant cause of mortality in the United States as well as worldwide. Even in European nations, cardiovascular disease (CVD) and stroke continue to be the leading causes of death, with coronary heart disease (CHD) and stroke accounting for maximum deaths [1]. It was reported that the accumulation of dietary Trans fatty acids (TFA) in plasma and various tissues can trigger inflammation and oxidative stress [2].

Hyperlipidaemia increases the production of peroxynitrite in the heart, resulting in myocardial dysfunction [3]. MMPs (matrix metalloproteinases) are a group of zinc-dependent endoproteases that are involved in tissue remodelling as well as

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protein degradation in extracellular matrix (ECM) [4]. MMPs are indicators of atherosclerotic plaque instability CHD. The future risk of both advanced cardiovascular disease (CAD) and acute coronary syndrome (ACS) could be predicted based on the increased levels of MMPs, indicating the value of MMPs as a prognostic tool and key biomarker of cardiovascular disease [5]. Circulating MMP-9 has been reported as a promising biomarker to predict atherosclerotic plaques, plaque instability as well as CVD [5-7]. Statins have pleiotropic effects in vivo, such as influencing signalling systems that inhibit MMPs [8]. The peripheral levels of MMP-2 and MMP-9 can be used as indicators of heart failure and for identifying individuals who would benefit from a specific therapeutic intervention involving the MMP pathway [9]. Findings reveal that individuals with hyperlipidaemia produce more reactive oxygen species (ROS) [10].

This could be due to a fat-rich diet increasing the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase genes [11], which is the primary enzyme responsible for ROS synthesis [12] resulting in increased ROS formation in cells [13]. Low-density lipoprotein (LDL) oxidation is triggered by the presence of ROS, which activates inflammatory cytokines, mediators and chemokines produced by invading resident macrophages. Both ROS and inflammation play a role in the onset and progression of CVD [14]. Atherosclerosis is considered as an inflammatory illness rather than a result of hyperlipidemia [15].

Recent research has suggested that immune response has a role in the aetiology of atherosclerosis, as evidenced by statins' anti-inflammatory action in the therapy of atherosclerosis [16]. The role of TNF-α in the affected heart tissue support both cardioprotective mechanisms and pathogenesis [17]. Despite the therapeutic benefits seen during targeting of TNF-α pathway in heart disease, it was discerned that a more specific and selective mechanism of TNF-α is required to reduce the disease pathogenesis. The current cholesterol-lowering drugs used to manage CVD pose several side effects which include toxicity and muscle-related problems [18, 19]. The risks associated with statins are myopathy, incidence of diabetes, hemorrhagic stroke, memory and cognitive impairment, hepatotoxicity renal toxicity and cataract [20-22]. There is a need for safe and viable therapeutic approach to combat CVD and associated problems. Many pivotal works have shown the importance and value of herbal medicines in treating CVD problems. Doxycycline induced shorter epicardium, decreased myocardial density with hyalinization and vacuolated swelling endocardium are signs of myofibril or functional cardiomyocyte loss and are responsible for left ventricular failure. Co-treatment with TAAqE (*Terminalia arjuna* aqueous extract) prevented the structural alterations. TAAqE appears to protect against Doxycycline-induced LV dysfunction[23]. *Terminalia arjuna* extract has cardioprotective properties, as evidenced by its ability to reduce heart damage induced by Isoproterenol (ISO), oxidative stress, apoptosis, and restore antioxidant state.

The scavenging of free radicals produced by Isoproterenol-induced stress is thought to be the mechanism of *Terminalia arjuna* extract cardio-protection. Cardioprotective and antioxidant properties of *Terminalia arjuna* extract could be seen through its ability to reduce heart damage induced by ISO, involving oxidative stress and apoptosis [24]. In this study, *T. arjuna* rendered cardiac protection by scavenging the free radicals generated by ISO. The cardioprotective effect of Alpinia zerumbet, a natural product with potential antioxidant action in the myocardium [25], shows that there are many avenues for detailed research exploration of molecular mechanisms exhibited by those potential phytochemical drugs.

The bark of *Terminalia arjuna*, a deciduous tree, has long been thought to be therapeutic for cardiac diseases in Indian medicine [26, 27]. Many ayurvedic remedies labelled as cardiotonics have it as an essential ingredient [28]. The increased understanding of inflammatory events in atherosclerosis provides a molecular framework for comprehending the therapeutic benefits of medications like statins and *T. arjuna*, which also have immunomodulatory properties [29]. *T. arjuna* extract increased endogenous antioxidant molecules in the rat heart to protect the myocardium from isoproterenol-induced myocardial ischemia-reperfusion injury [30]. Studies support the anti-diabetic properties of TA extract and *Emblica officinalis* aqueous stem bark extracts [31]. By reducing the expression of pro-

inflammatory cytokines and chemokines, decreasing oxidative stress, enhancing plasma zinc levels and gut microbiota structure, TA extract treatment alleviated disease activity in Trinitrobenzene sulfonic acid (TNBS) induced colitis [32]. Chronic high-fat diet (HFD) leads to disease development and decrease in cardiac adiponectin gene expression. Reduced cardiac *ADIPOQ* causes oxidative stress and inflammation, which leads to heart hypertrophy and reduced cardiac function [33]. A study reported the therapeutic role of *Moringa oleifera* ethanolic extract in the management of obesity and elimination of cardiometabolic abnormalities in female rats caused by high cholesterol diet. *M. oleifera* extract improved mRNA expression of leptin, resistin and *ADIPOQ* genes and functioned directly on visceral fat mass [34]. Intervention with TA 500 mg/kg could be a good substitute for NAC (N-acetyl cysteine) in rendering protection from APAP-induced (acetaminophen) hepatotoxicity [35]. Although evidence supports the traditional use of TA bark extract for cardio-protection, there is a paucity of animal studies to support the mechanism and cardioprotective benefits of this drug in a high-fat diet model. The aim of to investigate the protective effect of bark extract of *T. arjuna* on high fat diet induced cardiovascular alterations using immunohistochemical and gene expression analysis in rats.

MATERIALS & METHODS:

Animals: Wistar male rats weighing 180–280 g were used for this study. The rats were housed in polypropylene cages (3 rats per cage) with sterile paddy husk for a week to acclimatize to the laboratory environment (25±2ºC, 40-60% humidity and natural light/dark cycle). The rats were fed a pellet meal (Biogen, Bangalore) and given filtered water while being observed at the Centre for Laboratory and Animal Research (CLAR), Saveetha Institute of Medical and Technical Sciences, Chennai. The bedding husk material was changed on a regular basis to maintain a hygienic environment.

Ethics: The Protocol was approved by the Saveetha Medical College Institutional Animal Ethics Committee (SU/CLAR/RD/005/2019 Dated August 09, 2019). Animals were housed and maintained according to the standards of the "Committee for the Purpose of Control and Supervision of Experiments on Animals" (India).

Experimental Groups: Corn oil was given orally at a dose of 10 mL/kg six times a week as a high-fat diet for five weeks to induce heart damage to induce cardiac effects. A low dose of *T. arjuna* aqueous bark extract was orally administered at (125 mg/kg) six times a week for five weeks, and a high dose (250 mg/kg) of *T. arjuna* aqueous bark extract was orally administered six times a week for five weeks. The therapeutic doses were given 30 minutes after the corn oil was administered. The compounds were suspended in 2% DMSO for the administration of atorvastatin. Five groups of animals were used (6 rats each). The rats in Group 1 were used as a control group. For them, instead of corn oil, water was administered orally. The rats in Group 2 were provided with a high-fat diet. The groups from 3 to 5 were given a high-fat diet and atorvastatin (10 mg/kg), aqueous bark extract of *T. arjuna* (125 mg/kg) and aqueous extract of *T. arjuna* (250 mg/kg) respectively.

Sample Preparation: Twenty four hours after the last dose, the animals were anesthetized with isofluorane, the blood was collected in vacutainer tubes without anticoagulants from retroorbital puncture. It was allowed to coagulate for 20 minutes before being centrifuged at 3000 rpm at 4°C to separate the serum. The serum was separated and stored at -80°C. The animals were sacrificed by cervical dislocation and a vertical incision was made to access the thoracic cavity. The heart was taken out. The heart was gently cleaned in cold saline, blotted with a Whatmann no.1. filter paper and kept at -80°C for biochemical analysis in sterile plastic vials. The cardiac tissues were also preserved in 10% formalin solution for tissue morphological examinations.

***Terminalia arjuna* Stem Bark Aqueous Extract Preparation:** Herbal Care and Cure Centre (Chennai, India) provided the *Terminalia arjuna* bark powder. Two litres of distilled water was added to 1 kg fine powder of TA bark in a conical flask. It was boiled for 30 min and allowed to cool slowly. After cooling, it was thoroughly mixed at 37ºC by shaking continuously at 200 rpm. The contents were filtered using a muslin cloth. In a centrifugal evaporator, the filtered extract was

dried under reduced pressure at 40°C and refrigerated at 4°C for later use. Approximately 18 % of the yield was obtained. High Performance Thin Layer Chromatography plates were prepared in an optimal solvent system, dried in the air, then scanned at 254 nm with a CAMAG TLC scanner 3. To obtain the sample, the standard stock solution was prepared in HPLC grade methanol. The TA bark extract was dissolved in methanol and sonicated for 10 minutes before being diluted to a final volume of 5 mL. The required concentration of the samples was obtained by dilution of the stock solution. The Phenolic compounds, Flavonoids, Glycosides, Saponin and tannin present in *T. arjuna* were determined by HPTLC methods. IJPER Journal reviewed our paper (Data unpublished).

Biochemical Analysis: The MMP-9 kit was purchased from Ray Biotech Life Inc, (Peachtree Corners, Georgia). The MMP-9 activity was read at 450 nm using a microplate reader (MINDRAY model, India).

TNF-α Immunohistochemistry Study: TNF-*α* levels of cardiac tissues were determined by immunohistochemical analysis after the tissues were fixed in fixative for 4 hr and then cryoprocessed with 30% sucrose. The tissues were sectioned into 3-5 µm thick sections, which were then placed in an anti-freeze buffer. After that, the sections were treated with 1 % fixed tissue of H_2O_2 to quench the endogenous peroxidase activity, followed by blocking the nonspecific area by a 5% blocking buffer. After blocking, the sections were incubated overnight at 4°C with an anti-TNF-α antibody (1:1000). The sections were washed to remove the non-specific binding and incubated for 2 hr with a biotinylated secondary antibody (1:500). To visualise the immunopositive cells, the tissues in the slides were stained with diaminobenzidine. Under an inverted microscope, TNF-α immune positive regions were identified, and the acquired images were quantified using Image-J software.

Haematoxylin & Eosin Staining: Paraffin sectioning was used for histopathological evaluation and light microscopic examination of tissues. The tissues were then hydrated and dehydrated in a series of graded alcohols. It was then cleaned with Xylene and chloroform and then fixed in paraffin wax for a rotary microtome. Tissue sections (10µm) were then removed and stored overnight at room temperature. After that, the sections were deparaffinized and moistened with descending alcohol concentrations followed by distilled water. Using haematoxylin and eosin stain, the sections were stained and then washed with ascending concentration of alcohol. Permanent slide was prepared using a DPX mount. The slides were observed under a light microscope (40x) (Olympus microscope) and photomicrographs were taken using a digital camera (Sony RX100M3).

***ADIPOQ* Gene Expression Analysis:** Total RNA was extracted from tissue according to the directions provided by the RNA isolation kit obtained from Invitrogen by Thermo Fisher Scientific. A guanidium isothiocyanate/phenol-chloroform single step extraction kit purchased from Invitrogen by Thermo Fisher Scientific was used to extract total RNA from the tissues. Furthermore, each RNA sample's quality was confirmed using agarose-formaldehyde electrophoresis. RNA samples were kept at -80°C until they were analysed. Electrophoresis on a 1.5 % agarose gel containing ethidium bromide was used to detect polymerase chain reaction (PCR) products. A standard size marker, 100-bpladder (Applied Biosystems, Foster City, CA, USA), was used to confirm the location of the predicted product. After that, the gel was imaged using UV transillumination. A video image analysis system was used to measure the intensity of PCR products (Kodak Digital Science). The signal for each transcript was compared to the signal for -actin mRNA in each sample, and the results were reported as a ratio of the transcript to -actin mRNA.

Statistical Analysis: All values are displayed with mean± standard error. ANOVA was used to test for significance of data of the different groups with Bonforani "t" test for multiple comparison. The significance level was set to $P < 0.05$. For statistical analysis and graph plotting, SigmaPlot 14.5 (Systat Software Inc, USA) was utilized.

RESULTS:

Effect of TA on HFD Induced serum MMP 9 Expression Levels: The MMP 9 levels (mean ± SE) of control, HFD, HFD±ATV, HFD±TA125,

and HFD±TA250 groups were 123.05 ± 2.07, 344.66 ± 22.48, 165.6 ± 14.56, 197.33 ± 8.34 and 152.2 ± 17.72 ng/mL respectively. The MMP 9 levels of HFD group were 2.1 fold higher than the control. Also, the MMP 9 levels were higher than the reference range of rat species. However, in HFD+TA-treated groups, the MMP 9 levels were reduced as compared with HFD group **Fig. 1.**

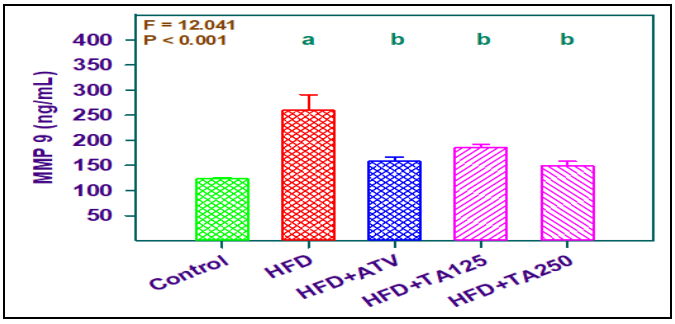


FIG. 1: EFFECT OF ATORVASTATIN (ATV), AND TEST DRUG *TERMINALIA ARJUNA* AQUEOUS BARK EXTRACT (TA) 125 AND 250 MG/KG ON SERUM MMP 9 EXPRESSION IN HIGH-FAT DIET (HFD) INDUCED RATS. [a]Significantly different from control group. [b]Significantly different from hfd group

In comparison with the HFD group, there was 1.6, 1.4, and 1.7 fold decrease in MMP 9 levels in HFD+ATV, HFD+TA 125 and HFD+TA 250 groups. The drug treatment groups showed almost similar results. Although TA250 showed a marginally better effect than TA125 and ATV, the difference was not statistically significant.

Effect of TA on HFD Induced TNF-α changes: The TNF-α positive cells (Mean±SE) of control, HFD, HFD ± ATV, HFD ± TA ± 125, and HFD ± TA250 were 3.31±0.48, 6.96±1.55, 6.22±1.97, 9.26±0.52 and 4.65±1.36 respectively. The TNF-α positive cells of the HFD group increased by 20-fold compared with the control. However, in HFD± TA treated groups, a decrease in the numbers of TNF-α positive cells were seen in **Fig. 2** as compared to HFD group. Compared to the HFD group, there was 3, 2 and 4-fold decrease in the TNF-α positive levels in HFD±ATV, HFD±TA 125 and HFD±TA250 groups, respectively.

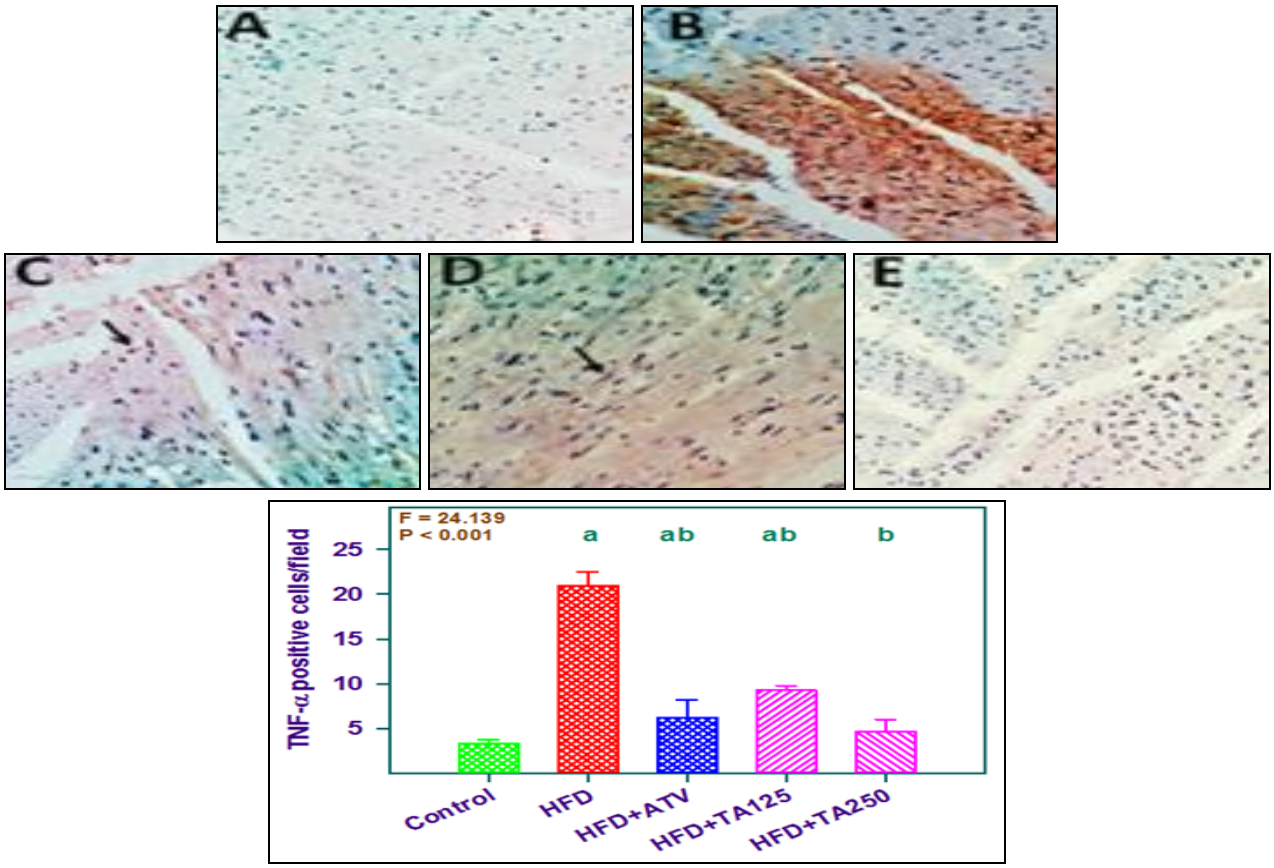


FIG. 2: REPRESENTATIVE IMAGES OF IMMUNOHISTOCHEMISTRY ANALYSIS FOR TNF-ALPHA. HEART TISSUE STAINED WITH DIAMINOBENZIDINE. X100. FIG. 2A. CONTROL, FIG. 2B. HFD, FIG. C. HFD + STANDARD DRUG, ATORVASTATIN. AND FIG. 2D. HFD + TA 125, FIG. 2E. HFD + TA 250. THE INTENSITY OF THE IMMUNOPOSITIVE SIGNAL IS AS FOLLOWS -HFD>TA125>ATV>TA250>CONTROL (REPRESENTATIVE BAR GRAPH). [a]Significantly different from control group. [b]Significantly different from hfd group.

Fig. 2 shows sections stained with diaminobenzidine, showing areas of immunoreactivity for TNF-alpha in heart tissue. Dark brown colour indicates expression of TNF-α positive cells. Samples from the control group did not show any marked areas of TNF-alpha positive expression **in Fig. 2A,** whereas immunostaining intensity was higher in high fat fed group **Fig. 2B**. Reduced TNF--α positive cells were seen in HFD+ATV **Fig. 3C**, HFD+TA125 **Fig. 3D** and HFD+TA250 **Fig. 3E** groups.

Effect of TA on Heart ADIPOQ Gene Expression Levels in HFD Induced Rats: The adiponectin gene expression of (mean±SE) of control, HFD.HFD±ATV and HFD±TA250, were 0.57±0.02, 0.19±0.02, 0.41±0.02 and 0.53±0.01 respectively. **Fig**. **3A** and **3B**.

The adiponectin gene expression of HFD group decreased by 5 fold compared with the control.

However, in HFD± TA treated groups, the decrease in the gene expression was 5 fold less than the HFD group, in comparison with HFD group, HFD±ATV and HFD±TA250 groups showed 4 and 5-fold decrease in the gene expression levels, respectively.

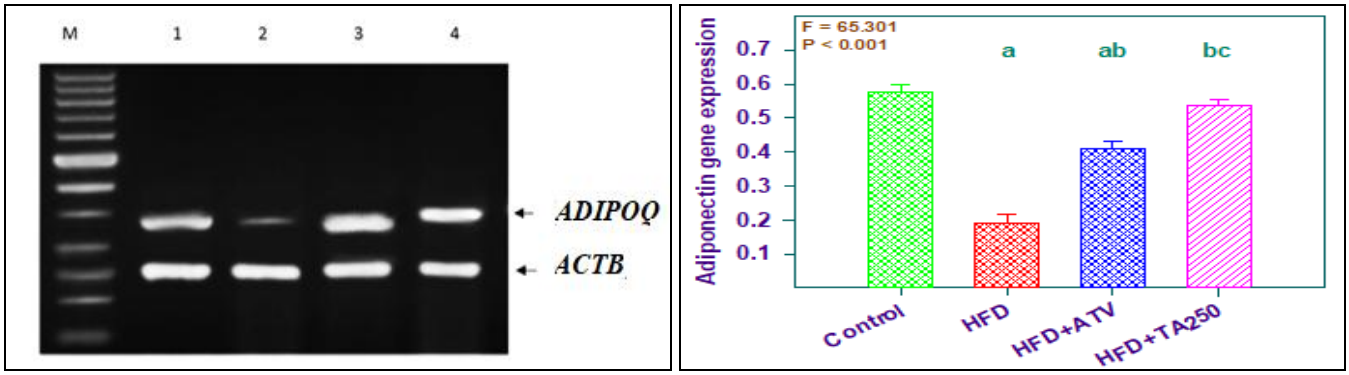
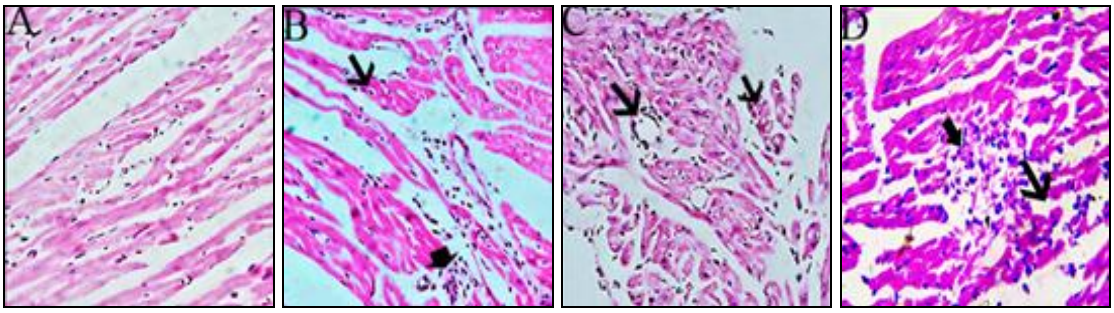


FIG. 3: GENE EXPRESSION STUDIES OF ADIPONECTIN (*ADIPOQ) BY* RT-PCR. THE HOUSE KEEPING GENE BETA-ACTIN (*ACTB*) WAS USED AS A LOADING CONTROL. LANE M- MARKER LANE (100-1000 BP); LANE 1 – CONTROL; LANE 2 –HFD GROUP; LANE 3 –HFD + STANDARD DRUG ATORVASTATIN TREATED; LANE 4 – HFD + TA 250 TREATED. THE REPRESENTATIVE BAR GRAPH BOTTOM FIG. 3B: DEPICTS THE VALUES OF FOLD CHANGE IN VARIOUS GROUPS.

Effect of TA on HFD Induced Histopathology Changes of Heart Tissue: Fig. 4 depicts the H&E stained heart tissues of control, HFD and drug-treated groups. Samples from the control group showed normal histology of cardiac myocardium **Fig. 4A**. Whereas in HFD induced group **Fig. 4B, C** & **D** the heart tissue section showed mononuclear cells (thin arrow) and neutrophil infiltration (arrow head) at the infarct site (**Fig. 4B**). In **Fig. 4B** the heart tissue section showed inflammation (long arrow), neutrophil infiltration (broad arrow). Moreover, in HFD group **Fig. 4D**, the heart tissue architecture was disrupted (long thin arrow) with necrosis of myocardial cells (thick broad arrow). Atorvastatin-treated rats showed less myofibrillar loss, reduced inflammatory cell infiltration and very little interstitial collagen fibres (arrows), as indicated in **Fig. 4E** and **Fig. 4F**. Meanwhile, treatment with *T. arjuna* extract (TA 125 and 250 mg/kg) showed minimal changes in the myocardial cells with few neutrophils (thick arrows) and absence of inflammatory infiltrates **Fig. 4G** and **Fig. 4H** respectively.



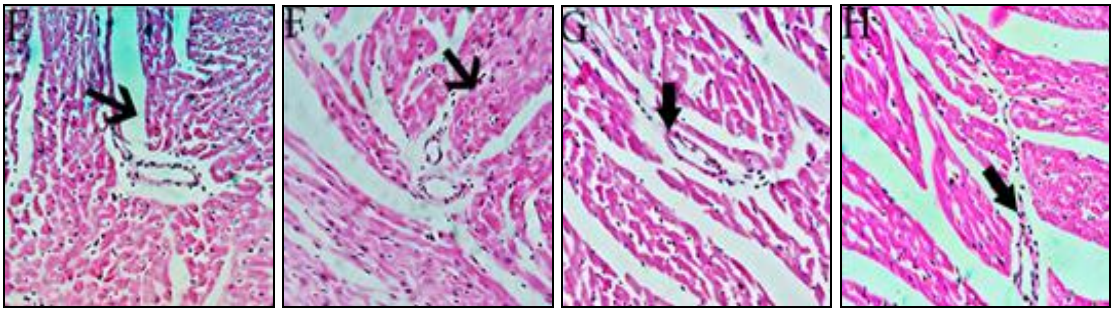


FIG. 4: REPRESENTATIVE IMAGES OF HISTOPATHOLOGICAL ANALYSIS OF HEART TISSUE STAINED WITH HEMATOXYLIN AND EOSIN CAPTURED AT X40 MAGNIFICATION. SECTION-A (CONTROL), SECTIONS – B, C & D (HFD FED), SECTION-E&F (HFD + STANDARD DRUG ATORVASTATIN TREATED) AND SECTION-G (HFD + TA 125 TREATED), SECTION-H (HFD + TA 250 TREATED)

DISCUSSION: Matrix metalloproteinase-9 (MMP-9) is critical in inflammation and fibrosis in cardiovascular disease [36]. High level and activity of MMP-9 has been linked to various cardiovascular disorders. This is because MMP-9 breaks type IV collagen, a major basal membrane constituent of vascular smooth muscle cells and endothelium.

MMP-9 retains a vital role in cell migration and infiltration in atherosclerosis. Furthermore, MMP-9's degradation of elastin protein has been associated with aneurysm as well as arterial wall stiffness, which can increase the risk of hypertensive crisis [38]. The present study shows that TA (250 mg/kg) high dose lowered MMP-9 levels more than ATV treatment, there by suggesting decreased risk of cardiovascular complications and disorders.

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine made by macrophages and monocytes. This protein is involved in a multitude of cell signalling events that can lead to necrosis or apoptosis [39]. TNF-α has been shown to play an important role in vascular dysfunction and constrict large blood vessels of the heart [40]. TNF-α gene polymorphism can induce hypertension [41].

Additionally, TNF-α has been shown to have cardio depressant effects on cardiac myocytes. By decreasing sarcoplasmic reticulum Ca^{2+} uptake as well as myofilament Ca^{2+} sensitivity, TNF-α can reduce cardiac contractility, thus decreasing cardiac output and tissue oxygen delivery [41, 42]. Inactivation of TNF-α can prevent progression of atherosclerotic lesions and improve heart failure patients' health [41]. In comparison to the sepsis group, the levels of inflammatory cytokines were significantly lower in the myocardial tissue of mice treated with sophoridine 20mg/kg, i.p. alone or in combination with dexmedetomidine 25μg/kg, i.p against lipopolysaccharides induced myocardial dysfunction [43]. The current study has substantiated that TA extract treatment can decrease TNF-α expression in heart tissue, similar to ATV.

ADIPOQ is an adipokine produced from adipose tissue. While it is usually present at 30 μg/mL in plasma, significantly decreased levels were observed in coronary artery disease [44]. Previous studies have shown that *ADIPOQ* exhibits cardioprotective effects through inhibition of inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. In addition, previous studies have demonstrated the *ADIPOQ* gene's ability to inhibit cardiomyocyte remodelling by reactive oxygen species [45].

The present study shows a statistically significant down-regulation of *ADIPOQ* gene expression in HFD rats and a statistically significant up-regulation of *ADIPOQ* gene expression in ATV and TA (250 mg/kg) treatments. This increase in *ADIPOQ* gene expression by the TA (250 mg/kg) treatment suggests that TA may play a role in cardio-protection.

Therapeutic intervention with *Terminalia arjuna* ethanol extract or *Terminalia arjuna* aqueous extract at 250 mg/kg body weight showed considerable cardio-protection in isoproterenol-induced myocardial infarction in rats to biochemical and histological data [46]. *Terminalia arjuna* extract may have protected myocytes from apoptosis and necrosis [47]. The histology evaluation showed minimal changes in the myocardial cells with few neutrophils and absence of inflammatory

cells in TA treated HFD-induced rats. Treatment with TA extract showed cellular organization of hepatocytes with reduced necrosis [48]. *T. arjuna* extract has also protected renal damage in hyperlipidemic rats [49]. Therefore, our results illustrate that the *Terminalia arjuna* bark extract can reduce the histological damage caused due to HFD.

CONCLUSION: In conclusion, the current investigation revealed that *T. arjuna* aqueous bark extract has therapeutic potential to ameliorate high fat diet-induced cardiovascular alterations and pathological changes.

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CONFLICTS OF INTEREST: There is no conflict of interest to disclose.

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