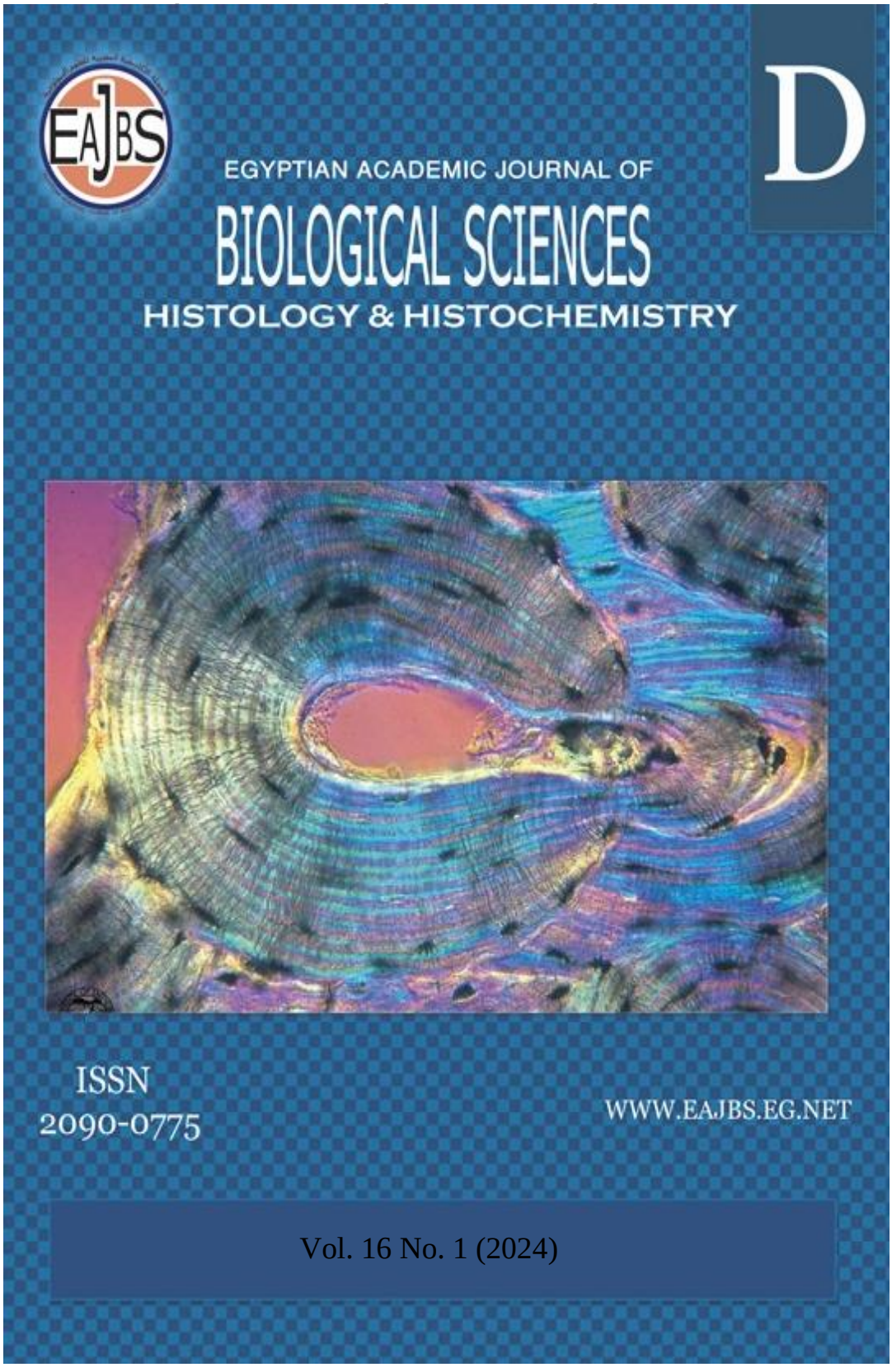

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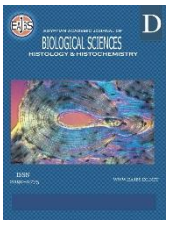

Prophylactic Effect of Combined Chia Seeds with Flaxseed Oil on Induced Aortic Atherosclerosis in A Rat Model

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ABSTRACT

Atherosclerosis is a leading cause of death due to cardiovascular diseases worldwide. Accumulation of lipids in the big arteries triggers inflammatory markers, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, that by turn induce endothelial injury. It is mandatory to find a prophylactic medication for this disease, especially from natural resources to avoid the side effects of drugs. Chia seeds and Flaxseed oil are natural herbal, each separate administration showed good results in ameliorating hyperlipidemia and reducing atherosclerosis inflammatory response. The combination of those two natural resources could have a synergistic prophylactic effect and better lessening of atherosclerosis and it is the aim of this study, to investigate the potential prophylactic effect of a combination of Chia seeds and Flaxseed oil on a rat model with induced atherosclerosis through high-fat diet. Using chemical and immunohistochemical techniques to explore the effect of this combination on serum lipid parameters level and to study the protein expression of inflammatory markers; IL6 and TNFα in aortic tissue with or without the administration of Chia seed and Flaxseed oil each separate or combined. Significant changes in the lipid profile and inflammatory markers were demonstrated in the combined group, where it improved lipid parameters, body weight, and mean arterial blood pressure, it also ameliorated aortic inflammation compared to Chia seeds or Flaxseed oil alone. This could be guidance to use Chia seeds combined with Flaxseed oil as prophylaxis against atherosclerosis to limit its unfavorable effect.

INTRODUCTION

There are over five million patients suffering from chronic heart disease and acute myocardial infarction due to inadequate treatment (Roger *et al.*, 2012). Atherosclerosis is a predisposing factor for cardiovascular diseases, such as ischemic heart disease and stroke (Hansson, 2005). Atherosclerosis disease is a leading cause of death worldwide (Weber & Noels, 2011). It is a progressive disease characterized by the accumulation of lipids in large arterial walls, like the aorta, coronary and cerebral arteries.

In early lesions of atherosclerosis, sub-endothelial deposition of macrophages occurs, which are the inducers of the atherosclerotic lesion through the accumulation of lipid-rich necrotic debris (Gerald *et al.*, 1992; Ridker *et al.*, 2009). In an advanced stage, lesions can spread more to form a thrombus or blot clot and block the blood flow, leading to myocardial infarction or stroke (Rodriguez-Granillo *et al.*, 2007). Many studies demonstrated the central role of the endothelium in mediating inflammation. Sub-endothelial accumulation of low-density lipoproteins (LDL) in the wall of blood vessels triggers monocyte recruitment and promotes the proliferation of smooth muscle cells (SMC) (Williams & Tabas, 1995; Kraehling *et al.*, 2016).

Inflammatory cytokines play a central role in the pathogenesis of atherosclerosis (Kleemann *et al.*, 2008; Ait-Oufella *et al.*, 2011; McLaren *et al.*, 2011), LDL induces expression of inflammatory cytokines in the vascular system (Witztum & Berliner, 1998; Dichtl *et al.*, 1999). In obesity, cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 accelerate atherosclerosis (Yudkin *et al.*, 1999). Macrophages secrete TNF-α to activate endothelium and induce SMC proliferation (Lei & Buja, 1996). It engaged in the pathogenesis of atherosclerosis (Brånén *et al.*, 2004; Boesten *et al.*, 2005). TNF-α has a great contribution to the severity of vascular diseases, it impairs the VE-cadherin barrier and enhances leukocyte infiltration across endothelium (Angelini *et al.*, 2006; Laviola *et al.*, 2013). IL-6 is also involved in endothelial dysfunction and atherosclerosis pathogenesis (Ridker, 2016).

Flaxseed oil reduced the development of atherosclerosis in murine models by suppressing inflammatory markers (Ali *et al.*, 2017). It also attenuated the progression of aortic atherosclerotic lesions and markedly reduced atherosclerotic plaque in a rat model by reducing platelet and endothelium activation (Francis *et al.*, 2013a; Haliga *et al.*, 2013). Moreover, it has an antihyperlipidemic effect on rats with hyperlipidemia (Hanan Elimam and Basma Kamal Ramadan, 2018). Chia seed oil also has a cardioprotective role against cardiotoxic drugs (Ahmed *et al.*, 2021), it reduces serum triglycerides levels and elevates high-density lipoprotein (HDL) in rat serum (Ayerza & Coates, 2005; Santos-López *et al.*, 2018).

A combination of Flaxseed oil and α lipoic acid had anti-inflammatory, anti-lipid effects and anti-oxidative stress on rats with atherosclerosis (Xu *et al.*, 2012a). Whether the combination of Flaxseed oil and Chia seed oil has a beneficial effect on atherosclerosis has not yet been studied and needs further investigation.

Atherosclerosis with its high morbidity and mortality is a major health problem worldwide, it is an imminent and urgent need for the development of a potent prophylactic agent for atherosclerosis rather than treatment of its complications. Finding a prophylactic medication for this disease is mandatory, especially from natural resources to avoid the side effects of medications. Chia seed and Flaxseed oil are natural herbal, each separate administration showed good results in reducing atherosclerosis inflammatory response and in ameliorating hyperlipidaemia. The combination of those two natural resources could have a synergistic prophylactic effect and better lessening of atherosclerosis.

Thus, this research was designed to evaluate the potential protective effects of combined Chia seed and Flaxseed oil on induced aortic atherosclerosis rat model using biochemical and Immuno-histochemical techniques. This would introduce a novel therapy for the prevention of the development of atherosclerosis and will give a chance for clinical trials on

patients with a high risk for developing atherosclerosis and vascular diseases.

MATERIALS AND METHODS

Experimental Protocol:

Thirty male Sprague-Dawley rats had been divided into 3 groups:

1: **Negative control**: (6 rats) had been fed on a basal diet only (the feed amount per each rat for all groups had been regulated to 25 g/day and water had been supplied ad libitum), then rats had been scarified after 12 weeks (to be compared with positive control and treated groups).

2: **Positive control (atherosclerosis induced with no treatment):** (6 rats) had been fed on a high-fat diet (HFD) containing 2.0% cholesterol and 0.5% cholic acid, mixed well with a normal diet for 12 weeks to induce atherosclerosis using the method of Qian *et al* (2016) (Qian *et al.*, 2016). The rats had been housed individually at a constant temperature (20–22°C) in specific pathogen-free conditions. Rats had been sacrificed after 12 weeks (to confirm the development of atherosclerosis), and to be compared with the treated group to test the effect of therapy on atherosclerotic rats).

3: **Treated (atherosclerosis induced with treatment):** (18 rats) had been given HFD + Flaxseed oil (purchased from Imtenan, Egypt; 270 mg/Kg/day; 6 rats) (Ali *et al.*, 2017) or Chia seed oil (purchased from Imtenan, Egypt; 2.5 ml/kg/day; 6 rats) (Ahmed *et al.*, 2021) or Flaxseed oil (270 mg/Kg/day) combined with Chia seed oil (2.5 ml/kg/day; 6 rats) orally daily for 12 weeks, then they had been sacrificed.

Body Weight and Mean Blood Pressure:

Body weight, systolic (SBP) and diastolic blood pressure (DBP) were measured. Blood pressure was deliberated by a non-invasive tail-cuff method (BP-98A; Softron, Tokyo) as previously described (Kuwahara *et al.*, 1991). Animals were rested in a quiet, dark and worm cylindrical, before measuring the blood pressure. Week 0 body weight was recorded as baseline (time 0) data for each animal, then weekly till scarification. SBP and DBP were measured prior to scarification at 12 weeks. The mean arterial blood pressure (MAP) was calculated using this equation: DBP + 1/3(SBP-DBP).

Specimen Collection:

At the assigned time (after 12 weeks), blood and aortic samples were collected after sacrifice by 1ml, i.p sodium thiopental administration, and assessed for the development of atherosclerosis and the effect of the treatment on atherosclerotic rats.

Blood Lipid Analysis:

Blood was obtained from rat tail veins from all groups. Plasma serum cholesterol, triglycerides (TG), HDL, and LDL were deliberated utilizing enzymatic methods and specific kits (Catalog number; MAK043, MAK266 and MAK045, Sigma Aldrich, Massachusetts, USA). All the manufacturer's recommendations were adhered to.

Tissue Processing, Histology, and Immunohistochemistry:

Thoracic aorta samples were cut, fixed, embedded in paraffin, and prepared for hematoxylin and eosin (HE) staining and immunohistochemistry. Collagen fiber deposition in atherosclerotic lesion areas was measured using Masson trichrome staining and the Image J graphic Analysis System.

Immuno-detections were performed on IL-6 and TNF-α to investigate endothelial activation/injury and atherosclerosis development in the rat model after 12 weeks and to follow up on the effect of therapy on atherosclerotic lesions. After the rats' scarification, the thoracic aorta was removed and washed, and 10 % formalin was used for fixation. Aortic samples were cut (5m), and stained with H&E for histopathological evaluation to confirm atherosclerosis generation in the HFD group. Specimens were blocked using serum goat, and primary antibodies; IL-6 (rabbit monoclonal #12912, Cell Signaling Technology, Massachusetts, USA), and TNF-α (rabbit monoclonal #8184, Cell Signaling Technology, Massachusetts,

USA) were used for staining following the recommended concentration, it was kept in the fridge overnight, a previously described protocol was followed (Ebrahim & Leach, 2016). Phosphate buffer saline (PBS) was used for washing, then secondary antibodies for incubation (goat anti-rabbit IgG conjugated HRP, ab6721, abcam, Waltham, Massachusetts, USA) for two hours at RT. Finally, the slides were washed in PBS three times and once in distilled water, mounted, and investigated using Olympus CX 31 light microscope.

Morphological Analysis:

The average area percentage of immune-histologically brown stained IL-6 and TNF-α sectors were estimated from nonoverlapped areas of four stained sections per group, utilizing the Image J program (National Institute of Health, Bethesda, Maryland, USA). Images stained with diaminobenzidine (DAB) and haematoxylin were saved in JPEG format, at × 200 magnification. Color deconvoluting was applied to separate colors, DAB was analysed, and the threshold was used to pick the area of positive immunohistochemical stained. The area percentage was measured, and all data were introduced as means ± standard error of the mean (SEM).

Statistical Analysis:

Statistical analyses were executed using (GraphPad Prism 9; and GraphPad Software Inc.) unpaired t-test for the two groups' comparison and One-way ANOVA was selected to compare quantitative variables in more than two groups' comparison. The significance level was counted as 0.05.

RESULTS

Body Weight And Mean Blood Pressure:

Table 1 exhibited the results of body weight, where there was a significant increase in the body weight at week 12 in HFD group compared to the control, **P < 0.01, while in the mixed Chia + Flaxeed, there was a significant reduction compared to the HFD and Flaxeed oil groups, #P < 0.05.

MAP displayed significant differences among experimental groups at 12 weeks. Significantly elevated MAP value in HFD was observed compared to the control group, **** P < 0.0001. Chia seeds and Flaxseed oil separately lowered the MAP compared to HFD. While the treatment with Chia + Flaxseed managed to lower the MAP significantly compared to HFD, flaxseed oil, and Chia seeds groups, ##P < 0.01.

Table 1. Body weights and mean blood pressure.

	Negative control	Positive control	Chia seeds treated	Flaxseed oil treated	Chia + Flaxseed treated
Body weights W0	185 ± 25.9	185 ± 23.7	193.5 ± 29	195 ± 28.5	195 ± 37
Body weights W6	235 ± 29.3	262.5 ± 39.3	265 ± 33.2	270 ± 19.4	232.5 ± 37.7
Body weights W12	247.5 ± 37.4	315 ± 19.2✱✱	295 ± 48.9	300 ± 40.9	227 ± 41#
MAP W12	90.3 ± 1.6	206.6 ± 7.7✱✱✱✱	163.3 ± 4.8✱✱✱✱	179 ± 7.9✱✱✱✱	124 ± 8.7##

Control, standard diet at 0 week and for 6 and 12 weeks (n = 6); High-fat diet at 0 week and for 6 and 12 weeks (n = 6); High-fat diet with Chia seeds at 0 week and for 6 and 12 weeks (n = 6); High-fat diet with Flaxseed oil at 0 week and for 6 and 12 weeks (n = 6); High-fat diet with Chia seeds and Flaxseed oil at 0 week and for 6 and 12 weeks (n = 6). **P < 0.01 vs. control group, #P < 0.05 vs. HFD and Flaxseed oil groups. Mean blood pressure at W12 in different experimental groups, **** P < 0.0001 vs. control group, ## P < 0.01 vs. HFD, flaxseed oil and the Chia seeds groups. Data are expressed as means ± standard deviation. HFD: high fat diet, W0: 0week, W6: 6 weeks, W12: 12 weeks, MAP: Mean blood pressure.

Lipid Profile:

The lipid profile revealed significant differences among the experimental groups. The level of cholesterol in the HFD group demonstrated a significant elevation as compared to the control group, **** P < 0.0001. Significant reductions in cholesterol levels in the Chia seeds and Flaxseed oil groups were observed compared to the HFD group, however, the combined chia and flaxseed oil treated group displayed a significant decline as compared to the Chia seeds,

Flaxseed oil and HFD groups, ##P < 0.01 (Fig. 1A). The level of TG was significantly elevated in HFD group compared to the control **** P < 0.0001 and declined significantly in combined chia and flaxseed oil as compared to the HFD, Chia seeds alone and Flaxeed oil alone, ##P < 0.01 (Fig. 1B). LDL level in HFD group illustrated significant elevation compared to the control, **** P < 0.0001. combined chia and flaxseed oil treated group illustrated significant reduction as compared to the HFD, Chia seeds, and Flaxseed oil groups, ##P < 0.001 (Fig. 1C). Whereas HDL in the HFD group demonstrated a significant alleviation as compared to the control, **** P< 0.0001. It then elevated significantly in the Chia seeds and Flaxseed oil groups as compared to HFD group, #P < 0.01. The combination of chia and flaxseed oil demonstrated significant elevation in HDL level as compared to the HFD group, ##P < 0.01, and the Flaxseed group, #P < 0.05, however, there were no significant differences with the Chia seeds group (Fig. 1D).

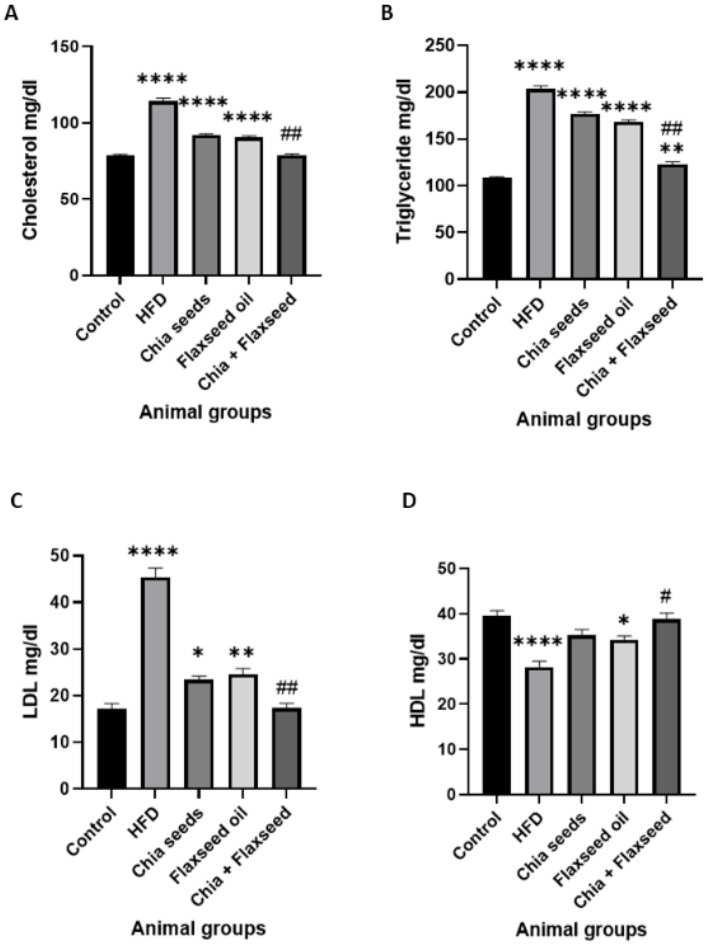

Fig. 1(A-D): Histograms for cholesterol, TG, LDL and HDL mean levels in the experimental grouping. (A) The level of cholesterol in HFD group illustrating significant upgrading compared to the control and elevated significantly also in the Chia seeds and Flaxseed oil groups as compared to the control group. It's demoted in the combined Chia and Flaxseed group compared to the HFD group. (B) Triglyceride level is significantly increased in in HFD group as compared to the control, and significantly increased also in the Chia seeds, Flaxseed oil and the mixed groups compared to the control. However, it's significantly alleviated only in the Chia seeds mixed with Flaxseed group compared to the HFD group. (C) LDL level in the HFD group shows significant increase as compared to control group, and in the Chia seeds and Flaxseed oil groups as compared to control group. It's then significantly relegated in the mixed group compared to the HFD group. (D) The level of HDL in the HFD, and Flaxseed oil groups demonstrate a significant decline comparing to control group. It's then shows significant raised in the combined Chia and Flaxseed oil group compared to the HFD group. Data are presented as mean ± SEM and n=6. * p< 0.05, ** p< 0.01, **** p< 0.0001, #P<0.05, ##P<0.01.

Histopathological Studies:

The aortic tissue of the control group demonstrated normal histological appearance with intact smooth endothelium and average thickness of the aortic layers; intima, media, and adventitia (Fig. 2A). Aortic sections in the HFD group illustrated partial intimal desquamation, thickening of the aortic wall and muscular layer with intimal bulging and foam cells (Fig. 2B). Chia seeds group aortic section showed a reduction in the thickness of the wall, but still thicker than the control with foam cells still presented (Fig. 2C). The Flaxseed oil group illustrated a thick muscular wall with intimal bulging (Fig. 2D). Treatment with both Chia seeds and Flaxseed oil demonstrated aortic wall thickness and architecture similar to the control (Fig. 2E).

Masson trichrome histopathological images demonstrated normal aortic walls with limited stains in the control group (Fig. 3A). While HFD group showed collagen deposition and lipid vacuolation (Fig. 3B). The Chia seeds treated group and the Flaxeed oil treated group demonstrated moderate collagen deposition and some lipid vacuoles are still present (Fig. 3C&D). However, the mixed Chia seeds + Flaxseed oil group illustrated restoration of the normal aortic appearance (Fig. 3E).

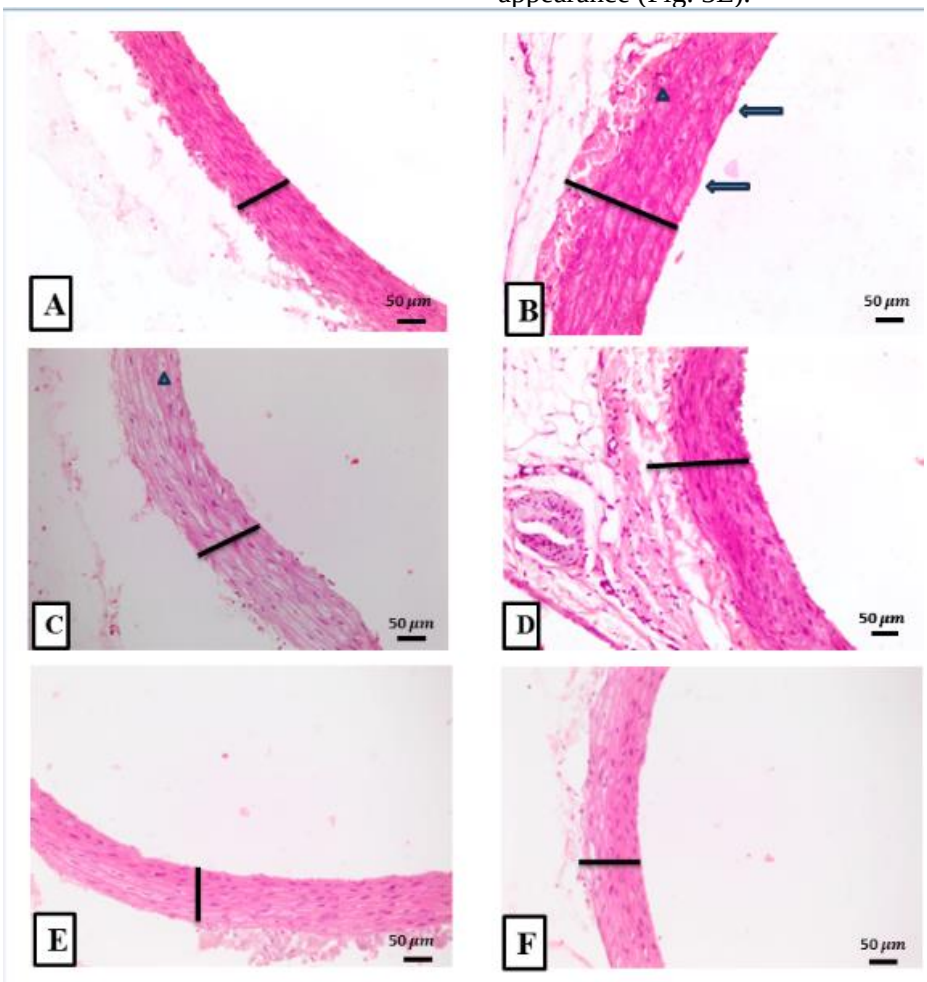

Fig. 2(A-F): Representative micrographs of the aortic sections. A) Control Aorta showing normal histological appearance and thickness of the aortic layers. (B) HFD group demonstrating partial intimal desquamation, thickening of the aortic wall with intimal bulging and projection (thin arrow), thickened muscular layer (line) with foam cells (arrowhead). (C) Chia seeds group aortic section showing reduction in the thickness of the wall, but still thicker than the control (line) with foam cells still present (arrowhead). (D) Flaxseed oil group illustrating thick muscular wall with intimal bulging. (E&F) Combined Chia and Flaxseed group showing similar aortic wall thickness and appearance like the control. H&E image magnification= 100x.

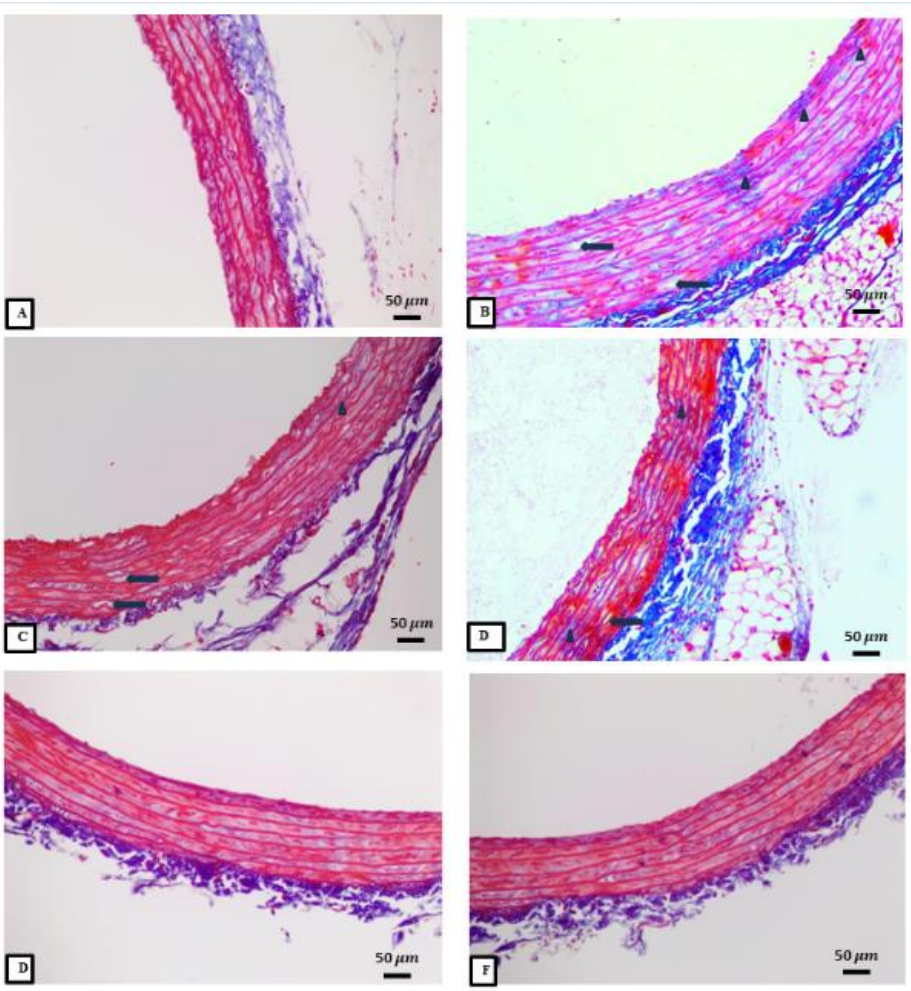

Fig. 3: Micrographs of Masson trichrome histopathological images. A) The control group demonstrating normal aortic wall. (B) HFD group showing collagen deposition and lipid vacuolation, arrow heads show collagen fibres, and black arrows indicate lipid vacuoles. (C) The Chia seeds treated group, and (D) the Flaxseed oil treated group, where there is moderate collagen deposition and some lipid vacuoles still present. (E) The mixed Chia with Flaxseed group illustrating restoration of the normal aortic agriculture. Image magnification=50µM.

Immunohistochemistry of IL6 and TNFα:

IL6 was detected in the aortic adventitia and aortic media layer, and it was enhanced in the HFD-treated group, and less intense in the control, and the mixed Chia seeds +Flaxseed oil-treated groups. In the Chia-treated and Flaxseed-treated groups, there was moderate IL6 stain compared to the control and the HFD groups (Fig. 4A–E). In the HFD-treated group, the intensity of IL6 area percentage staining was significantly increased compared to the control ($P<0.00001$), and it reduced significantly in the Chia seeds group ($P<0.01$), and Flaxseed oil group ($P<0.0001$) compared to HFD group. While in the mixed Chia seeds with Flaxseed oil group, it was significantly attenuated compared to the HFD group ($P<0.0001$), the Chia seed group, and the Flaxseed oil group ($P<0.001$), (Fig. 4F).

TNFα was localised in the aortic media and adventitia layers, there was a basal expression in the control group. However, in the HFD-treated group, an intense stain was observed compared to the control (Fig. 5A&B). In the Chia-treated and Flaxseed-treated groups, there was moderate TNFα stain compared to the control and the HFD groups (Fig. 5C&D). The mixed Chia seeds and Flaxseed oil-treated group showed diminished stain compared to the HFD group (Fig. 5E). In TNFα area percentage in the HFD-treated group, the intensity of staining was significantly increased compared to the control ($P<0.0001$), and in the mixed Chia + Flaxseed group, it was significantly alleviated compared to the HFD group ($P<0.0001$), the Chia seed group, and the Flaxseed oil group ($P<0.001$), (Fig. 5F).

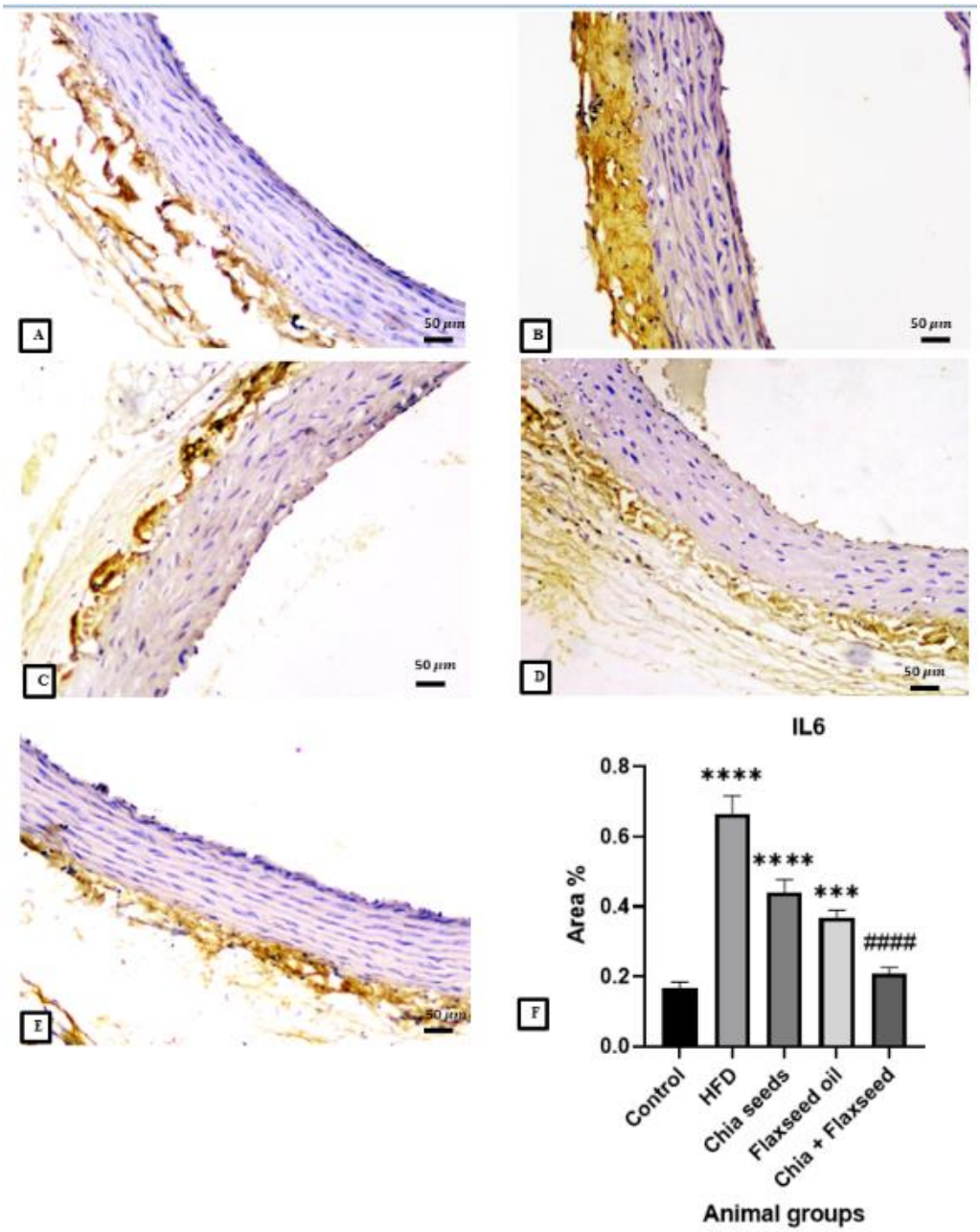

Fig. 4: Micrographs immunolocalize IL6 in rat's aorta (A–E). (A) The control group demonstrating limited stain. (B) HFD group significantly upregulates the IL6 positive cells, compared to the control group. (C) The Chia seeds treated group, and (D) the Flaxseed oil treated group, where the number of IL6 positive cells are reduced as compared to the HFD group, but not similar to the control. (E) The mixed Chia and Flaxseed group illustrating magnificent reduction in IL6 stain compared to HFD group. (F) Histogram showing the quantitative analysis of the intensity of IL6 stain in different experimental groups. The HFD, Chia and Flaxseed treated groups demonstrating a statically significant upregulation compared to the control group, ****P<0.0001. While in the mixed Chia and Flaxseed treated group showing a significant reduction in IL6 intensity compared to HFD group, ####P<0.0001.

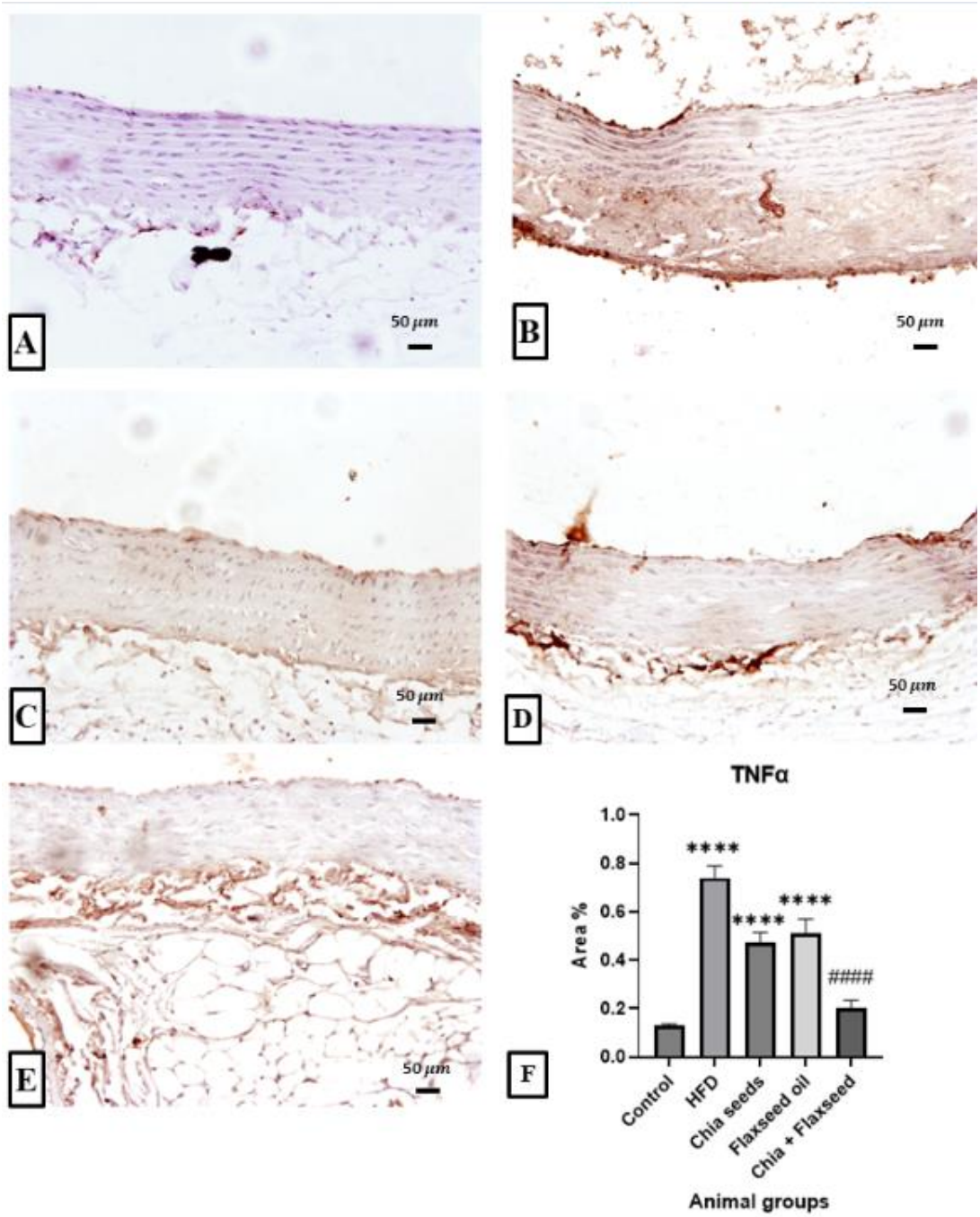

Fig. 5: Micrographs immunolocalize TNFα in rat's aorta (A–E). (A) The control group demonstrating less stain. (B) HFD group significantly alleviates the TNFα positive cells, compared to the control group. (C) The Chia seeds treated group, and (D) the Flaxseed oil treated group, where TNFα stain is moderate compared to the HFD group. (E) Treatment with both Chia and Flaxseed shows amelioration in TNFα stain compared to HFD group. (F) Histogram of the quantitative analysis of TNFα stain intensity in different experimental groups. The HFD, Chia and Flaxseed treated groups demonstrating a statically significant elevation compared to the control group, ****P<0.0001. While in the mixed Chia with Flaxseed treated group shows a significant decrease in TNFα intensity compared to HFD group, ####P<0.0001.

DISCUSSION

Atherosclerosis is a major health problem, causing marked morbidity and mortality worldwide, it is well known to be accelerated by a high-fat diet. High serum lipids and vascular inflammation play a significant role in atherosclerosis pathogenesis and its subsequent cardiovascular complications. Atherosclerosis prevention is based on lipid-lowering agents, including medications, foods, and dietary supplementation (Duffield *et al.*, 1983).

In the present study, the prophylactic effect of combined Chia

seeds and Flaxseed oil dietary intervention on an atherosclerosis-induced rat model fed a high-fat diet, was explored for the first time, using certain parameters including body weight, MAP, lipid profile, histological and immunohistochemical studies. The data revealed that treatment with both Chia seeds and Flaxseed oil significantly ameliorated the body weight, MAP, lipid profile and inflammatory biomarker compared to HFD, Chia seeds alone and Flaxseed oil alone.

HFD was noticed to increase body weight, MAP, lipid profile and inflammatory markers compared to the control, and this certainly predisposes to atherosclerosis. Experimental models were induced using atherogenic diets containing high fat and cholesterol (Gisterå *et al.*, 2022). Atherosclerosis is a chronic inflammatory disease of the large and medium-sized arteries (Cagnina *et al.*, 1999). At week twelve in this study, HFD significantly increased the body weight and MAP compared to the control. This was concomitant with the elevation in the serum cholesterol, triglycerides, and LDL, while HDL was reduced significantly compared to the control. The lipid profile showed a similar pattern at week twelve in another study after the administration of HFD (Wang *et al.*, 2011). The aorta in our study appeared thicker with intimal desquamation, bulging, more collagen deposition, foam cells, and lipid vacuolation by histopathological examination. Moreover, the immunohistochemical study revealed enhanced expression of IL6 and TNFα inflammatory biomarkers in the aortic tissue of the HFD group as compared to the control. This matched what was illustrated in another study (Shatoor *et al.*, 2019), where IL6 and TNFα protein levels showed significant upregulation in the HFD group as compared to the control.

Chia seeds showed a reduction in body weight, and MAP compared to HFD group. Regarding the lipid profile, the chia seeds group illustrated a significant elevation in cholesterol, triglycerides and LDL as compared to the control and a significant reduction when compared to the HFD group, while the reverse took place in HDL. Histological examination in the Chia seeds group revealed a reduction in the thickness of the aortic wall, but still thicker than the control and still also associated with foam cells and some collagen deposition and lipid vacuolation. The addition of Chia seed oil to the HFD in a rabbit model demonstrated significant attenuation in lipid concentration and aortic thickness (Sierra *et al.*, 2015), it showed a similar effect on the lipid profile in the murine model (Grancieri *et al.*, 2022). Moreover, IL6 and TNFα area percentage demonstrated significant upregulation compared to the control, whereas it reduced significantly also compared to HFD. Chia seed consumption was also shown to decrease IL-1β inflammatory markers protein level in a rat model in another study (Da Silva *et al.*, 2019).

Flaxseed oil demonstrated amelioration in the body weight and MAP compared to HDF. It was noticed that flaxseed oil also decreased rats' weight and aortic thickness after HFD in another study (de França Cardozo *et al.*, 2014). Aortic wall thickness decreased in this study but didn't return to normal, some collagen fibers were still localized. The lipid profile in our study showed improvement in the Flaxseed oil group in comparison with HFD. Flaxseed oil in other studies showed a similar hypolipidemic effect (Ratnayake *et al.*, 1992; Prasad *et al.*, 2020). The inflammatory biomarkers: TNFα and IL6 area percentage demonstrated elevation in the flaxseed group as compared to the control, and showed reduction as compared to the HDF group. Flaxseed oil demonstrated an anti-inflammatory effect on a murine model aortic tissue by reducing IL6 expression and inhibiting atherosclerosis (Dupasquier *et al.*, 2007a). It induced atherosclerosis plaque regression in a rabbit model (Francis *et al.*, 2013b). It has a significant effect in attenuating atherosclerosis in a mouse model by reducing cholesterol levels and suppressing IL6 expression (Dupasquier *et al.*, 2007b).

A combination of the Chia seeds and the Flaxseed oil reduced the body weight, and MAP compared to HFD, Chia seeds alone and Flaxseed oil alone, this combination also reduced the lipid profile compared to the same groups. Flaxseed grain showed a maximum lipid-lowering effect in goats than flaxseed oil, this might be related to the extra component in the grain more than the oil (Liu *et al.*, 2021). Two different variants of Flaxseed oil demonstrated different effects on lipid profile and

atherosclerosis regression in the aortic rabbit' model (Bujok *et al.*, 2021).

A combination of the Chia seeds and the Flaxseed oil returned aortic architecture to the normal and reduced inflammatory markers; IL6 and TNFα compared to HFD, Chia seeds alone and Flaxseed oil alone. IL6 is a vascular inflammatory biomarker, mostly related to the acute phase response in vascular injury, atherosclerosis, and its harmful sequelae (Szekanecz *et al.*, 1994). Therapeutic targeting of vascular inflammation including atherosclerosis should include IL6 mediated inflammation inhibition (Omoigui, 2007). A combination of Flaxseed oil with α-lipoic acid in an HFD rat model showed better action in ameliorating atherosclerosis (Xu *et al.*, 2012b). A combination of flaxseed oil and astaxanthin also ameliorated atherosclerosis in HFD-fed rats by decreasing the lipids and IL6 inflammatory markers (Xu *et al.*, 2014). Mixtures of Flaxeed/Sesame and Flaxeed/Peanut seeds showed better effects in lowering lipid parameters in rats (Makni *et al.*, 2010). This could support our hypothesis, that mixing components could have a synergistic effect and gain more benefits in lowering lipid profile and improving inflammation and thus ameliorating atherosclerosis.

Our data provides proof of the potential role of mixing Chia seeds with Flaxseed oil in attenuation of the lipid profile, and regression of aortic inflammation with its subsequent atherosclerosis in rats fed a high-fat diet. Further future studies need to be focused on results validation with additional techniques including protein and gene expression. Additional studies are still also required to standardize the Chia seeds and flaxseed oil dose to justify its use in an appropriate pharmaceutical form.

In conclusion, the current study anticipates biochemical and immunohistochemical evidence of the essential prophylactic role of Chia seeds combined with Flaxseed oil in lipid profile amelioration that resulted in lessening of inflammatory markers, aortic injury, and limitation of atherosclerosis. There is a synergistic effect of mixing the Chia seeds with the Flaxseed oil on the alleviation of atherosclerosis associated with a high-fat diet. This could be guidance to use this mixture of Chia seeds and Flaxseed oil as a nutritional supplement in the prophylaxis against the sequelae of high fat-diet ingestion including atherosclerosis and cardiovascular diseases. This could have great benefits on human quality of life, by reducing the risk of atherosclerosis development and its damaging effect on various body systems.

Declarations:

Ethical Approval: This is an experimental case-control study; it was conducted following the principles of laboratory animal care and the use of lab guidelines. Thirty male Sprague-Dawley rats, each weighing 150 –250 gm were purchased from Mansoura Medical Experimental Research Centre to be used in this study, where also the experiment was conducted. All the experimental procedures were approved by the institutional review board (IRB), Mansoura University, Faculty of Medicine (Ref: R.22.07.1770).

Conflict of Interests: No conflicts of interest to declare.

Contributions of the Authors: NE outlined the study design, and AM and NE conducted laboratory experiments and collection of data. SE was responsible for the rats' manipulation. ZE carried out the biochemical investigation. The histological and immunohistochemical assessment and statistical study conducted by NE. NE, AM and ZE were assigned for the elucidation of the results. The manuscript was drafted by NE and was critically revised by AM and ZE.

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Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

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ARABIC SUMMARY

التاثير الوقائي لدمج بذور الشيا مع زيت بذور الكتان علي تصلب الشريان التاجى المستحث في نموذج الجرزان

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1-قسم التشريح و علم االجنة - كلية الطب - جامعة المنصورة

-2قسم الفسيولوجي -كليه الطب -جامعة المنصورة

-3مركز ابحاث الحيوان-كليه الطب -جامعة المنصورة

يعد تصلب الشرايين سببًا رئيسيًا للوفاة بسبب أمراض القلب والأوعية الدموية في جميع أنحاء العالم. يؤدي تراكم الدهون في الشرايين الكبيرة إلى ظهور علامات التهابية، مثل α-(TNF) والإنترلوكين 6-(IL)، اللذين يؤديان بدورهما إلى إصابة بطانة الأوعية الدموية. ومن الضروري إيجاد دواء وقائي لهذا المرض وخاصة من الموارد الطبيعية لتجنب الآثار الجانبية للأدوية. تعتبر بذور الشيا وزيت بذور الكتان من الأعشاب الطبيعية، وقد أظهرت كل جرعة منفصلة نتائج جيدة في تحسين فرط شحميات الدم وتقليل الاستجابة الالتهابية لتصلب الشرايين. يمكن أن يكون الجمع بين هذين المصدرين الطبيعيين تأثير وقائي تآزري وتقليل تصلب الشرايين بشكل أفضل، والهدف من هذه الدراسة هو دراسة التأثير الوقائي المحتمل لمزيج من بذور الشيا وزيت بذور الكتان على نموذج فأر مصاب بتصلب الشرايين المستحث من خلال اتباع نظام غذائي عالي الدهون. استخدام التقنيات الكيميائية والمناعية الكيميائية لاستكشاف تأثير هذا المزيج على مستوى معايير الدهون في الدم ودراسة التعبير البروتيني للعلامات الالتهابية؛ IL6 وTNFα في أنسجة الشريان التاجى مع أو بدون إعطاء بذور الشيا وزيت بذور الكتان كل على حدة أو مجتمعة. تم إظهار تغييرات كبيرة في صورة الدهون وعلامات الالتهاب في المجموعة المشتركة، حيث تحسنت معايير الدهون ووزن الجسم ومتوسط ضغط الدم الشرياني، كما أنها خففت من التهاب الشريان التاجى مقارنة ببذور الشيا أو زيت بذور الكتان وحده. يمكن أن يكون هذا دليلاً لاستخدام بذور الشيا مع زيت بذور الكتان كعلاج وقائي ضد تصلب الشرايين للحد من تأثيره السلبي.