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# Flaxseed Oil Effectively Reduces the Risk of Development of Atherosclerosis in Rats Fed on High Cholesterol Diet

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### Authors' contributions

This work was carried out in collaboration between all authors. Author ZYA designed the study, wrote the protocol and the first draft of the manuscript. Authors ZYA, MEY and MT managed the analyses of the study and the literature searches. Authors HHA and MYE conducted the experiment and performed the statistical analysis. All authors read and approved the final manuscript.

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## ABSTRACT

**Background:** The quantity and type of dietary fatty acids play an important role in the risk development of cardiovascular disease.

**Aims:** The current study was designed to investigate fatty acid profile of flaxseed oil (FO) and assessment the possible cardiovascular protective potentials of FO on Sprague-Dawley rats fed on high cholesterol diet (HCD) for 12 weeks and explores the possible mechanism of action.

**Methodology:** Fatty acid profile of FO was investigated by Gas Chromatography- Flame Ionization

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Detector (GC/FID). Animals were divided randomly into equal five groups as follows: Group 1: fed on the basal diet and served as control group. Group 2: fed on HCD for 12 weeks. Group 3-4: fed on HCD along with FO at two doses of 270 and 540 mg/Kg b.w/day, respectively. Group 5: fed on HCD and received a human equivalent dose of rosuvastatin, approved drug that slow plaque buildup in arteries.

**Results:** GC/FID analysis revealed that FO has a unique and healthy fatty acid profile with 67.4 percent as  $\alpha$ -linolenic acid (ALA), giving a very favorable omega-6:omega-3 ratio of 0.147:1. HCD is effective in triggering hyperlipidemia with elevation of serum myocardial diagnostic enzymes, and enhancement of myocardial inflammatory response and alteration in the redox state. However, daily co-administration of FO at two doses and HCD for 12 weeks significantly preserved all these biochemical changes in dose dependent manner. The histopathology examination of the aortic tissue was in parallel with the biochemical results. This beneficial cardioprotective effect was more pronounced in rosuvastatin followed by FO at a dose of 540 mg/Kg/day, which equivalent to human recommended doses of 6 g of flaxseed oil containing 4.04 g of ALA supplements per day. Histological examination of aortic tissues supports our biochemical results.

**Conclusion:** Flaxseed oil enriched with ALA, an omega-3 fatty acid effectively reduces the risk development of atherosclerosis in a dose dependent manner in rats through anti-inflammatory mechanism. Further studies still needed to standardize the flaxseed oil to justify its use in a suitable pharmaceutical form to choose the appropriate dose for human.

*Keywords: Atherosclerosis; flaxseed oil; high cholesterol diet; lipid profile; inflammation; antioxidant.*

## 1. INTRODUCTION

Cardiovascular diseases (CVD), mainly including atherosclerosis, hypertension, cardiac hypertrophy, myocardial infarction and heart failure, are the principal cause of death worldwide. Although the development of pharmacotherapies to treat, CVD has contributed to a decline in cardiac mortality. In the past few decades, CVD is estimated to be the cause of one-third of deaths globally [1,2]. Atherosclerosis is a chronic inflammatory disease affecting large and medium arteries and is considered a major underlying cause of CVD. Atherosclerotic vascular disease results from the pathological deposition of lipids, such as cholesterol and triglycerides, within the walls of arterial blood vessels. These leading to calcification, inflammation, recruitment of macrophages and foam cells, and eventually thrombosis [3]. Inflammatory response is considered as a predominant driving force in atherosclerotic plaque formation, growth and progression towards instability and rupture. Notably, accumulation of macrophages in the intima and emergence of a pro-inflammatory milieu are a characteristic feature of plaque progression and these processes can be modulated by adaptive immune responses [4].

Dyslipidemia and vascular inflammation are essential mechanisms for coronary artery atherosclerosis. Deposition of oxidized low-density lipoprotein cholesterol (oxLDL-c) in

arterial wall resulting in vascular endothelial dysfunction, together with the consequent vascular inflammatory disorder contributes to the progression of coronary atherosclerotic plaques [5]. Statins are the most commonly used cholesterol-lowering agent. They reduce the circulating LDL-c level by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase, the enzyme involved in the rate-limiting step during cholesterol biosynthesis [6]. However, high-dose statin therapy is associated with adverse effects, such as muscle pain and hepatic abnormalities [7]. Therefore, alternative therapeutics are needed that can either be taken alone or in combination with statins.

Lifestyle behaviors such as physical activity, smoking and diet, and environmental factors contribute to cardiovascular health. In addition, individual behavioral choices modify traditional risk factors such as lipid profiles, body mass index, and blood pressure are believed to play a role in the development and progression of atherosclerosis [8].

Natural products have played an important role throughout the world in both treatment and prevention of human from several diseases. Lipid-lowering medicinal plants may reduce hyperlipidemia, preventing atherosclerosis and vascular endothelial damage due to their potential of targeting multiple steps involved in pathogenesis, and fewer side effects [9]. Present curiosity in traditional medicine has led to the

exploration and development of many herbal drugs for the management of atherosclerosis [10]. The role of omega-3 highly unsaturated fatty acids (HUFAs) in human mental health has been widely studied in the last two decades [11]. Dietary intake of PUFAs is vital because they cannot be synthesized *in-vivo*. Fish oil as an animal source and flaxseeds as plant source, are rich sources of omega-3 PUFAs.

Flaxseed (*Linum usitatissimum* L.) is a rich source of nutritive and bioactive compounds that can potentially improve health. Recently, flaxseeds were also incorporated into food with other nutraceuticals or food ingredients to improve the nutritional quality of food [12]. Flaxseed oil is a vegetarian source of omega-3 FAs. Flaxseed oil contains both omega-3 and omega-6 fatty acids, which are needed for health. Flaxseed oil contains the essential fatty acid alpha-linolenic acid (ALA), which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [13]. All of these omega-3 fatty acids help decrease inflammation, which is a trigger for heart disease. ALA is a potent biological antioxidant that is found naturally in the human body at very low concentrations, primarily in the mitochondria [14]. It was reported that DHA synthesized from ALA can provide sufficient DHA for the adult brain [15]. As a functional food ingredient, flaxseed oil has been incorporated into baked foods, juices, milk and dairy products, muffins, dry pasta products, macaroni and meat products [16]. Therefore, the current study has been designed to evaluate the possible cardiovascular protective potentials of flaxseed oil from the development of atherosclerosis and reduce the incidence of CVD in Sprague-Dawley male rats fed on high cholesterol diet and explores the possible mechanism of action.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

Seeds of *Linum usitatissimum* L. were purchased from a local herbal store and the specimen was identified in the department of Botany, Faculty of Agriculture, Cairo University, Egypt.

### 2.2 Flaxseed Fixed Oil

Powdered seeds were weighed accurately (2 kg), crushed and subjected to cold pressing. The obtained oil was filtered through a fine cloth and kept in dark brown bottles in refrigerator.

Saponification of the petroleum ether extract of seeds and preparation of the fatty acid methyl esters were carried out according to standard methods [17,18].

### 2.3 Gas Chromatography- Flame Ionization Detector (GC/FID) Analysis

Gas Chromatography – Flame Ionization Detector (GC/FID) analysis of flaxseed oil was carried out on a Gas-Chromatograph Agilent 689 equipped with a flame ionization detector (Agilent 5973). A fused silica capillary column (HP-5MS) 5% phenyl methyl siloxane as a nonpolar stationary phase (30 m x 0.25 mm, 0.25  $\mu$ m) was used. Temperature programming for fatty acid methyl esters was adjusted from 120°C to 200°C at a rate of 20°C/min., then completed to 300°C with a rate of 10°C/min. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (GC/FID) and the percentages were obtained by FID peak area normalization method.

### 2.4 Animals

Male albino Sprague-Dawley rats weighting 150  $\pm$  20 g were obtained from National Organization for Drug Control and Research (NODCAR). Animals were housed in standard cages with controlled temperature (20 - 25°C) and 12/12 hours light/dark. They kept free access to water and normal diet. The animals were acclimatized to the experimental conditions for a week before starting the experiment. The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

### 2.5 Induction of Hyperlipidemia

Hyperlipidemia was induced in rats by feeding on high cholesterol diet (HCD) containing cholesterol (2%) and cholic acid (0.5%), well mixed with normal laboratory diet [19,20].

### 2.6 Experimental Design

A total of 40 rats was divided randomly into equal five groups (8 rats each). Eight rats fed on the normal diet and served as a control for 12 weeks (Group 1). While, the remaining animals fed on HCD and divided equally as follows: Group 2:

untreated rats served as a positive control. Group 3-4: received flaxseed oil per oral at a low dose (LD) of 270 mg/Kg/day and double dose (DD) of 540 mg/Kg/day, which equivalent human dose of 3 and 6 g FO /day. Group 5: received a human equivalent dose of rosuvastatin (0.9 mg/kg b.w/day, p.o., for 12 weeks), approved drug that slow plaque buildup in arteries.

## 2.7 Serum and Tissue Processing

At the end of the experiment, the rats were fasted overnight; blood was drawn from retro orbital plexus under light ether anesthesia with ether. Serum was separated by centrifuge at 3000 rpm, and 4°C for 15 minutes and kept in -20°C until biochemical analysis. Then, all the animals were anesthetized with mild anesthesia and sacrificed by cervical dislocation. Hearts were quickly removed and immediately washed with ice-cold saline (0.85% sodium chloride). A 5% tissue homogenate of cardiac tissue was prepared in ice-cold saline solution. Heart homogenates were centrifuged at 1000 g and 4°C for 15 min, and the supernatants were kept in -20°C until biochemical analysis was performed. Aorta tissue was preserved in 10% neutral buffered formalin for histological analysis.

## 2.8 Biochemical Analysis

### 2.8.1 In serum

Serum total cholesterol (T.C) [21], triglycerides (TG) [22], high density lipoprotein cholesterol (HDL-c) [23] were investigated. Whereas, low-density lipoprotein cholesterol (LDL-c) and very low-density lipoprotein (VLDL) were calculated by Friedewald formula:  $LDL-c = T.C - (HDL-c + VLDL)$ ,  $VLDL = TG/5$  [24]. Atherogenic index (A.I) =  $(T.C-HDL-c)/HDL-c$ . Quantitative determination of creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH) and aspartate transaminase (AST) enzyme activities were followed the method of Wurzburg et al. [25], Henry et al. [26] and Reitman and Frankel, [27], respectively. While, C-reactive protein (CRP) and Total antioxidant capacity (TAC) were determined according to the method of Price et al. [28] and Koracevic et al. [29], respectively.

### 2.8.2 In heart homogenate

Enzyme linked immunosorbents assays (ELISA) were used for quantifying inflammatory markers as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin (IL)-6 and IL-10 using Quantikine Rat Immunoassay ELISA kit (R&D systems,

Inc., USA). The myocardial enzyme activities of myeloperoxidase (MPO), xanthine oxidase (XO) were measured following the methods of Bradley et al. [30] and Bergmeyer et al. [31] respectively. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was assayed according to Sigma assay kit (Sigma, USA). Superoxide dismutase (SOD) [32], catalase (CAT) [33], reduced glutathione (GSH) [34], nitric oxide (NO) [35], malondialdehyde (MDA) [36] and protein carbonyl (PC) [37] were also investigated.

## 2.9 Histopathological Study

Autopsy samples were taken from the aorta of all rats and fixed in 10% formalin saline for twenty-four hours, then washing with tap water. Serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for twenty-four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, de-paraffinized, and stained by hematoxylin & eosin (H&E) stain for routine examination through the light electric microscope [38].

## 2.10 Statistical Analysis

All results are presented as mean  $\pm$  S.E for n = 8. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison test using Software Package for the Social Sciences (SPSS) version 22.0 (Chicago, IL, USA). A value of  $^{\#}P < 0.001$  versus control group, while  $^{\#}P < 0.05$ ,  $^{**}P < 0.01$  and  $^{***}P < 0.001$  versus HCD group.

## 3. RESULTS

### 3.1 GC/FID Analysis

The data depicted in Table 1 and Fig. 1 representing the GC/FID analysis of fatty acid methyl esters of flaxseed oil that reveals the identification of five compounds constituting 97.97% of the total fatty acids. In which the saturated and unsaturated fatty acids represent 11.61% and 88.39% respectively. The monounsaturated fatty acids represent 11.08% and polyunsaturated fatty acids 77.31% of the total unsaturated FAs. The major identified fatty acid was linolenic acid (67.43%) followed by oleic (11.08%).

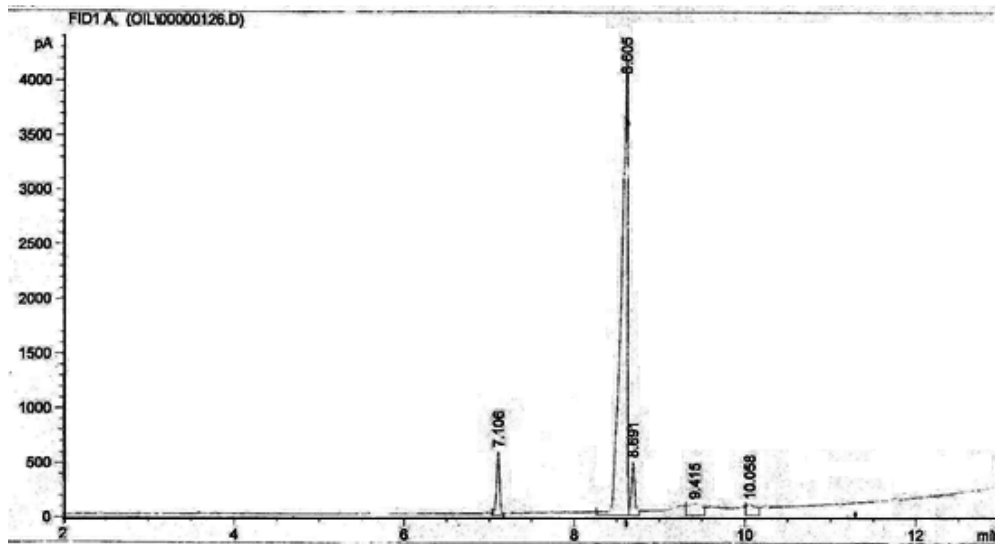


Fig. 1. The chromatogram from GC/FID analysis of the flaxseed oil

Table 1. GC/FID of the fatty acids of flaxseeds oil represented as methyl ester

Rt	Compounds	Saturation	Family name <sup>a</sup>	Formula	Rel%
7.11	Oleic acid	Monounsaturated	Omega-9	C18:1 $\omega$ -9	11.08
8.61	$\alpha$ -Linolenic acid	Polyunsaturated	Omega-3	C18:3 $\omega$ -3	67.43
8.69	Linoleic acid	Polyunsaturated	Omega-6	C18:2 $\omega$ -6	9.88
9.42	Palmetic acid	Monounsaturated	Omega-7	C16:1 $\omega$ -7	6.60
10.06	Stearic acid	Saturated	--	C18:0	5.01

Rt: Retention time (minute); Rel %: relative area percent; a: The family name shows the position of the first double bond in the carbon chain or backbone of the fatty acid, marked from the methyl end with an omega symbol ( $\omega$ ).

Omega-6/omega-3 ratio is 0.143

### 3.2 Effect on Serum Lipid Profile

The data depicted in Table 2 represents the effect of flaxseed oil supplementation on serum lipid profile after 12 weeks incomparable to rosuvastatin in rat fed on high cholesterol diet (HCD). Feeding on HCD for 12 weeks, resulting in a significant ( $P < 0.001$ ) increase in serum TG, T.C, LDL-c and VLDL levels by 3.2, 1.9, 3.0 and 3.2- fold, respectively above the normal levels, a combined with significant ( $P < 0.001$ ) decrease in HDL-c by 24.9% as compared to control group. Furthermore, atherogenic index (A.I) showed a significant ( $P < 0.001$ ) in the untreated group fed on HCD by 4.1- fold above the normal level. Meanwhile, co-administration of FO at low dose with HCD for the same period was significantly decreased serum TG, T.C, LDL-c and VLDL levels by 31.0%, 15.6%, 18.0% and 31.0%, respectively and a combined with a significant increase in HDL-c by 3.8% versus HCD fed group. Furthermore, co-administration of FO at double dose with HCD for the same period was significantly decreased serum TG, T.C, LDL-c

and VLDL levels by 57.5%, 37.3%, 47.6% and 57.5%, respectively and a combined with a significant increase in HDL-c by 15.7% versus HCD fed group. Rosuvastatin showed more pronounced improvement effect.

### 3.3 Effect on Serum Diagnostic Markers of Heart Function

Data represented in Table 3, illustrated the effect of FO on serum diagnostic markers of heart function. HCD-fed group exhibited a significant elevation in serum AST, LDH and CK-MB by 1.92, 1.86 and 6.77- fold above the normal level. All these changes were significantly preserved in both FO and rosuvastatin- treated groups. As compared with untreated HCD- fed group, AST was significantly decreased by 10.6%, 25.4% and 41.6%, LDH was significantly decreased by 20.3%, 30.3% and 35.3%, and CK-MB was decreased by 45.1%, 67.2% and 70.9%, respectively in both doses of FO (LD and DD) and rosuvastatin- treated groups, respectively.

**Table 2. Effect of FO and rosuvastatin on serum lipid profile after 12 weeks**

Groups	TG (mg/ dl)	T.C (mg/ dl)	HDL-c (mg/ dl)	VLDL (mg/ dl)	LDL-c (mg/ dl)	A.I
Control	42.5 ± 0.63	77.5 ± 1.79	37.9 ± 0.77	8.50 ± 0.12	31.1 ± 1.47	1.05 ± 0.04
HCD	135.8±3.08 <sup>###</sup>	148.6±3.17 <sup>###</sup>	28.4 ± 0.61 <sup>###</sup>	27.2 ± 0.28 <sup>###</sup>	93.0 ± 3.14 <sup>###</sup>	4.25 ± 0.19 <sup>###</sup>
HCD + FO (LD)	93.8 ± 1.38 <sup>***</sup>	125.4 ± 2.89 <sup>***</sup>	29.5 ± 0.71 <sup>ns</sup>	18.8 ± 0.28 <sup>***</sup>	76.2 ± 2.58 <sup>***</sup>	3.13 ± 0.11 <sup>***</sup>
HCD + FO (DD)	57.7 ± 1.23 <sup>***</sup>	93.2 ± 1.40 <sup>***</sup>	32.9 ± 0.74 <sup>***</sup>	11.6 ± 0.25 <sup>***</sup>	48.8 ± 1.73 <sup>***</sup>	1.85 ± 0.08 <sup>**</sup>
HCD + Rosuvastatin	49.9 ± 1.99 <sup>***</sup>	82.8 ± 1.47 <sup>***</sup>	35.6 ± 0.60 <sup>***</sup>	9.97 ± 0.40 <sup>***</sup>	37.2 ± 1.18 <sup>***</sup>	1.33 ± 0.04 <sup>***</sup>

All values are mean ± Standard error of means of eight rats. The statistical analysis was performed by one-way ANOVA, followed by Tukey test. A value of <sup>###</sup>P < 0.001 versus control, <sup>\*\*</sup>P < 0.001 and <sup>\*\*\*</sup>P < 0.01 versus HCD; ns: non-significant. Abbreviations: HCD: high cholesterol diet; FO: Flaxseed oil; LD: low dose; DD: double dose; TG: triglyceride; T.C: total cholesterol, HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; VLDL: very-low-density lipoprotein; A.I: atherogenic index

**Table 3. Effect of FO and Rosuvastatin on serum diagnostic markers of heart function after 12 weeks**

Groups	AST (U/ml)	LDH (IU/L)	CK-MB (ng/ml)
Control	47.9 ± 1.04	314.9 ± 9.66	1.26 ± 0.04
HCD	92.0 ± 1.18 <sup>###</sup>	587.1 ± 14.5 <sup>###</sup>	8.60 ± 0.19 <sup>###</sup>
HCD + FO (LD)	82.3 ± 1.79 <sup>***</sup>	467.8 ± 16.0 <sup>***</sup>	4.73 ± 0.26 <sup>***</sup>
HCD + FO (DD)	68.6 ± 1.13 <sup>***</sup>	409.4 ± 12.6 <sup>***</sup>	2.82 ± 0.11 <sup>***</sup>
HCD + Rosuvastatin	53.7 ± 0.99 <sup>***</sup>	379.3 ± 9.83 <sup>***</sup>	2.50 ± 0.07 <sup>***</sup>

All values are mean ± Standard error of means of eight rats. The statistical analysis was performed by one-way ANOVA, followed by Tukey test. A value of <sup>###</sup>P < 0.001 versus control, <sup>\*\*</sup>P < 0.001 versus HCD. Abbreviations: HCD: high cholesterol diet; FO: Flaxseed oil; LD: low dose; DD: double dose; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CK-MB: creatine phosphokinase-MB

### 3.4 Effect on Markers of Inflammatory Markers

As shown in Fig. 2, HCD triggered significant elevation in serum CRP and myocardial TNF- $\alpha$  and IL-6 after 12 weeks by 2.1, 2.0 and 2.1- fold and depletion in IL-10 by 48.1% versus normal control group. This significant elevation was suppressed in FO and Rosuvastatin- treated groups. As compared with untreated HCD- fed group, serum level of CRP decreased by 15.3%, 28.3% and 35.9%, respectively, TNF- $\alpha$  also decreased by 9.5%, 19.8% and 31.3%, respectively, as well as IL-6 also decreased by 22.4%, 26.5% and 32.2%%, respectively with administration of FO (low & double doses) and Rosuvastatin respectively. In contrast, IL-10 was significantly enhanced in FO (LD & DD) and rosuvastatin- treated groups by values of 28.3%, 58.8% and 79.7%, respectively.

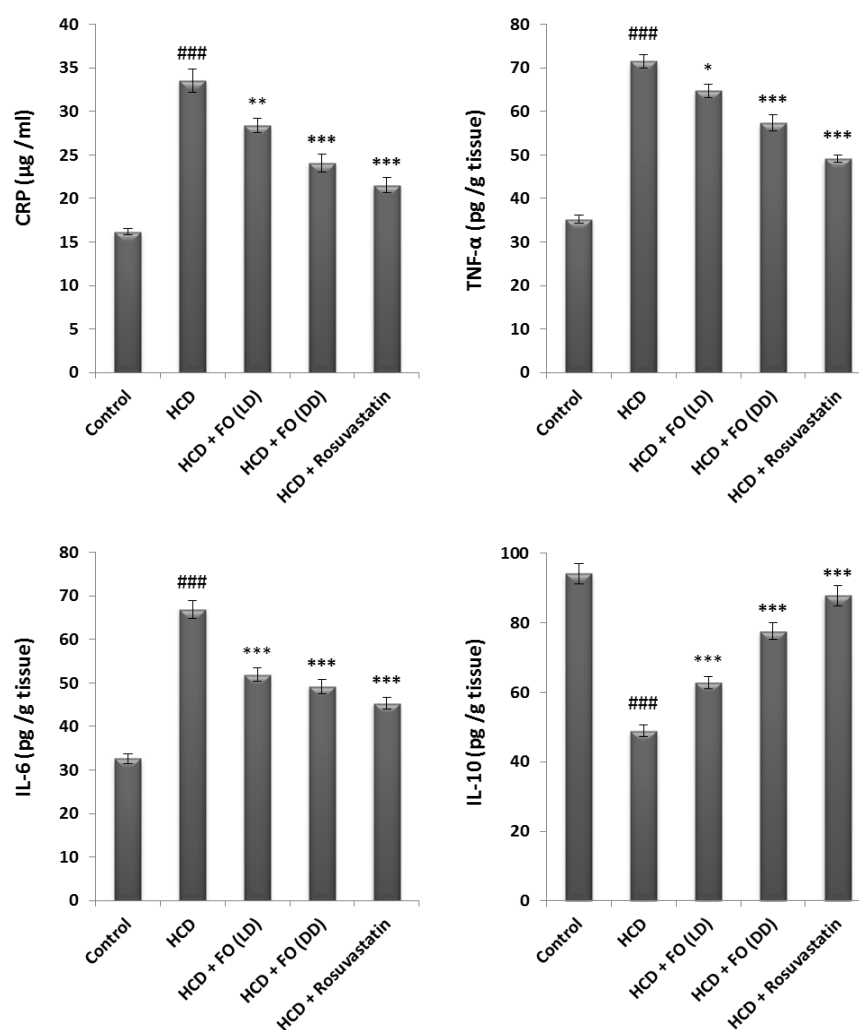
### 3.5 Effect on Myocardial Oxidant Enzymes and NO

As illustrated in Table 4, HCD- fed group had a significant enhanced myocardial enzyme activities of MPO, XO, NADPH oxidase and NO by 2.75, 2.30, 2.58 and 2.34- fold above the

normal level. These marked elevation were significantly decreased after administration of two doses of flaxseed oil and Rosuvastatin for 12 weeks in the order of: Rosuvastatin > double dose of FO > low dose of FO as compared with HCD- fed group. There was a negative correlation between these enzyme activities and the diagnostic markers of heart injure (AST, LDH and CK-MB), and a positive correlation with inflammatory markers (CRP, TNF- $\alpha$  and IL-6).

### 3.6 Effect on Myocardial Markers of Oxidative Stress

Compared with control group, HCD- feeding group for 12 weeks exhibited a significant decrease in enzyme activities of myocardial SOD and CAT by 34.0% and 28.8%, respectively associated with a significant depletion in the myocardial content of GSH by 38.3% versus control group (Fig. 3). However, compared to the untreated HCD group, flaxseed oil supplementation at two doses (LD and DD) for 12 weeks resulted in a significant elevation in myocardial SOD and CAT enzyme activities and GSH content and depletion in the markers of oxidative stress (MDA and PC) in dose dependent manner.



**Fig. 2. Effect of FO and rosuvastatin on inflammatory markers**

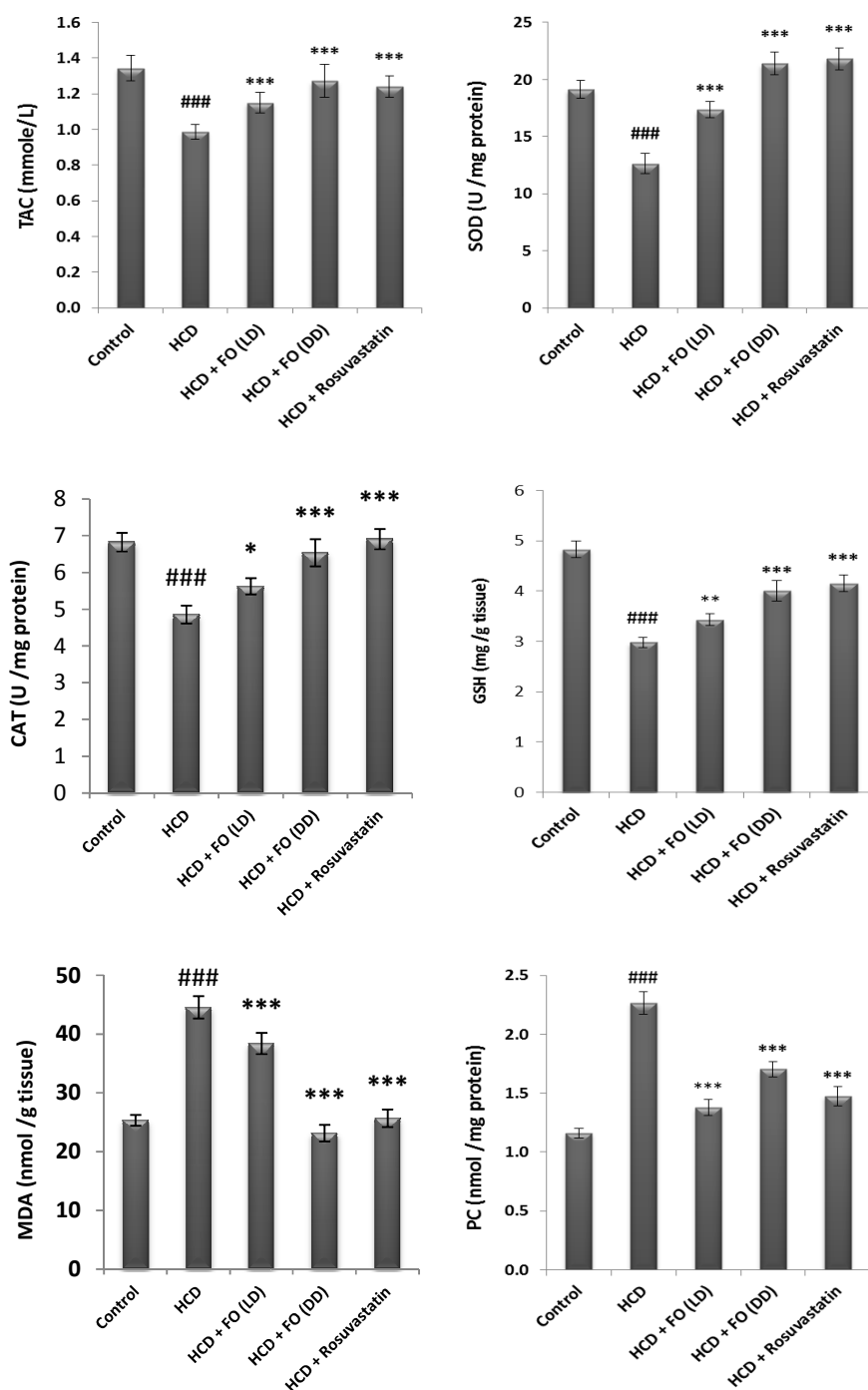
All values are mean  $\pm$  Standard error of means of eight rats. The statistical analysis was performed by one-way ANOVA, followed by Tukey test at #### $P < 0.001$  vs control, \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.05$  vs HCD. Abbreviations: HCD: High cholesterol diet; FO: Flaxseed oil; LD: Low dose; DD: Double dose; CRP: C-reactive protein; TNF- $\alpha$ : Tumor necrosis factor-alpha; IL: Interleukin

**Table 4. Effect of FO and Rosuvastatin on myocardial MPO, XO, NADPH oxidase and NO**

Groups	MPO (U/mg protein)	XO (U/mg protein)	NADPH oxidase (U/mg protein)	NO ( $\mu$ mol/mg tissue)
Control	0.77 $\pm$ 0.05	1.80 $\pm$ 0.05	12.1 $\pm$ 0.55	8.50 $\pm$ 0.12
HCD	2.12 $\pm$ 0.03###	4.13 $\pm$ 0.06###	31.2 $\pm$ 1.34###	27.2 $\pm$ 0.28###
HCD + FO (LD)	1.75 $\pm$ 0.11***	3.65 $\pm$ 0.10***	23.2 $\pm$ 1.06**	18.8 $\pm$ 0.28***
HCD + FO (DD)	1.53 $\pm$ 0.04***	2.93 $\pm$ 0.06***	19.7 $\pm$ 0.90***	11.6 $\pm$ 0.25***
HCD + Rosuvastatin	1.48 $\pm$ 0.03***	2.77 $\pm$ 0.05***	13.6 $\pm$ 0.64***	9.97 $\pm$ 0.40***

All values are mean  $\pm$  SEM. The statistical analysis was performed by one-way ANOVA, followed by Tukey test at #### $P < 0.001$  vs. control, \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. HCD group.

Abbreviations: HCD: high cholesterol diet; FO: Flaxseed oil; LD: low dose; DD: double dose; MPO: myeloperoxidase; XO: xanthine oxidase; NADPH oxidase: Nicotinamide adenine dinucleotide phosphate; NO: nitric oxide



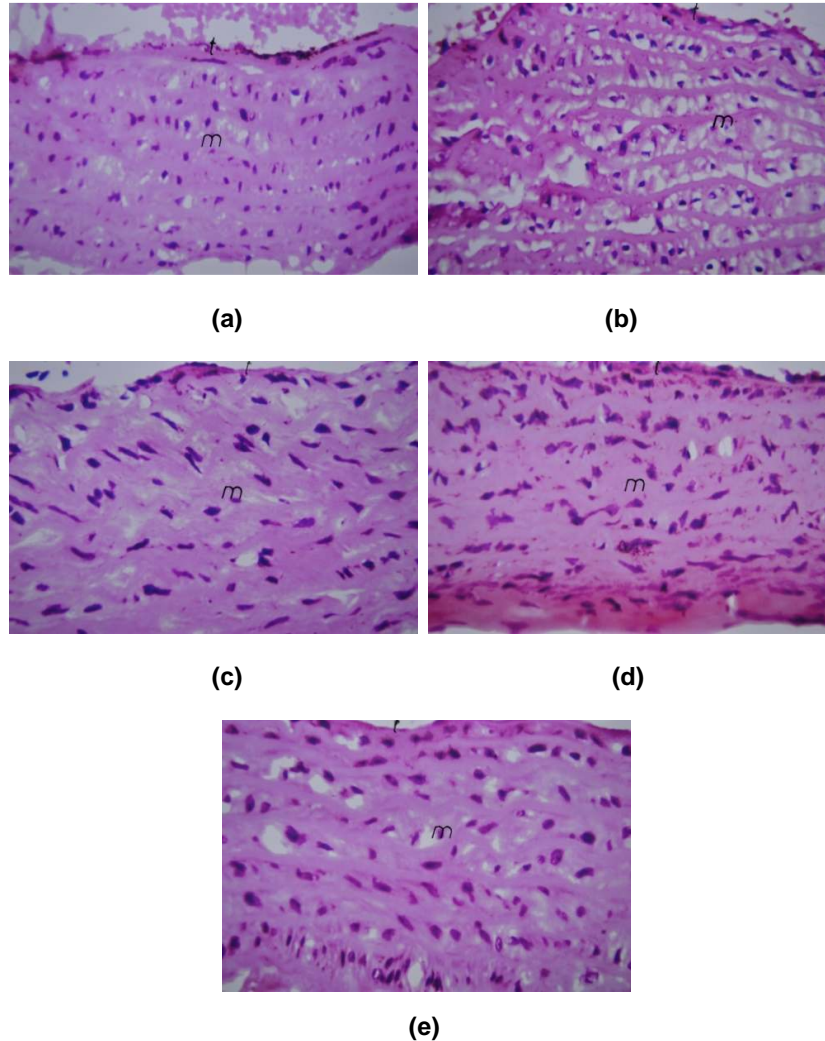
**Fig. 3. Effect of flaxseed oil supplementation on myocardial markers of oxidative stress**  
 All values are mean  $\pm$  Standard error of means of eight rats. The statistical analysis was performed by one-way ANOVA, followed by Tukey test at ###P < 0.001 vs. control; \*P < 0.01 and \*\*\*P < 0.001 vs. HCD. Abbreviations: HCD: high cholesterol diet; FO: Flaxseed oil; LD: low dose; DD: double dose; FO: Flaxseed oil; LD: low dose; DD: double dose; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathione; MDA: malondialdehyde; PC: protein carbonylation



### 3.7 Histological Examination

Histological examination of aorta sections of all examined groups illustrated in Table 5 and Fig. 4(a-e). Fig. 4(a) illustrated that the aortic tissue consists of three basic layers named: Tunica intima, the most inner layer, the second is tunica media and the outermost layer is tunica adventitia. On the other hand, the aortic tissue of

HCD-fed rats (Fig. 4b) revealed focal vacuolated areas, replacing dissolved fat in sub-endothelial layer accompanied with moderately oedema formed in the tunica adventitia indicating premature atherosclerosis. However, no histological alteration in aorta tissues (Fig. 4c-e) of both flaxseed oil and Rosuvastatin- treated groups confirming their effective cardiovascular protection.



**Fig. 4. Histological sections of aortic tissues of all tested groups (H&E x80)**

(a): Aorta section of normal control rats showing normal histological structure of the Tunica intima (t) and tunica media (m). (b): Aorta section of HCD-fed rats showing vacuolation in the tunica media (m). (c & d): Aorta section of rats treated with FO at LD and DD showing normal histological structure. (e): Aorta section of rats treated with Rosuvastatin showing no histological alteration

**Table 5. Histological examination of all treated groups after 12 weeks**

Groups	Control	HCD	HCD + FO (LD)	HCD + FO (DD)	HCD + Rosuvastatin
Vacuolization	-	+++	-	-	-

Abbreviations: HCD: High cholesterol diet; FO: Flaxseed oil; LD: Low dose; DD: Double dose.

No change (-), Sever (+++)

#### 4. DISCUSSION

Flaxseed oil (FO) is the richest plant source of omega-3 fatty acid, alpha-linolenic acid (ALA) which has been suggested to have a positive impact on CVD. In the present study, the fatty acid analysis of FO reveals unique feature of flaxseed oil in the accumulation of large amounts of linoleic acid (C18:3 $\omega$ -3), followed by oleic acid (C18:1 $\omega$ -9), and linoleic acid (C18:2 $\omega$ -6) which provides an excellent omega-6:omega-3 fatty acid ratio of approximately 0.15 : 1. The amount of total unsaturated fatty acids in flaxseed oil was 95%, while the amount of total saturated fatty acids was 5%. These findings are in agreement with the previous studies [39,40]. ALA metabolizes to longer-chain (n-3) PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [13]. Oleic acid is the principal member of monounsaturated fatty acid (MUFA) in human diet. MUFA consumption has been associated with decreased low-density lipoprotein (LDL) cholesterol, and possibly increased high-density lipoprotein (HDL) cholesterol [41]. MUFA can be beneficial over the short term (postprandial lipemia), primarily in relation to triglycerides metabolism, and over the long term in association with improvements in the plasma lipid profile [42].

The present results revealed that the rat model of dietary-induced hypercholesterolemia, which used in this investigation is effective in triggering hyperlipidemia and inflammatory reactions with alteration in the myocardium redox state. The present study provides evidence that flaxseed oil enriched with omega-3 Fatty Acids effectively reduces the risk of development of atherosclerosis in dose dependent manner in rats fed on HCD for 12 weeks. The highest effect was provided by FO at a dose of 540 mg/Kg/day, which equivalent to human recommended doses of 6 g flaxseed oil containing 4.04 ALA supplement per day. Our results are in agreement with the previous reports of many governments and public health authorities who recommended daily amount 1.6 g of ALA/day for males and 1.1 g/day for females [43,44] and with various expert committees who recommended 2- 3 g of ALA per day to achieve cardiovascular protection [16,45].

Lipid dysfunction is one of the major risk factors for many cardiovascular diseases [20]. Hypercholesterolemia in combination with elevated LDL-cholesterol concentration represent a major risk factor for the development and

progression of atherosclerosis and consequently of CVD. Atherosclerosis resulting from the pathological etiologies and risk factors like hyperlipidemia, hypertension, diabetes mellitus and obesity. Pathophysiology of atherosclerosis is very intricate, which is known to involve several mechanisms such as oxidation of LDL, endothelial cell dysfunctioning, lipoprotein level modification, adhesion of molecules, SMCs migration, plaque formation etc. [10]. Therapeutic agents available for the treatment of atherosclerosis produce their effect, mediated through one or more aforementioned mechanisms.

Preventive treatments of atherosclerosis are based on lipid lowering strategies, which also involve functional foods and dietary supplementation. Serum cholesterols and triglycerides play a key role in the pathogenesis of cardiovascular disease not only due to the development of atherosclerosis but also due to the modification of the composition, structure, and stability of cellular membranes. In this investigation, hyperlipidemia was induced by feeding rats with HCD supplemented with both cholesterol and cholic acid for 12 weeks. Cholic acid aids cholesterol absorption, and suppresses conversion of cholesterol to bile acids [46]. It was demonstrated that disruption of lipid homeostasis leads to cholesterol accumulation, formation of foam cells and progression of atherosclerosis [3]. In this concept, disturbance in serum lipid profile was detected in the animals fed on HCD as evidenced from marked elevation in serum concentration of TG, T.C, VLDL and LDL-c, and depletion in HDL-c result in more than 4-fold increase in the atherogenic index above normal level. These findings are in accordance with the previous studies [20,47], who demonstrated that high cholesterol and fat diet induce a disturbance in serum lipid profile. Whereas, simultaneous co-administration of either flaxseed oil at two doses or rosuvastatin and HCD for 12 weeks were significantly suppressed the lipotropic effects of HCD in heart tissue. Moreover, flaxseed oil increased the serum level of HDL-cholesterol, leading to dose-dependent improvement of the atherogenic index. Therefore, the obtained results suggested that flaxseed oil effectively reduces the risk of development of atherosclerosis in rats fed on atherogenic diet.

Elevated level of LDL-c in untreated HCD group is one of the early events in promoting atherosclerosis and cardiovascular events [3].

LDL serves as the primary source of lipid accumulation in the arterial wall during atherosclerotic lesion development. Meanwhile, HDL-c has atheroprotective functions stimulating cholesterol efflux and catabolism [48]. Therefore, the observed reduction in LDL-c and increase in HDL-c in FO-treated groups are important mechanisms for cardiovascular protective effect of FO. These were accompanied by down regulating circulated TG, T.C and VLDL resulting in significant decrease in atherogenic risk factor in dose dependent manner. These findings confirmed the potential effect of FO against HCD induced atherosclerosis to decrease the risk development of CVD. In addition, previous study confirmed that the protective effect of HDL-c is partly mediated through anti-inflammatory activity. It was demonstrated that HDL-c inhibited the proinflammatory polarization of macrophages as assessed by marker genes as TNF- $\alpha$  and IL-6 [49]. Our results are in agreement with the previous studies reviewed by Paola et al. [11] who reported that polyunsaturated fatty acids (PUFAs) slow coronary atherosclerosis by optimizing cholesterol concentrations and lowering plasma triglyceride levels.

The prevention of CVD has been linked to the consumption of fresh food items and plants rich in natural antioxidants because of their superior efficacy and safety compared to synthetic products [50]. Myocardium contains high concentrations of diagnostic markers included serum levels of AST, LDH and CK-MB. Once myocardial cells are damaged or destroyed, the cardiac membrane becomes permeable or may rupture, resulting in leakage of cytosolic enzymes into the blood stream with concomitant increases of their serum concentrations [47]. In addition, serum level of CK-MB isoenzyme activity is an important specific diagnostic indicator for cardiac function test, owing to its marked abundance in myocardial tissue and its virtual absence from most other tissues. Therefore, increased activity of serum CK-MB isoenzyme reflects the alterations in the plasma membrane integrity and permeability. Consistent with previous studies [50], in the present study, the elevation of serum levels of AST, LDH and CK-MB enzyme activities in HCD- group is clear indication of cardiac muscle injury and the weakness of the myocardial plasma membrane that induced by HCD [20,47]. Whereas, co-administration of FO with HCD for 12 weeks resulted in a dose dependent reduction in these serum myocardial diagnostic markers, concomitant with improvement in serum lipid profile as compared

to the untreated atherogenic animals. These findings could be attributed myocardial membrane stabilizing effect of flaxseed oil that maintains membrane integrity, thereby limiting the leakage of these biomarkers. By this mechanism, flaxseed oil supplement could possibly suppress the lipotropic effects of HCD on heart and suggested that consumption of flaxseed oil can have a positive effect on the myocardial membrane integrity.

Inflammatory factors play a crucial role in plaque initiation and progression [51]. It was demonstrated that macrophages play a central role in the pathogenesis of atherosclerosis, through actively participate in LDL uptake and lipid accumulation in the arterial wall [48]. Therefore, various pro-inflammatory biomarkers as TNF- $\alpha$  and IL-6 have been extensively investigated as a potential enhancement of cardiovascular risk and reflect different pathophysiological pathways underlying CVD [52]. In addition, C-reactive protein (CRP), a classical protein marker that is significantly elevated in the acute phase of inflammation, infection, and tissue damage [53]. Meanwhile, IL-10, an anti-inflammatory cytokine down-regulates the expression of pro-inflammatory cytokines as TNF- $\alpha$  and consequently reduces the damage caused by pro-inflammatory cytokines [9].

In the present study animals subjected to the induction of experimental atherosclerosis by feeding on HCD for 12 weeks displayed a marked decrease in IL-10 and elevation in the levels of inflammatory markers as CRP, TNF- $\alpha$ , IL-6, MPO and NO, associated with multiple biochemical sequels as disturbance in serum lipid profile, increase in serum diagnostic markers of heart injury and atherogenic index as compared with control group. The observed high levels of MPO activity can be used as an indicator of neutrophil accumulation and progression of atherosclerosis. Our findings is consistent with that of Orekhov et al. [54], who conducted immunohistochemical analysis of human aorta and proved the presence of proinflammatory macrophage marker (TNF- $\alpha$ ) in higher concentration in atherosclerotic lesions than in normal areas. Furthermore, our results are in line with previous reports, which demonstrate that cytokines are produced and act synergistically on almost all cells involved in the pathogenesis of atherosclerosis and participating in all steps of the process, from the early endothelial dysfunction to the late formation and disruption of a vulnerable plaque [52]. All these

inflammatory reactions were significantly preserved in FO or rosuvastatin- treated groups after 12 weeks as compared with HCD group. Therefore, these results suggested that supplementation of FO for decreased this infiltration of neutrophils, suppressed the production of proinflammatory cytokines as TNF- $\alpha$  and IL-6 and enhanced the anti-inflammatory cytokine (IL-10), confirmed the anti-inflammatory potential of FO [48]. In addition, FO suppresses the production of inflammatory mediator as nitric oxide (NO) is a clear reflects the inhibitory effect of FO on inducible NO synthase (iNOS). These findings indicated that FO exerts anti-inflammatory effects in animals fed on HCD in dose dependent manner by modulating signaling pathways that down regulate macrophage production of proinflammatory cytokines. Consistent with our results, Harding et al. [14] provided more evidence for using alpha-lipoic acid in reducing lipoprotein and inflammatory related atherosclerotic risk.

ROS can generate in vascular cells by oxidases such as NADPH oxidases, xanthine oxidase, lipoxygenases, cytochrome P450, or by uncoupling of the mitochondrial respiratory chain [55]. NADPH oxidases are considered as a major source of ROS in the vasculature and are key players in mediating redox signaling under physiological and pathophysiological conditions [56]. Overproduction of ROS results in increased oxidative stress. Oxidative stress accounts for oxidative modification of LDL. These modified LDL can trigger the development of the immune response and induce lipid accumulation in the arterial wall. Numerous evidence from the previous studies [3,9] reported that oxidation of LDL represents the important risk factors in the initiation and progression of atherosclerosis. Overproduction of ROS enhanced the macrophages to adhere to the artery wall. Atherosclerosis is caused by the accumulation of macrophages containing cholesterol (foam cells) in artery walls (in the intima). Meanwhile, under normal physiological conditions, this process is counter balanced by antioxidant agents, NADPH oxidase inhibitors [56] and XO [57].

In accordance with recent studies [47,50], the present study revealed that HCD enhanced myocardial oxidative stress as evidenced by enhancing ROS-generating enzymes (MPO, XO, NADPH oxidase and NO synthase as evidence from elevated level of NO) and suppressed the activities of antioxidant defense system (TAC, SOD and CAT) and depletion in GSH content as

compared to control group. As a consequence, the myocardial tissues become more susceptible to free radical damage and at a risk of atherosclerosis.

Previous studies [55,58], confirmed that ROS especially superoxide radicles, mediate several pathophysiological responses in the vessel wall. Superoxide radicles can react extremely fast with NO to form the more potent peroxy nitrite anion ONOO<sup>-</sup>, which has injurious effects on vascular cells. On the other hand, H<sub>2</sub>O<sub>2</sub> derived from O<sub>2</sub> is involved in vascular smooth muscle cell proliferation, apoptosis and migration. Vascular generation of ROS and NO plays a crucial role in the blood vessel and their production is tightly regulated to maintain vascular homeostasis. An imbalance in vascular ROS-production and scavenging system in heart tissues contributes to stimulating lipid peroxidation (MDA), protein oxidation (PC) and endothelial dysfunction, which is associated with cardiovascular disease. In the present study, the elevated level of myocardial MDA (a product of lipid peroxidation), PC (a product of protein oxidation) and NO is a clear manifestation of excessive formation of free radicals and oxidative stress [58,59]. Meanwhile, the decrease in myocardial GSH content in atherogenic rats may be due to its increased utilization to counteract overproduction of reactive radicles. Consistent with our results, other investigators reported an increase in lipid peroxidation in hyperlipidemia along with alternations of the activities of the enzymes involved in the peroxide metabolism [47,60].

Meanwhile the current study reveals that FO supplementation at dose two doses with HCD for 12 weeks could be able to prevent atherosclerosis as confirmed from inhibition of the NADPH oxidases, xanthine oxidase enzyme activities, strength the total antioxidant capacity, and preserve or increase synthesis of GSH in dose dependent manner. Consequently, FO appears to have a profound inhibitory effect on the ROS production induced by atherogenic diet. In the present study, a negative correlation could be observed between the activity of ROS-generating enzymes and serum levels of heart diagnostic enzymes confirming the potential protective effect of flaxseed oil against harmful effect of HCD. These results suggested that flaxseed oil enriched with omega-3 FA provide cardiovascular protection through antioxidant mechanism. The bioactive constituents in flaxseed oil strength the myocardial antioxidant defense system consequently, flaxseed oil can

inhibit the generation of an oxidative modified LDL, rebalance vascular redox state and efficiently early decline the development and progression of atherosclerosis. These findings are in agreement with the results of Xu et al. [61] who confirmed that flaxseed oil and alpha-lipoic acid combination ameliorates hepatic oxidative stress and lipid accumulation in comparison to lard. In addition, Xu et al. [62] reported that a combination of flaxseed oil and astaxanthin improves hepatic lipid accumulation and reduces oxidative stress in high fat-diet fed rats.

Furthermore, the obtained biochemical results were corroborated with the histological examination of aorta tissues of tested groups. Consistent with our results, Collins et al. [51] showed that atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the inner layer of the vessel wall. Meanwhile, FO treated rats possess protective effect on the aortic tissues as compared to untreated HCD-fed group. The cardiovascular health benefits of antioxidant and omega-3 fatty acids have been described in several epidemiological and clinical studies. Our results are consistent with previous studies carried out by Kim et al. [63] who found the traditional herbal formula made from *Scutellariae Radix* (SR), the root of *Scutellaria baicalensis*, possessing an anti-atherosclerotic potential by effective inhibition of LDL oxidation. The presence of oxLDL within the intima of the artery triggers an inflammatory response in the neighboring endothelial cells, which start producing proinflammatory cytokines and chemokines [2,9]. As mentioned above, flaxseed oil is rich in a number of chemical constituents which, work through different mechanisms included antioxidant and anti-inflammatory activities, was shown to reduce the risk of atherosclerosis in rats fed on HCD. In addition, reduction effect of FO in the incidence of myocardial inflammatory response is also related to lowered omega-6/omega-3 ratio of FO [64,65]. However, in the current study, rosuvastatin was more effective than FO in the reduction of the risk of development of atherosclerosis in animals fed on HCD. This improvement effect of rosuvastatin is also mediated by hypolipidemic, anti-inflammatory and antioxidant mechanisms. In addition, a number of clinical trials have indicated that statin therapy can improve the composition of plaques and delay or even reverse the progression of plaques [5].

## 5. CONCLUSION

Flaxseed oil in dose dependent manner effectively reduces the risk of development of atherosclerosis in rats fed on high cholesterol diet. This protection mediated through anti-inflammatory and antioxidant mechanism. Flaxseed oil exhibited effective cardiovascular protection at a dose of 540 mg/ Kg body weight / day, which equivalent to a human dose of 6 g FO/ day containing 4.04g ALA. Further studies still needed to standardize the flaxseed oil to justify its use in a suitable pharmaceutical form.

## ETHICAL APPROVAL

The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by Research Ethics Committee (REC) for Animal Experimentation with serial number of PT (804), at Faculty of Pharmacy, Cairo University, Cairo, Egypt.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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