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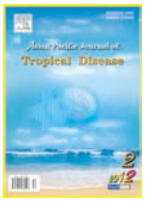

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Moringa oleifera Lam. A herbal medicine for hyperlipidemia: A pre-clinical report

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ABSTRACT

Objective: Herbal medicine is a use of plant's seeds, roots, barks, leaves, berries or flowers for medicinal purposes. In this present study, leaves of *Moringa oleifera* Lam were evaluated for its hypolipidemic, antioxidant, anticoagulant, platelet antiaggregatory and antiinflammatory activity in experimental animals. **Methods:** The experimental animals were divided into five groups of male Wistar rats each. The hydroalcoholic extract of *Moringa oleifera* (HEMO) was prepared and administered orally to hyperlipidemic rats for a period of 28 days. **Results:** The results showed that oral administration of HEMO at two different dose level (100 and 200mg/kg/b.wt) showed significant (P<0.001) reduction in elevated levels of body weight, total cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein and similarly significant (P<0.001) increase in high density lipoprotein level. Atherogenic index was significantly reduced in the *Moringa oleifera* treated groups at tested dose levels. The antioxidant parameters of SOD, Catalase, MDA was significant (P<0.001) in vivo. The intravenous administration of HEMO delayed the plasma recalcification time in rabbits and also inhibited ADP induced platelet aggregation *in vitro*, which was comparable to commercial heparin. The significant (P<0.001) inhibition of proinflammatory cytokines like TNF-α and IL-1α by HEMO were taken as it s antiinflammatory activity. **Conclusions:** All these results revealed the therapeutic potential of HEMO against vascular intimal damage and atherogenesis leading to various types of cardiovascular complications. Thus, *Moringa oleifera* can be prescribed as food appendage for coronary artery disease patients along with their regular medicines.

1. Introduction

Herbal medicines also known as phytomedicine or botanical medicine refers to use of plant's seeds, roots, barks, leaves, berries or flowers for medicinal purposes. Herbal medicine is fetching more in clinical research in treating and preventing various diseases. Plants had been used for medicinal purposes from ancient days. During 19th century, chemical analysis was available first and scientists instigated to extract and amend the active ingredients from plants. Later, chemists started making their own version of plant compounds and over time, the use of herbal medicines declined in favor of drugs [1,2]. Recently, the World Health Organization (WHO) appraised that 80% of people worldwide have faith on herbal medicines for some part of their primary health care. In Germany, nearby 600 – 700 plant-based medicines are available and are prescribed by 70% of German physicians. In the last 20 years in the United States, public frustration with the cost of prescription medications, combined with an interest in returning to natural remedies, has led to an increase in herbal medicine use.

Moringa oleifera Family: Moringaceae known as Drumstick tree in English. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods have been claimed in traditional literature to be valuable against a wide variety of diseases. Indian Materia Medica describes the uses of *Moringa oleifera* roots in the treatment of a number of ailments including asthma, gout, lumbago, rheumatism, enlarged spleen or liver, internal deep seated inflammations, dermic infection, gastrointestinal infection and calculous affections. In recent decades, the extracts of leaves, seeds and roots of *Moringa oleifera* have been extensively studied for many potential uses including wound healing, antitumour, antifertility, hypotensive and analgesic activity, antipyretic, antiepileptic, antiinflammatory, antiulcer, antispasmodic, diuretic,

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hypocholesterolaemic, antifungal, antibacterial, antifungal, aphrodisiac, cholagogue, antioxidant, hepatoprotective, immunomodulattors, cardiotonic and as cardiac and circulatory stimulants. It has been shown to have potential therapeutic values against cancer, diabetes, rheumatoid arthritis and other diseases and is being employed for the treatment of different ailments in the indigenous system of medicine particularly in South Asia [3,4,5]. Because of these wide varieties, the plant earned the name of "Miracle tree" and "Wonder tree" in Thailand.

Atherosclerosis is a disorder of blood vessels which preferentially affects the large and medium sized arteries and therefore, generally called as "hardening of the arteries". The main arteries affected are the aorta, coronary artery and it may also affect capillaries after a prolonged period. The term "atherosclerosis" is derived from the Greek word, "athero," meaning gruel or porridge, and "sclerosis," meaning hardening [6]. It is referred to as a "silent killer" and is one of the leading causes of death for both sexes in the developed countries and is on the rise in the developing countries like India [7]. The American heart association has identified the primary factors associated with atherosclerosis as elevated levels of cholesterol and triglycerides in the blood. The normal range for total blood cholesterol is between 140 and 200 mg per decilitre (mg/dL) of blood. Levels between 200 and 240 mg/dL indicate moderate risk and levels surpassing 240 mg/dL indicate high risk. Atherosclerosis is characterized by the accumulation of fatty substances, cholesterol, cellular waste products, calcium and other substances in the inner lining of the arteries. As these substances are deposited along the artery walls, fatty streaks develop and lead to decreased blood flow. These fatty streaks cause platelet aggregation and release platelet derived growth factor (PDGF), which is a powerful chemotactic agent and vasoconstrictor. The other autocrine and paracrine factors present in platelets and atheroma cells include endothelial growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), tumor necrotic factor - alpha (TNF-α), Interleukin-1 (IL-1) and T-lymphocytes which are mitogenic towards vascular monocytes. This formation of fatty streaks leads to the migration of smooth muscle cells from media to intima and its proliferation, all of which leads to the formation of more advanced lesions called as "fibrous plaques". These fibrous plaques are soon covered by a thick dome of connective tissue embedded with smooth muscle cells called "Fibrous cap", that have a core of foam cells, extra cellular lipids and necrotic cellular debris. These atherosclerotic plaques may be stable or unstable. Stable plaques regress, remain static or grow slowly over several decades and become calcified, stiffened and undergo further changes leading to partial or total block of the blood flow through the artery. Whereas the unstable plaques are vulnerable to spontaneous erosion, fissure or rupture and cause acute thrombosis which leads to the formation of clot or the broken piece of plaque called "atheroma" which may be carried by the blood and lodged in distal sides (embolisation) or block a narrow artery. Most clinical events result from unstable plaques, which lead to coronary or ischemic heart diseases, which are recognized as leading causes of morbidity and mortality in developed countries [6]. WHO predicted that heart diseases and stroke are becoming more deadly, with a projected combined death toll of 24 million by 2030. Moreover, the current predictions estimate that by the year 2020, cardiovascular diseases notably atherosclerosis will become the leading global cause of total disease burden [2]. Present study is a scientific approach to reestablish the traditional uses of *Moringa oleifera* and evaluates its hypolipidemic, antioxidant, anticoagulant, platelet antiaggregatory and antiinflammatory properties.

2. Materials and methods

2.1. Collection of plant material and extraction

The leaves of the plant *Moringa oleifera* were shade dried, milled and ground into coarse powder with the help of a mixer. The powdered material was subjected to cold maceration using sufficient quantity of ethanol and distilled water (1: 1) for ten days with intermittent shaking in a round bottomed flask. On tenth day, it was strained and marcs were pressed. The expressed liquids were added to the strained liquids and the combined liquids were clarified by filtration and the filtrate was subjected to distillation at temperature 600C for removing the ethanol and water. After distillation, the semi solid obtained was kept in a vacuum dessicator for drying [8]. For pharmacological experimentation, a weighed amount of dried extract was freshly suspended in solvent.

2.2. Animals

Healthy adult male albino rats of Wistar strain weighing around 120 to 150g were procured from the central animal house, J.S.S. College of Pharmacy, Ootacamund, Tamilnadu. The animals were housed under laboratory conditions (relative humidity 85±2%, temperature 22±1℃ and 12h light and 12h dark cycle). They were fed with standard rodent pellet diet (Gold Mohar, Lipton - India, Ltd.,) and distilled water ad libitum before the experiments. The study was approved by the institutional animal ethics committee for animal care and use. (JSSCP/IAEC/M.Pharm/ Ph.cology /10/2008-09).

2.3. Hypolipidemic activity

Hyperlipidemia was induced in rats by feeding them with atherogenic diet. The atherogenic diet (AD) consisted of 2g cholesterol, 8g saturated fatty oil (vanaspathy), 100mg calcium and 90g of powdered standard commercial pellet diet. All these ingredients were thoroughly mixed and made into pellets and fed to rats for 28 days. Along with their atherogenic diet, the rats were fed with weekly challenge

of oral vitamin-D3 [9]. Group 1 was fed with normal saline (10ml/kg p.o.). Atherogenic diet was given to groups 2, 3, 4 and 5 to induce Hyperlipidemia. Groups 3, 4 and 5 were administered with 2mg/kg, p.o. Atorvastatin and 100mg/kg, 200mg/kg, p.o. of hydro alcoholic extract of *Moringa oleifera* respectively for a period of 28 days. Group 2 served as hyperlipidemic control.

2.4. Antioxidant study

After blood collection, animals were sacrificed with excess doses of anaesthesia and heart was quickly removed and washed in ice cold saline. The heart (0.8g) were sliced into pieces and homogenized in ice cold tri-hydrochloride buffer (pH 7.2). The homogenates were centrifuged at 3200 rpm for 10mins. Supernatant obtained was used for estimation of reactive oxygen metabolites in terms of lipid peroxidation, superoxide dismutase (SOD) and catalase (CAT) [10].

2.5. Antiinflammatory activity

Estimation of proinflammatory cytokines like TNF-α, IL-1α were performed using ELISA Protocol [11]. The blood was collected by tail vein under light ketamine anesthesia and centrifuged at 3000rpm for 10 minutes for serum separation.

2.6. Anticoagulant activity

Anticoagulant activity was calculated by plasma recalcification method. Blood was collected from normal rabbits through the ear vein in 0.1M EDTA added tubes. The plasma was separated by centrifugation at 1000 rpm for 5min. 200μl of 0.01M calcium chloride was added to 100μl of the plasma. The time taken for the formation of a firm clot was noted immediately using a stopwatch. Similarly the plasma recalcification time was noted 10 min after the separate intravenous administration of heparin (1mg/kg/b.wt) and *Moringa oleifera* (100 and 200mg/kg/b.wt) individually [12,13].

2.7. Platelet antiaggregation activity

Platelet rich plasma (PRP) was prepared by centrifugation of normal rat blood at 1000rpm for 5min. 1.5 ml of acid citrate dextrose (ACD) was used as anticoagulant for every 8.5 ml of blood and this PRP was taken into glass cuvettes. Platelet poor plasma (PPP) collected by centrifugation (3000 rpm × 5 min) was kept as reference. The cuvettes were incubated at 37 ℃ for 5 min. The aggregation was initiated by adding 20μl of ADP (10μM) to 1ml of PRP. The aggregation was recorded for 5 min at 600 nm using spectrophotometer. The effect of different concentrations of *Moringa oleifera* on platelet aggregation was studied by incubation of PRP at 37 ℃ for 5 min before the addition of ADP [13,14]. Commercial heparin (20μg/ml) was used as reference standard.

2.8. Statistical analysis

Data were expressed as mean± SEM and subjected for One way Analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and Two way repeated measures ANOVA followed by Bonferroni post test by using Graphpad Prism Version 5.01(GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Qualitative and quantitative analysis of extract

The hydroalcoholic extract of *Moringa oleifera* (HEMO) was tested for preliminary qualitative phytochemical screening. The reports revealed the presence of alkaloids, carbohydrates, glycosides, saponins, proteins, phytosterols, tannins, phenolic compounds and flavonoids. The quantitative analysis of HEMO for 毬-sitosterol by LC-MS indicates the presence of 90mg/g of ◎-sitosterol.

3.2. Hypolipidemic activity

The weight gain in atherogenic diet group was significantly (P<0.001) higher than in normal control group, reflecting the influence of atherogenic diet on day 28. Experimental hyperlipidemia in rats was associated with an increase in serum lipid profile. Treatment with HEMO significantly (P<0.001) changed the lipid parameters (Table 1). Administration of HEMO for a period of 30 days was associated with significant (P<0.001) decline in TC, TG, LDL and VLDL with significant (P<0.001) increase in HDL levels. The atherogenic index was highly significant.

3.3. Antioxidant activity

Atherogenic diet fed animals showed a significant (P<0.001) reduction in SOD and significant (P<0.05) reduction in catalase levels while significant (P<0.001) elevation in MDA levels when compared to control group (Table 2). Treatment with *Moringa oleifera* (200mg/kg/b.wt) showed significant (P<0.001, P<0.05) elevation in SOD and catalase levels respectively with significant (P<0.001) reduction in MDA levels.

3.4. Antiinflammatory activity

Table 3 shows the quantitative measurements of TNF-α and IL-1α levels. The report reveals the significant (P<0.001) increase in serum TNF-α and IL-1α in atherogenic diet fed rats. Treatment with *Moringa oleifera* (100 and 200mg/kg/b.wt) significantly (P<0.001) decreased these parameters which is taken as the antiinflammatory activity of the *Moringa oleifera*.

3.5. Anticoagulant activity

Table 4 depicts the normal plasma recalcification time noticed in rabbits was 56± 3 Sec. Administration of

Table 1

Effect of *Moringa oleifera* on day 28 serum lipid parameter levels in atherogenic diet induced hyperlipidemic rats

Groups	Biochemical parameters (mg/dL)					
	TC	TG	HDL-C	LDL-C	VLDL-C	AI
1	98.76±0.526	54.82±1.516	46.59±0.316	58.72±0.265	10.86±0.226	2.03±0.280
2	246.82±1.146###	124.62±0.918###	34.62±0.918###	145.82±0.184###	24.62±0.149###	6.13±0.058#
3	118.04±1.320***	56.68±0.632***	42.85±0.516***	98.38±0.751***	11.33±0.224***	1.42±0.102***
4	136.12±1.268***	82.68±1.677***	38.62±1.254*	128.50±0.596***	16.53±0.081**	2.52±0.115*
5	121.08±2.331***	78.17±1.760***	41.00±0.516***	116.8±0.463***	15.63±0.180**	1.95±0.044***

# $P<0.005$, # # # $P<0.001$ as compared to control; *$P<0.05$, **$P<0.01$, ***$P<0.001$ as compared to sham control. Data expressed as mean± SEM, $n=6$; Two Way ANOVA followed by Bonferroni post test. Group: 1-Vehicle control; Group: 2- Hyperlipidemic control; Group: 3- Atorvastatin (2mgkg/b.wt, p.o); Group: 4- HEMO (100mg/kg/b.wt, p.o); Group: 5 - HEMO (200mg/kg/b.wt, p.o).

Table 2

Effect of *Moringa oleifera* on day 28 serum antioxidant parameters and thiobarbituric acid reactive substance in atherogenic diet induced hyperlipidemic rats

Groups	SOD (U/mg protein)	CAT (U/mg protein)	MDA (n mol/mL)
Vehicle control	12.12±0.512	7.99±0.061	230±0.002
Hyperlipidemic control	6.79±0.303###	3.23±0.116#	330±0.006###
Atorvastatin 2mg/kg	9.76±0.432	5.91±0.032	328±0.008
Moringa oleifera 100mg/kg	10.11±0.432*	6.18±0.074	324±0.002***
Moringa oleifera 200mg/kg	11.87±0.443***	7.68±0.081*	296±0.004***

# $P<0.005$, # # # $P<0.001$ as compared to control; *$P<0.05$, **$P<0.01$, ***$P<0.001$ as compared to sham control. Data expressed as mean± SEM, n=6; One Way ANOVA followed by Tukey's multiple comparison test.

heparin delayed the recalcification time to 148± 2 Sec and administration of the *Moringa oleifera* (100 and 200mg/kg/b.wt) delayed the time to 130± 5, 136± 4 Sec respectively which is much greater than the recalcification time of normal plasma.

Table 3

Effect of *Moringa oleifera* on day 28 serum Tumor necrosis factor alpha (TNF- α) and Interleukin 1 alpha (IL-1 α) levels in atherogenic diet induced hyperlipidemic rats

Groups	Concentration (pg/mL)	
	TNF- α	IL-1 α
Vehicle control	300±1.247	600±7.516
Hyperlipidemic control	650±9.763###	1100±3.543###
Atorvastatin 2mg/kg	619±5.728***	1074±4.857***
Moringa oleifera 100mg/kg	562±8.753***	950±7.428***
Moringa oleifera 200mg/kg	528±4.591***	850±4.652***

###$P<0.001$ as compared to control; ***$P<0.001$ as compared to sham control. Data expressed as mean± SEM, $n=6$; One Way ANOVA followed by Tukey's multiple comparison test.

3.6. Platelet antiaggregation activity

The platelet aggregation was induced by the addition of ADP to plasma of normal rat blood. The extent of aggregation of ADP induced platelet was shown in Fig 1 which indicate decrease in absorbance over a 5 minutes period with the decrease being drastic up to third minute indicating enhanced aggregation during first three minutes followed by a maintenance in next two minutes. Addition of *Moringa oleifera* (100 μg/mL) prevented the aggregation in first and second minutes as indicated by increased absorbance in first and second minutes. However absorbance decreased from third minute towards 0.5 and below it indicating capability of *Moringa oleifera* in prevention of platelet aggregation in initial stages. *Moringa oleifera* (200 μg/mL) inhibited platelet aggregation throughout the 5 minutes period as mentioned by increased absorbance and its maintenance including dose dependent prevention of platelet aggregation.

Table 4

Plasma recalcification time on day 28 serum of rats fed with atherogenic diet

Sample	Plasma recalcification time (in seconds)
Normal rabbit blood	56±3
Heparin	148±2
Moringa oleifera 100mg/kg	130±5
Moringa oleifera 200mg/kg	136±4

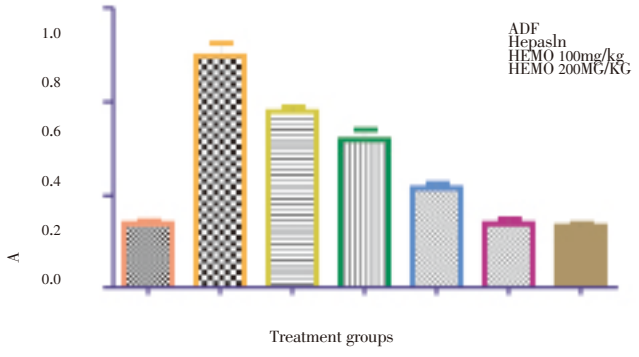

Fig. 1 - Effect of *Moringa oleifera* on ADP induced platelet aggregation inhibition (Treatment groups Vs Absorbance values)

4. Discussion

The quantitative analysis of hydroalcoholic extract of *Moringa oleifera* leaf for β-sitosterol by LC-MS, shown the presence of 0.09% β-sitosterol. Plant sterols inhibit the absorption of dietary cholesterol. β-sitosterol is one of the plant sterol which lowers the cholesterol level by lowering plasma concentration of LDL and by inhibiting the reabsorption of cholesterol from endogenous sources in association with a simultaneous increase in its excretion into faeces in the form of neutral steroids [15]. Therefore it can be concluded that β-sitosterol may be a bioactive phytoconstituent in the leaves of *Moringa oleifera* which may be responsible for its lipid lowering effect [16].

Atherogenic index indicates the disposition of foam cells or plaque or fatty infiltration or lipids in heart, coronary artery, aorta, liver and kidneys. Higher the atherogenic index, higher is the risk of the above organs for oxidative damage [10]. Treatment with *Moringa oleifera* (100 and 200mg/kg/b.wt) significantly reduced the atherogenic index in atherogenic diet fed rats on day 28.

Hyperlipidemia plays a major role in atherogenesis and it is an important risk factor for atherosclerosis. Diabetes mellitus is well known to be associated with an increase in the synthesis of cholesterol, which may be due to the increased activity of HMG CoA reductase [17]. The condition of hyperlipidemia during diabetes mellitus is well documented with profound alterations in the serum lipid levels along with an increased risk of premature atherosclerosis [18,19]. Therefore, in the present study, the lipid parameters were measured in atherogenic diet fed and as well as in diabetes induced rats. The study reveal the clear-cut abnormalities in lipid metabolism as evidenced from the significant elevation of TC, TG, LDL, VLDL and reduction in HDL levels in the atherogenic diet fed and diabetes induced rats on day 28.

In the present study, SOD, catalase, MDA levels were measured in atherogenic diet fed rats. Since high fat diet induces oxidative stress it leads to the generation of free radicals. These free radicals cause the peroxidation of lipids especially LDL thereby producing oxidized LDL. These oxidized LDL is taken up by the endothelial cells and macrophages and thus accelerates the atherosclerotic process. The antioxidant enzymes, mainly superoxide dismutase and catalase are first line defensive enzymes against these free radicals [20].

In hyperlipidemia, there are high levels of lipids and phospholipids. Due to this, there is increased production of arachidonic acid and PGs with the help of phospholipase A2 and LOX enzymes. Oxyradicals are produced during production of PGs. MDA is the end product of lipid peroxidation. Therefore, measurement of MDA gives an indirect evidence of LDL oxidation. The present work shows that *Moringa oleifera* treated groups have higher levels of antioxidative parameters like SOD and Catalase and decreased levels of lipid peroxidation indicating its efficacy to reduce the LDL-cholesterol oxidation.

It is well documented that flavonoids and polyphenols are natural antioxidants and have been also reported to significantly increase SOD and catalase activities [21]. High fat diet brings about remarkable modifications in the antioxidant defense mechanism against the process of lipid peroxidation. Potential antioxidant therapy should, therefore, include either natural free radical scavenging enzymes or agents which are capable of augmenting the activity of the antioxidants. A number of studies have investigated the ability of flavonoid-rich fraction to act as antioxidants. Flavonoids can directly react with superoxide anions and lipid peroxyl radical and consequently inhibit or break the chain of lipid peroxidation. This radical scavenging activity of extracts could be related to the antioxidant nature of polyphenols or flavonoids, thus contributing to their electron/hydrogen donating ability. Higher the molecular weight phenolics have more ability to quench free radicals and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups. The antioxidant activity of β-sitosterol has also been already reported. In the present study, the quantification of β-sitosterol has been done and from the above review it can be concluded that β-sitosterol in *Moringa oleifera* may be responsible for its hypolipidemic and as well as antioxidant properties. However, further studies are required to isolate the other major flavonoids present in *Moringa oleifera* for its potent antioxidant properties. The elevated levels of both SOD and catalase with *Moringa oleifera* treatment could be due to the influence of flavonoids and polyphenols. The present study has also indicated that hydroalcoholic extract of *Moringa oleifera* showed the presence of flavanoids, polyphenols and sesquiterpinoids. Studies revealed 8 μg/ml of phenolic and 27 μg/ml of flavonoid contents in hydroalcoholic extract of *Moringa oleifera*. It is well known that flavanoids, polyphenols and sesquiterpinoids are natural antioxidants. Thus it can be concluded that the antihyperlipidemic and antioxidant activities of *Moringa oleifera* may due to be the presence of these phytoconstituents. The several reported studies on Moring oleifera also evidenced the similar findings [3,15,21,22].

Atherosclerosis is also an inflammatory disease and does not result simply from the accumulation of lipids. Cytokines play an important role in atherosclerosis. Recruitment of circulating cytokines like TNF-α and IL-1α into vessel wall is crucial for the initiation and progression of atherosclerotic lesion [23,24]. In the present investigation, both quantitative and qualitative analysis of serum TNF-α and IL-1α were performed. The result reveals that treatment with *Moringa oleifera* significantly reduced the elevated levels of serum TNF-α and IL-1α in atherogenic diet fed animals.

The present study also reveals the platelet antiaggregatory and anticoagulant activity of *Moringa oleifera*. The obtained results were comparable to that of the standard heparin. Recent published studies have supported to the evidence for a prethrombotic state in hyperlipidaemia [25]. The consequence of plaque disruption in a coronary artery will depend partly upon the magnitude of the thrombotic response to this event. This is the rational for the antiplatelet and anticoagulant therapy in patients with CHD. Lipid lowering therapy may also be beneficial in this respect by reversing changes in the clotting pathway, fibrinolytic system and in blood platelets of hyperlipidaemic patients. [12,13]. Platelets play an important role in the process of atherosclerosis by adhering to the damaged regions (caused by reactive oxygen species) of the endothelial surface. The activated platelets form platelets to platelets bonds, binds also to leucocytes bringing them into a complex process of plaque formation and growth. The antiplatelet therapy constitutes the best available tool for ameliorating the mechanisms related to atherogenesis.

5. Conclusion

From the present investigation it is concluded that the hydroalcoholic extract of *Moringa oleifera* has hypolipidemic, antiinflammatory, antioxidant, anticoagulant and platelet antiaggregatory properties. Based on the results of our study and other previous studies, it can be suggested that *Moringa oleifera* has high therapeutic potential and it may serve as a safe and cheap source for the prevention of atherosclerosis and cardiovascular diseases. Since this plant has long been used as food and vegetable in many Asian countries and moreover without any side effects being reported *Moringa oleifera* can be prescribed as food supplement to CAD patients along with their regular lipid lowering medicines. In spite of these, further investigation and clinical studies are warranted to examine the mechanism of *Moringa oleifera* for their activities in other invivo and invitro models before declaring this as antiatherogenic agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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