Journal of Medicinal Plants Research Vol. 5(13), pp. 2670-2676, 4 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

The effects of cornelian cherry on atherosclerosis and atherogenic factors in hypercholesterolemic rabbits

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Accepted 15 April, 2011

Atherosclerosis results from gradual deposition of lipids in medium and large arteries and is the leading cause of death world wide. Due to the side effects of chemical compounds more attention has been paid to herbal remedies. This study was aimed to evaluate the curative effects of *Cornus mas* on atherosclerosis and its risk factors in hypercholesterolemic rabbits. Twenty five male New-Zealand rabbits weighing 2 to 2.5 kg were randomly desigrated into five groups of five and were fed for 60 days using the following diet regimens: Standard diet, standard diet plus *C. mas* (1 g/kg bw, daily), hypercholesterol diet (1%), hypercholesterol diet plus *C. mas* (1 g/kg bw, daily), hypercholesterol diet plus lovastatin (10 mg/kg bw, Daily). The concentration of total cholesterol (TC), LDL cholesterol, triglycerides (TG) HDL cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), antioxidant capacity, malondialdehyde (MDA) and fibrinogen were determined in rabbits in the beginning and at the end of the experiment. Fatty streak formation was also evaluated. *C. mas* powder significantly increased antioxidant capacity and decreased MDA, fibrinogen and AIP (AIP = log TG/HDL) in plasma. It also decreased TC, LDL and TG levels and atherosclerotic lesion in the aorta, although the reduction was not statisticant. Results showed that consumption of *C. mas* might be beneficial in hypercholesterolemic patients due to its antioxidant and anti-inflammatory properties.

Key word: Atherosclerosis*, Cornus mas*, hypercholesterolemia, rabbit.

INTRODUCTION

Atherosclerosis and its complications are the leading cause of death world wide (Yusuf et al., 2001). Atherosclerosis usually leads to damage and alteration in the blood vessels. Different environmental and genetic factors are involved in the progress of the disease (Braunwald, 2001). Although, the precise mechanism of atherosclerosis is still unknown, it has been suggested that inflammation and high cholesterol play key role in the formation and development of this disease (Pentikäinen et al., 2000). In studies carried out in 19 developed and developing countries it was found that a significant relationship exists between the level of diet cholesterol and the occurrence of cardiovascular disease (Moarreaf, 2004). Synthetic lipid lowering drugs (statins) and synthetic antioxidants are effective in treating

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hyperlipidemia and atherosclerosis (Lankin et al., 2003). However, due to their side effects (Heuer et al., 2000) attention has been drawn to the use of herbal drugs. Cornelian cherry (*Cornus mas)* is a member of the Cornaceae family which grows in most parts of Europe and Asia.

The fruit contains large amounts of anthocyanin, vitamin C, sugar, tannin and organic acids (Demir and Kalyoncu, 2003). Pelargonin and cyanidin are the main anthocyanin compounds of this fruit (Tural and Koca, 2008). *C. mas* fruit has anti-inflammatory and anti-oxidant activities and therefore, may enhance vessel function and affect atherosclerosis by affecting inflammatory and vascular factors (Kang et al., 2007). Considering the beneficial and effective properties of this plant on factors leading to atherosclerosis, in this study we aimed to evaluate the effects of *C. mas* on the development of atherosclerosis in hypercholesterolemic rabbits and also to determine its impact on hematologic factors of this disease.

MATERIALS AND METHODS

The fruits of *C. mas* were purchased from the vegetable market. The identity was made with the help of a botanist in herbarium unit of Shahrekord Medical Plants Research Center. A specimen with the code 201 was kept there for further reference. After the fruits were washed and the seeds removed, they were dried and finally used as powder.

Categorizing and treating the rabbits

Twenty five adult male New-Zealand rabbits weighing 2 to 2.5 kg were purchased from Razi institute in Karaj, Iran, and were transferred to the animal house in Shahrekord University of Medical Sciences. The rabbits were fed a basic diet for two weeks in order to adapt to the new environment. They were then randomly designated into 5 groups of five and fed with the following diet regimens for 60 days.

The first group fed with the basic diet, the second group fed with the basic diet along with *C. mas* powder (1 g/kg bw daily), the third group fed with a high cholesterol (1%) diet, the fourth group fed with a high cholesterol (1%) diet along with *C. mas* powder (1 g/kg bw daily) and the fifth group had a high cholesterol (1%) diet along with lovastatin (10 mg/kg bw).

The animals had free access to food and water during this period. The rabbits were given *C. mas* powder along with water by gavage. In the high cholesterol diet, cholesterol (Merck Company, Germany) was mixed with olive oil and fed to the rabbits by gavage. Due to potential effects of olive oil, the same amount of this oil was gavage fed to rabbits in the other groups. Blood samples were collected in the beginning and at the end of the period. Blood was centrifuged at 3000 rpm for 15 min and the serum was separated and stored in Eppendorf micro tubes at -17°C. The procedures in this study were approved by the ethics committee of the Cardiovascular Research Centre in Esfahan.

Biochemical tests

Blood glucose level was measured using Biosystem kits and the amount of HDL cholesterol, LDL cholesterol, triglyceride, total cholesterol, alanin amino transferase (ALT) and aspartate amino transferase levels were determined using Pars-azmoon commercial kits (Iran) on the Hitachi 902 Autoanalyzer (Japan). For plasma fibrinogen measurement, blood was collected and stored in citrate tubes and the plasma was immediately separated by centrifugation. Fibrinogen was meas6ured by the coagulation method using Mahsayaran kit (Iran). Antioxidant capacity and Malondialdehyde (MDA) levels were determined using spectrophotometer (Kostner et al., 1997; Miki et al., 1987). Atherogenic index of plasma (AIP), a marker of plasma atherogenicity on the basis of plasma lipids, was determined in all groups using the AIP=Log (TG/HDL) formula (Dobiasova, 2004).

Atherosclerotic lesion evaluation

After blood collection, the animals were anesthetized with chloroform and the aorta was dissected and washed with physiologic serum and stored in formalin 15%. Sections of aorta were stained with Haemotoxylin and Eosin to determine the grade of the atherosclerotic plaque. Atherosclerotic lesions were graded according to the Chekanov index and the thickness was assessed in the following categories (Chekanov, 2003):

Grade 1 – Plaque thickness less than half that of the aorta media (moderate forms of malfunction).
Grade 2 – Plaque thickness almost half that of the aorta media (abundant connective tissue in the plaque).
Grade 3 – Plaque thickness equal to that of the aorta media.
Grade 4 – Plaque thickness more than that of the aorta media.

Evaluation of antioxidant activity

Antioxidant activity of the serum was determined using ferric thiocyanate method (Masude et al., 1992). In this method, 500 µg of the serum was added to a reaction mixture containing 2.88 ml of 2.5% linoleic acid and 9 ml of 40 mM phosphate buffer in a vial. The vials were incubated at 40°C for 96 h. During incubation (every 12 h), 0.1 ml of each vial was diluted with 9.7 ml of 75% ethanol, 0.1 ml ammonium thiocyanate and 0.1 ml $FeCl_2$. The absorbance of samples was measured at 500 nm and the percent of inhibition (the capacity to inhibit the peroxide formation in linoleic acid) was determined using the following equation (A high inhibition percent indicates a high antioxidant activity).

Percent of inhibition=[1- (absorbance of sample)/(absorbance of control] x 100.

Determination of the major constituents of phenolic extract

To standardize the ethanolic extract of *C. mas*, total flavonoid and phenolic contents of the extract were determined as follows.

Total flavonoids determination

The amount of total flavonoids in the extract was determined using colorimetric method as described by Chang and colleagues (2002). 0.5 ml of the extract or Rutin (standard flavonoid compound) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm using Rutin solutions at concentrations of 25 to 500 ppm in methanol. The experiment was repeated for three times. Total flavonoids were expressed in terms of Rutin equivalent (mg/g), which is a common reference compound.

Total phenolic compounds determination

The amount of total phenolic compounds in the extract was determined colorimetrically using Folin–Ciocalteu assay as described by Kim and coworkers (2003). Briefly, 5 ml of the extract or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M).

The mixture was allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. A standard curve was prepared using 0, 50, 100, 150, 200, 250 mgL^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Total phenol value was expressed in terms of gallic acid equivalent (mg g^{-1}), which is a common reference compound. The experiment was repeated for three times.

Statistical analysis

Results were expressed as Mean ± standard deviation. Biochemical data were analyzed and compared between groups using the Kruskal-Wallis test with SPSS 15 software.
$P<0.05$ was considered statistically different.

RESULTS

Each 100 g of *C. mas* powder yielded 11 ± 0.4 g dried ethanolic extract. The amount of flavonoids (expressed as mg Rutin) and the total phenolic content (expressed as mg gallic acid) were found to be 17.98 ± 1.9 mg/g, and 215.56 ± 2.88 mg/g, respectively. As shown in (Table 1) the high cholesterol diet caused a significant increase in triglyceride, LDL, atherogenic index, blood glucose and AST levels ($P< 0.05$). Antioxidant capacity and HDL level had none significant reductions while ALT and MDA showed none significant increase.

A significant decrease in MDA, fibrinogen and atherogenic index levels and a significant increase in antioxidant capacity were observed in high-cholesterol plus *Cornus mas* group compared to that of high cholesterol group ($P<0.05$). In the former group also, there was an increase in HDL level and a decrease in blood glucose, TG, ALT, AST levels compared to that of the high cholesterol group, although not statistically different.

Histological sections of aorta artery stained from the 3 groups are shown in (Figure 1). Atherosclerotic changes were absent in normal diet group, whereas in the intima surface of the aorta artery from high-cholesterol diet group, many fat-laden macrophages plaque thicknesses were seen equal to degree 3 of Chekanov scale. In the high cholesterol plus *C. mas* diet group plaque degree was 2. (Figure 2) shows the average degree of fatty streak formation in the aorta of experimented groups. The degree of fatty streak formation was significantly higher in the group fed with high cholesterol diet compared to that of the group fed with normal diet ($P<0.05$). The intake of *C. mas* along with the high cholesterol diet caused a not significant decrease in the lesions compared to that of the group under high cholesterol diet.

DISCUSSION

Results showed that the group fed with *C. mas* had a significant decrease in MDA, fibrinogen and atherogenic index levels and a significant increase in the antioxidant capacity compared to that of the high cholesterol-fed group. Research shows that roughly 92% of the antioxidant activity of all fruits is carried out by two phenolic compounds anthocyanins and hydrolyzed tannins (Gil et al., 2000). Anthocyanins are the main phenolic compounds in *C. mas* (Seeram et al., 2002). The anthocyanin content of some species of *C. mas* is 10 to 15 times higher than that of other fruits containing anthocyanin (Vareed et al., 2006). Also, *C. mas* is an important source of vitamins C and E which have potent antioxidant activity (Tural and Koca, 2008). The vitamin C content of *C. mas* is two folds that of orange and some other fruits containing this vitamin (Seeram et al., 2002) (Demir and Kalyoncu, 2003).

Antioxidants reduce free radicals activity and prevents lipid peroxidation by affecting superoxides, hydrogen radicals and chelating metallic anions (Gülçin et al., 2005) and the beneficial effects of *C. mas* on lipid profile, atherosclerosis index or atherosclerosis lesions seem to be, at least in part, related to its antioxidant activity. Vitamin E strengthens the vitamin E present in LDL and acts by directly affecting superoxide removal and prevention of lipid peroxidation (Qing, 2002). *In vitro* studies also show that flavonoids are effective in delaying oxidation changes in ascorbic acid (Hughes and Wilson et al., 1977).

The aqueous and alcoholic extracts of *C. mas* have anti-cyclooxygenase effects and inhibit lipid peroxidation (Vareed et al., 2006). Malondialdehyde level in plasma which was decreased in *C. mas* treated rabbits, is an important index for free radicals injury and lipid peroxidation. Injury to vascular endothelial cells leads to activation of the prostaglandin pathway through cyclooxygenase (COX) enzyme and increases platelet activity which causes adherence and aggregation of these cells. Prostaglandin activation and platelet aggregation trigger the release of several compounds including MDA (Marrow, 1999). Research on the function of anthocyanins of *C. mas* revealed anti-inflammatory properties of this plant through inhibition of COX1 and COX2 enzymes. In addition, it has been shown that delfinidin glucoside is the main inhibitor of these enzymes in *C. mas* fruit (Seeram et al., 2002).

It has been shown that consumption of antioxidants increase antioxidant capacity and reduce the risks of elevated fibrinogen and cardiovascular disease (Caen et al., 1993). Fibrinogen is an inflammatory and coagulative factor and plays an important role in the process of atherosclerosis (Roitman et al., 2004). Induction of platelet aggregation and fibrin clot formation in the damaged vessels are the primary roles of fibrinogen in homeostasis (Crabtree, 1987). It has been shown that

Table 1. Effects of Cornus mas on atherosclerosis risk factors in hypercholesterolemic rabbits compared to control groups.

		Groups				
Risk factors	**Measure time**	**Basic diet**	**Basic diet+ *Cornus mas***	**High cholesterol**	**High cholesterol +*Cornus mas***	**High cholesterol +Lovastatin**
TC	Start	37.96±7.5	39.8±2.72	40±5.3	70±19.85	63.8±4.79
	End	38.57±7.41	22.6±3.09	1676±585.2	2224±450.6	1168±268.8
TG	Start	88.2±4.66	91.4±9.6	95±10.91	125.40±27.67	115.6±5.8
	End	88.8±4.04	81 6.8	772±199.93	458±47.47	594±122.7
HDL-C	Start	18.6±1.74	16.6±1.8	23±2.21	26±7.71	27.4±3.26
	End	15.8±2.2	14.2±1.06	30.4±1.5	101±4	71.12±10.96
LDL-C	Start	12.8±3.89	13.6 1.9	12.2±2.37	32.8±12.74	24.6±9.96
	End	7.6±1.69	5.8 ±1.7	1370±256	1028±66.36	682±190.4
ALT	Start	35.4±4.2	34±5.4	40.4±3.26	33±4.61	30±4.1
	End	30.8±2.2	25.8±3.6	104±4	72±20.75	138±33.6
AST	Start	21.4± 4.6	36±7.07	32.2±5.6	27.2±1.31	23.8±1.8
	End	40.4±2.3	31.4±5.3	152±47.15	104±18.6	144±19.13
Glucose	Start	79.6±5.5	93.2±5.7	78.4±8.23	97±18.9	89.2±8.5
	End	93±5.4	117±5.8	172±32.69	179±13.28	122±14.28
Fibrinogen	Start	256± 12	261 8.8	265±12.4	279±7.1	281.2±12
	End	283±18.6	253±10.5	356±18.15	223±18.16*	254.8±14.9
Malondealdehyde	Start	0.24±0.03	0.16 0.02	0.14±0.03	0.21±0.02	0.20±0.03
	End	0.31±0.20	0.2±0.26	0.45±0.06	0.24±0.03*	0.30±0.05
Antioxidant activity	Start	20.52±1.35	22.55 2.25	26.68±1.47	23.42±2.17	23.32±3.21
	End	23.41±2.77	44.34±10.34	3.16±0.74	48.34±12.39*	17.23±8.01
Weight	Start	2130± 88	2180±80	2080±37	2160±92	2100±77
	End	2644±113	2484±80	2662±77	2666±51	2574±71
Daily amount of consumed food	Start	90±4.4	80.6±0.87	90.8±3.6	90±4	78±3.7
	End	139.4±4.28	124.4±4.8	122.4±2.5	120±3.3	108.2±3.7
AIP	Start	0.6560±0.05	0.7380±0.06	0.5980±0.04	0.5875±0.16	0.6480±0.08
	End	0.7640±0.08	0.9680±0.08	1.3220±0.11	0.6202±0.04*	0.8920±0.18

Results are expressed as mean± SEM. *C. mas* powder significantly increased Antioxidant capacity and decreased Malondialdehyde (MDA), Fibrinogen and AIP (AIP = log TG/HDL) in the plasma ($P<0.05$), However, it had no significant effect on Total cholesterol (TC), Low density lipoprotein (LDL), Triglycerides (TG), High density lipoprotein (HDL), Total cholesterol (TC), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST).

oxidation of fibrinogen and the remnants of its fibrinolysis induce platelet aggregation and increase IL-6 levels (Roitman et al., 2004). Thus the effects of *C. mas* fruit in reducing fibrinogen might be beneficial in cardiovascular diseases.

In this study, in the group fed with *C. mas* the antioxidant capacity increased and the MDA produced in inflammatory pathways reduced, therefore, it is safe to say that anti-inflammatory agents and antioxidants present in this fruit play a key role in reducing fibrinogen

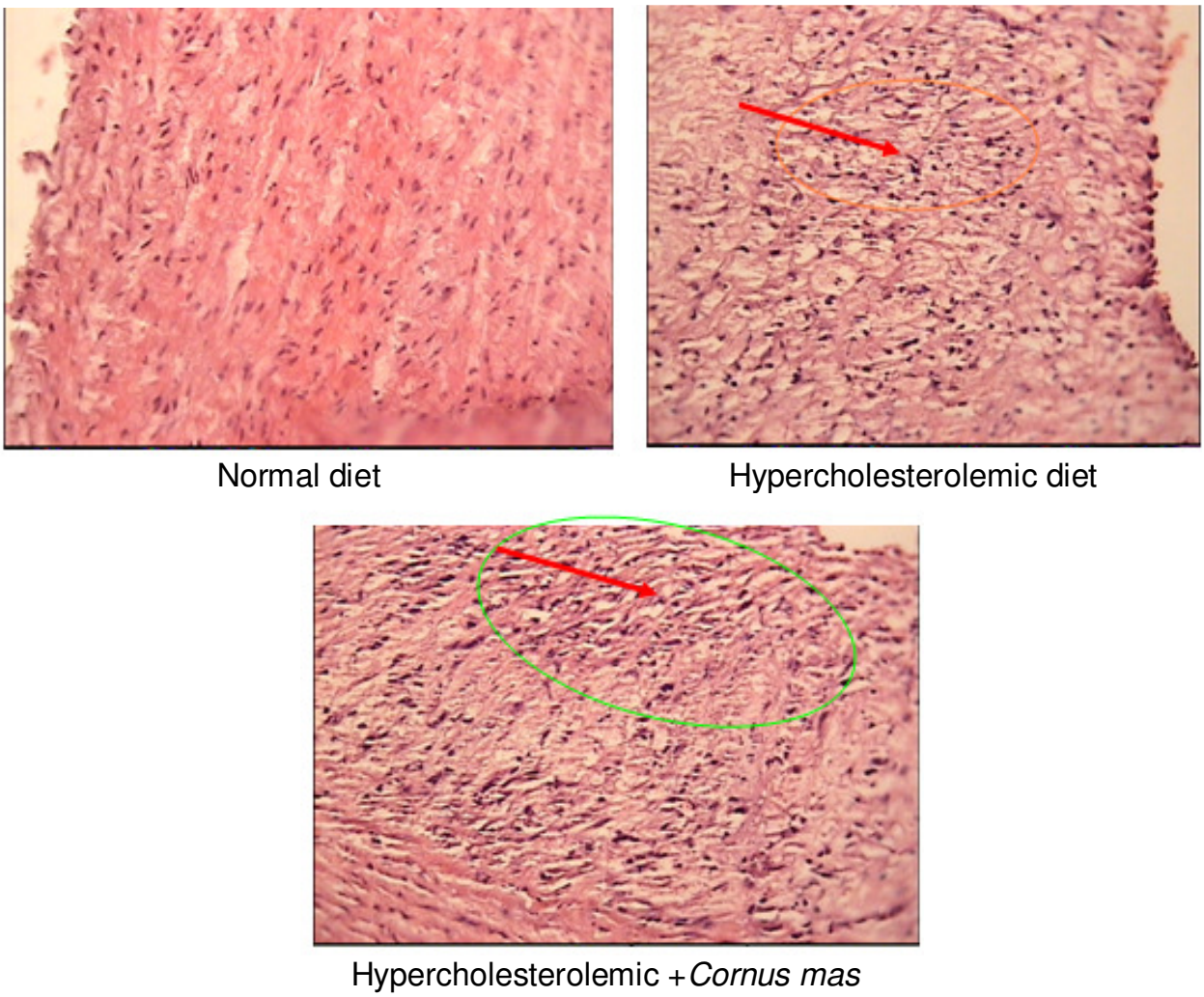

Figure 1. Aorta artery intima cross-section in the studied groups at the end of 2 months treatment in rabbits.

levels.

In our study, the atherogenic index, defined as the ratio of TG to HDL significantly decreased. Also there was an increase in HDL levels and a decrease in TG and LDL levels, although not statistically different. In studies carried out on mice fed with high cholesterol diet along with *Corni fructus*, it was found that this diet significantly reduced atherogenic index, cholesterol and lipid peroxidation (Park et al., 2009). Studies on the anthocyanin present in powdered fruits such as cherry also indicate that this diet reduces the amount of cholesterol, glucose, insulin, and TG and liver fat levels (Caen et al., 1993). It also increases the antioxidant capacity of plasma (Seymour et al., 2008). PPARs are a transcription factor that regulates tissue lipolysis and blood lipids metabolism. Recent studies have shown that extracts abundant in anthocyanins and phenolic compounds will enhance the function of these receptors (Munoz-Espada and Watkins, 2006; Xia et al., 2005). *C. mas* extract decreases lipid aggregation in the liver and enhances metabolic parameters related to high fat diet (Jayaprakasam et al., 2006). Even though the anthocyanin levels used in the latter study was higher than those in our study. However, it is hypothesized that phenolic compounds found in fruits in the same family as *C. mas* and cherry such as Quercetin and melanin may have stabilizing and syngergic effects on anthocyanins. Also a hypothesis exists that the low pH in cherry and other sour fruits leads to the stabilization and prolonging of anthocyanin effects (Seymour et al., 2008).

The two mentioned hypothesis may also be applicable to the effects of the diet containing *C. mas* powder which have a low pH (Karadeniz, 2002) and contain important phenolic compounds such as Quercetin (Pawlowska et al., 2010). Histologic results showed that *C. mas* consumption can reduce lesions in the aorta of rabbits fed cholesterol along with *C. mas* compared to that of the group under high cholesterol diet although it was not

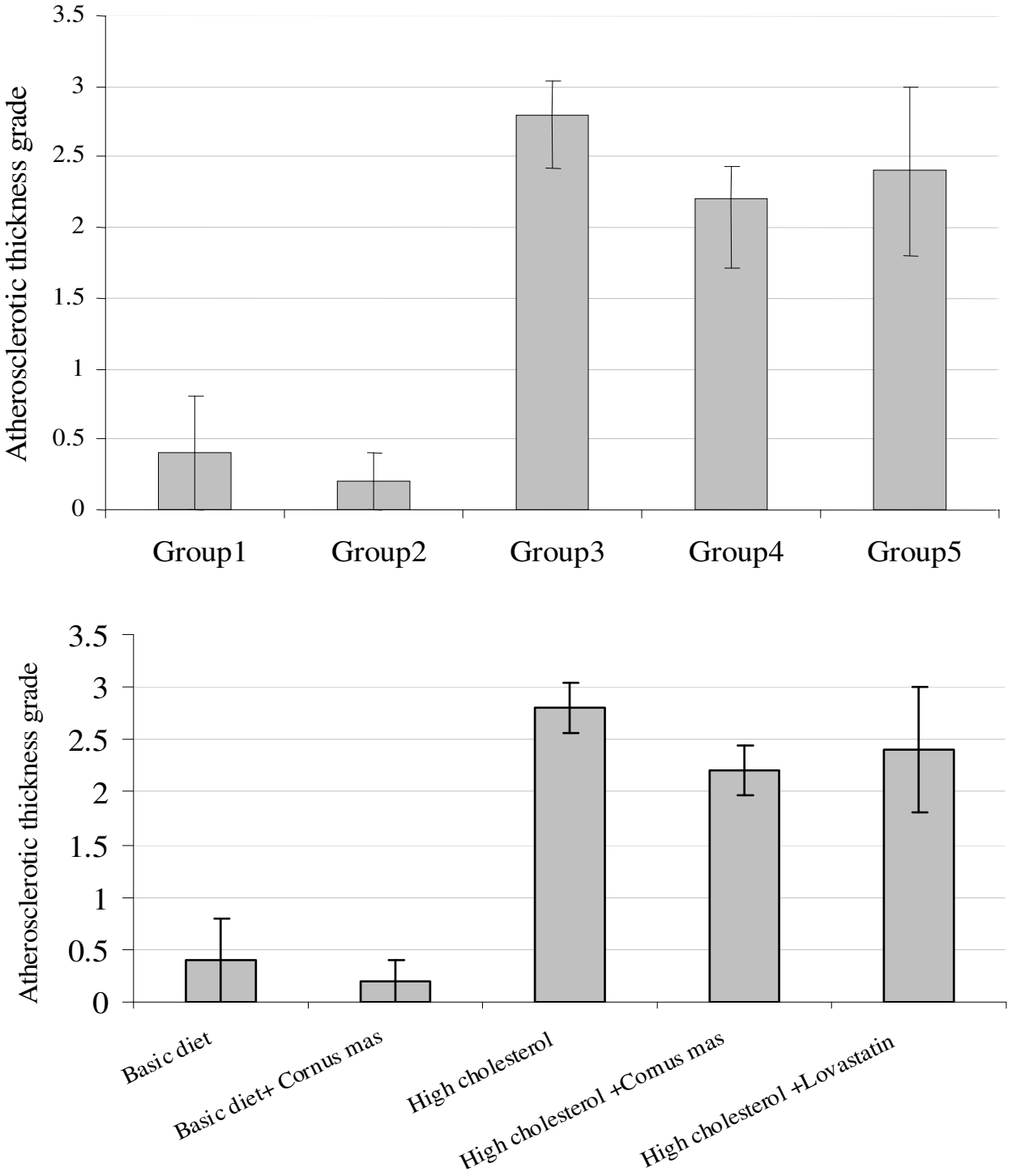

Figure 2. Mean of atherosclerotic thickness grade in studied groups.

statistically different. Research carried out on the function of *C. mas* extract showed that this compound acts by affecting the No/cGMP signaling pathways in the endothelial cells and causes expansion of the smooth muscles in the vessel walls (Kang et al., 2007). It was also found that these compounds inhibit the expression of vascular adhesive molecules and also reduce the expression of MCP-1 (as an inflammatory factor) in the human endothelial cells (Kang et al., 2007*).

Anthocyanins as important antioxidant compounds reduce the risk factors of cardiovascular disease by affecting the inflammatory process (Youdim et al., 2002;

Wang and Mazza, 2002) and preventing the coagulation of blood platelets (Renaud et al., 1992). Poly-phenolic compounds also in addition to their antioxidant properties exert protective effects on the cardiovascular system by enhancing the function of the endothelium, prevention of angiogenesis, proliferation and migration of cells in the blood vessel (Stoclet et al., 2004). In the present study the decrease in malondialdehyde and fibrinogen levels and the increase in antioxidant capacity as inflammatory and coagulative factors indicated the effectiveness of this fruit in reducing the risk factors for atherosclerosis disease. Some of the factors measured in this study were not statistically significant perhaps because the number of animals in each group was quite small. Further research is necessary to determine the effective dosage of this fruit as a medicinal plant for the treatment of atherosclerosis and diabetes.

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