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Study of Hypocholesterolemic and Antiatherosclerotic Properties of *Medicago sativa* L. Cultivated in Egypt

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ABSTRACT

Alfalfa was reported to be hypocholesterolemic and antiatherosclerotic. Saponin glycosides were suggested to be responsible for this activity by neutralizing cholesterol in the stomach, enabling it to be excreted from the body. This makes alfalfa superior to the current anti-cholesterol medications which act by blocking cholesterol synthesizing apparatus. This study was undertaken in an attempt to prepare an alfalfa extract with high saponin content and free or low content of other toxic constituents found in alfalfa (canavanine and coumestrol), which causes serious side effects. A patent process was used to render alfalfa toxin free and to produce a drug present in the international market. Our study is based on monitoring the Egyptian crop at two different localities for its saponin, canavanine and coumestrol contents at different growth stages. The study showed that the tested extract of the chosen stage to be biologically studied (hypocholesterolemic and antiatherosclerotic effects) on the basis of the highest saponin content was just before fruiting stage of the locality A. This stage was free from both coumestrol and canavanine. The study proved that the Egyptian crop of alfalfa was found to safely reduce natural cholesterol and to possess a strong antiatherosclerotic activity. This extract (I) produced the most significant decrease in total cholesterol and LDL-cholesterol by 85.1 and 88%, respectively, of the corresponding levels in hypercholesterolemic rabbits. This decrease is more significant than that produced by gemfibrozil (73 and 74%) upon concomitant administration with a cholesterol enriched diet using the same animal model at the tested dose level. Also, it was obvious that all alfalfa preparations produced significant antioxidant properties. All alfalfa extracts possessed antiatherosclerotic activity as observed by the almost normalization of the aortic sections upon concomitant use of alfalfa extracts with cholesterol-enriched diet.

Key words: alfalfa, saponins, canavanine, coumestrol, cholesterol, LDL, hypercholesterolemia, atherosclerosis

INTRODUCTION

Alfalfa, *Medicago sativa* L.[1,2] is a widely cultivated, environmentally tolerant forage crop. Alfalfa is also known as Lucerne, Buffalo Herb and Purple medic[2]. The whole plant material contains many important substances, including steroidal saponins, sterols, coumarins, flavonoids, isoflavones and coumestrol analogues, alkaloids, acids, enzymes, vitamins, amino acids, sugars, proteins (25% by weight), minerals, trace elements, and other nutrients[3]. Sprouts and seeds contain canavanine, a toxic amino acid analogue of arginine derivative[4].

The alfalfa saponins of the aerial parts and roots have been identified as mono-, bis-, or tridesmosides of medicagenic acid, hederagenin, zanhic acid and soysapogenol B[5]. Among them, medicagenic acid and hederagenin glycosides have been recognized as the "biologically active" saponins. The most often used methods of determining saponins are based on the inhibition of the growth of *Trichoderma virde* and plant seedlings or on the haemolytic activity[6].

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Several reports suggested that saponins are responsible for the reduction of cholesterol absorption and prevention of atherosclerotic plaque formation in experimental animals[7-9]. Alfalfa meals prevented hypercholestrolemia, triglyceridemia and atherogenesis in cholesterol fed chicks[9], rabbits[10] and Cynomolgus monkeys[11].

Toxicity reported of alfalfa is due to its content of alkaloid *l*-canavanine and the coumistan coumestrol[12]. Many commercial alfalfa products contain coumestrol (20-190 ppm), which lead to pathological side effects. Canavanine has been associated with systemic lupus-like erythmatosus due to an alteration in red blood cells[12,13]. A preparation derived from a technically advanced patented extraction process, which removed coumestrol and canavanine from alfalfa leaf and provided an extremely potent form of saponins had been shown to reduce serum cholesterol levels without serious adverse effects[12].

Therefore, for the great medicinal value of this plant and its potential in reducing hypercholesterolemia, the Egyptian crop was studied with the aim of following up the bioactive constituents at the different growth stages. One of our main targets is to reach a proper extract that satisfies

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the criteria of being effective at proper concentrations and has enough specificity for the indicated use.

Since saponin glycosides were shown to be responsible for the hypocholestrolemic effect[7-9], it was necessary to estimate the percentage of saponins and to test the presence or absence of canavanine and coumestrol in the collected samples at the different growth stages.

MATERIALS AND METHODS

I. *Plant Materials*

The seeds bought from the Egyptian market were germinated in Experimental Station of College of Pharmacy, Cairo University (locality A). The seeds bought from Fayoum Governorate were cultivated in the same place (locality B). Different crops of the herb were collected every 45 days from both localities starting from mid-November 2002 to mid-June 2003. Identification of the plant material was carried out by Dr. H. A. Hosny, Professor of Taxonomy, Faculty of Science, Cairo University.

II. *Preparation of Plant Extracts for Biological Evaluation*

Samples for biological evaluation were prepared by reflux of 5 kg of the plant material (combined of samples 4 and 5 of locality A) with 25 L of 30% ethanol (extract I) and 4 kg of the plant material with 20 L of water (extract II) for 1.5 hr twice. The combined extracts of each alcoholic and water extracts were evaporated and kept in dissecator.

III *Chemical Analysis*

(I) *TLC Analysis for the Presence of Canavanine Alkaloid*

Ten grams of each of the collected samples were extracted by reflux twice with 100 mL of 30% methanol, each time for 1.5 hr. The concentrated aqueous extract was purified by extraction with chloroform followed by *n*-butanol saturated with water.

The concentrated *n*-butanol extract was tested for the presence of canavanine on silica gel TLC precoated plates and using *n*-butanol/acetic acid/water (80:20:20, v/v/v, system 1) and chloroform/methanol/ammonia (40:40:20, v/v/v, system 2) as solvent systems against authentic sample of canavanine (limit of detection 5 μg/mL). The spots were visualized by spraying with ninhydrin reagent.

(II) *TLC Analysis of Coumestrol*

Ten grams of each of the collected samples were extracted by reflux twice with 100 mL of 90% methanol, each time for 1.5 hr. The combined extracts were purified by shaking with petroleum ether. The volume was adjusted to 100 mL in a volumetric flask with methanol. The methanolic extract was used to test for the presence of coumestrol using TLC on silica gel plates against authentic sample of coumestrol (limit of detection 1 μg/mL) using chloroform/methanol (9:1, v/v, system 3) as developing solvent. Coumestrol was visualized under UV 366 nm blue fluorescent spot.

(III) *HPLC Analysis of Coumestrol*

HPLC analyses were performed on HP 1100 liquid chromatograph with binary pump equipped with 1100 series variable wavelength UV detector. Separation was performed on RP-18 column 5 μm (250 × 4.5 mm I.D.). Chromatographic runs were carried out with water/methanol (2.1:1, v/v) at a flow rate of 1 mL/min and monitored at 260 nm. Injection volume was 10 μL. Standard coumestrol (0.8 mg/10 mL) was used.

The purified extract prepared above was filtered through a membrane filter (45 μm) and 10 μL of the sample was injected in the system adopting the conditions mentioned above. Retention times and UV absorption patterns of the tested samples were compared with the authentic standard analyzed under the same condition (limit of detection 5 ng/injection).

(IV) *Determination of the Saponins Content*

1. *Haemolytic Method for Determination of Saponins*

Samples from both localities at the different growth stages were assayed using the method of El-Hossary G. A.[14] The percentages of the haemolytic saponins calculated as white saponin of Merck are given in Table 1.

2. *Colorimetric Methods for Determination of Saponins*

A. *Colorimetric Method Using Acetic Acid/Sulfuric Acid Reagents*

The procedure adopted in this assay was as described by Honerlagen and Tretter[15].

B. *Colorimetric Method Using Vanillin/Sulfuric Acid Reagents*

The procedure described by Hiai *et al*.[16] was followed.

Table 1. Percentage of saponins in the different growth stages of the herb collected from the two localities (calculated as white saponin of Merck)

Stage	Experimental station (locality A)	Fayoum (locality B)
Nov. 2002 (sample 1)	0.13	0.06
Jan. 2003 (sample 2)	0.12	0.07
Mar. 2003 (sample 3)	0.15	0.12
May 2003 (sample 4)	0.19[a]	0.19[a]
Jun. 2003 (sample 5)	0.20[a]	0. 11

[a]The highest haemolytic saponin content calculated as white saponin of Merck.

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IV. *Biological Analysis*

(I) *Evaluation of Hypocholesterolemic Effect of the Different Extracts*

The protective effect of the alfalfa extracts against hypercholesterolemia and development of atherosclerosis in hyper-cholesterolemic rabbits were tested. Induction of hypercholesterolemia was carried out by feeding rabbits [70 male New Zealand rabbits (*Oryctolagus cuniculus*), weighing around 1.5-2 kg] 2% cholesterol-enriched diet for 28 days. Rabbits were classified into 7 groups. The first group received cholesterol only for 28 days and served as a control (hypercholesterolemic group). Four groups received cholesterol-enriched diet containing the different forms of alfalfa extracts [1 & 2% of extract I (30% alcoholic) and 1 & 2% of extract II (aqueous) of combined samples 4 and 5 of locality A, collected just before fruiting] for a period of 28 days. A group received the cholesterol-enriched diet for 28 days containing 40% ground seeds. An additional group of rabbits was fed plain chow diet only and served as a normal group.

In the end of the feeding experiments, food was withheld for 16-18 hr and all animals were killed and dissected. Blood and aorta were separated before following investigations were performed:

1. Plasma lipid profile including total cholesterol (TC)[17], triglycerides (TG)[18], HDL- & LDL-cholesterol (HDL-C & LDL-C)[19].
2. Histopathological examination of the aorta for assessment of atherosclerosis.
3. Plasma malondialdehyde (MDA) level, as an index of lipid peroxidation was determined colorimetrically based on the reaction of thiobarbituric acid with the acid hydrolysed product of MDA[20].

(1) *Histopathological Examination of the Rabbit Aorta*

Staining of a paraffin section with Hematoxylin and Eosin (Hx and E) was done according to the method of Carleton (1967)[21].

The aorta was removed from the formol saline and a block of hard paraffin with the tissue in its center was prepared for sectioning. The paraffin (wax around the organ) was dissolved by putting the slide in xylol solution for 3 min, and then the xylol was replaced by alcohol by putting the slide in absolute alcohol. The slide was hydrated by putting it in descending grades of alcohol (in 100% alcohol then in 90% then in 70% alcohol and finally distilled water) for 3 min in each step. The section was stained in Hematoxylin for 7 min. This basic stain will stain the nuclei and the basophilic structures of the cytoplasm with a blue color. The slide was put in tap water for 5 min to blue the section. Since the nuclei will appear darker after tap water. The section was stained in Eosin for 1 min. Eosin stains the acidophilic structures of the cytoplasm with red color. The slide was then washed in distilled water for 3 min. Dehydration of the slide was done by putting it in ascending grades of alcohol (for 1 min in 70% alcohol, then for 3 min in 90% and for another 3 min in 100% alcohol). The slide was put in xylol in order to clear it from alcohol and to allow it to be miscible with Canada balsam. A drop of Canada balsam was put on a clean cover and the slide was removed from xylol with its face downwards and was put quickly on the cover. The stained section was thus ready for microscopical examination.

(2) *Determination of Intimal Thickness*

To avoid error due to pathological heterogeneity of atherosclerosis, a total of ten specimens from the aorta of one animal were subjected to the measurement of intimal thickness (μm) by an image analyzer. This measurement was done in Histology department, Faculty of Medicine, Cairo University. The mean of the intimal thickness from different sections represented the intimal thickness of the animal. The average intimal thickness for 10 animals was calculated for each group.

(II) *Statistical Analysis*

Data are presented as mean ± SEM. Statistical significance between groups was evaluated using Student's *t*-test.

RESULTS

I. *Chemical Analysis*

It was found that all samples obtained from locality A contain higher percentage of saponins than the corresponding samples collected from locality B. Sample 4 from both localities and sample 5 from locality A, collected just before fruiting (May-June) were the samples containing the highest saponin content. Results are shown in Table 1.

TLC analysis revealed the presence of canavanine alkaloid (as pink spot with ninhydrin reagent; R_f 0.12 in system 1) only in the seeds bought from the market and cultivated in locality A. Canavanine alkaloid was absent from all other samples. TLC analyses of all herb samples as well as seeds bought from market showed weak or very weak bluish-violet fluorescent spot parallel to authentic coumestrol (UV light, R_f 0.74, system 3), while the seed sample from locality B showed absence of coumestrol. However, HPLC analyses confirmed the absence of coumestrol in all samples from both sources.

II. *Biological Analysis*

(I) *Effect of Cholesterol-enriched Diet*

Feeding a 2% cholesterol-enriched diet to rabbits for 28 days resulted in a significant increase of plasma TC, HDL-C and LDL-C levels reaching up to 1563, 260 and

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Table 2. Effect of alfalfa extracts on plasma lipid parameters and malondialdehyde levels

Plasma parameter[a]	Normal	Hypercholesterolemic	Extract I (locality A)		Extract II (locality A)		Seeds 40%
			1%	2%	1%	2%	(locality B)
TC	88.1 ± 4.7	1376.0[b] ± 172.4	812.8[c] ± 73.0	204.5[c] ± 23.2	943.0 ± 137.8	374.9[c] ± 80.1	324.1[c] ± 39.1
HDL-C	27.1 ± 3.0	70.4[b] ± 15.3	31.1[c] ± 4.8	22.7[c] ± 1.5	26.9[c] ± 3.0	18.8[c] ± 1.6	14.8[c] ± 1.5
LDL-C	34.5 ± 6.1	1280[b] ± 166.9	764.7[c] ± 74.3	153.3[c] ± 22.3	895.9 ± 135.9	337.8[c] ± 76.7	290.1[c] ± 39.8
VLDL-C	26.6 ± 1.6	25.5 ± 3.1	18.1 ± 2.3	28.4 ± 14.0	20.3 ± 2.2	18.4 ± 3.4	19.2 ± 2.3
TG	132.9 ± 8.0	127.6 ± 15.5	93.3 ± 11.8	142.1 ± 14.0	101 ± 13.6	92.1 ± 17.0	96.0 ± 11.5
MDA	2.1 ± 0.14	5.8[b] ± 0.22	2.6[c] ± 0.17	3.0[c] ± 0.39	1.9[c] ± 0.29	2.3[c] ± 0.17	1.3[c] ± 0.17

[a]TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol; TG: triglycerides; MDA: malondialdehyde. Values are mean ± SEM (TC, HDL-C, LDL-C, VLDL-C& TG are in mg/mL and MDA in nmol/mL).
[b]Represents significant difference from normal at $p < 0.05$.
[c]Represents significant difference from hypercholesterolemic control at $p < 0.05$.

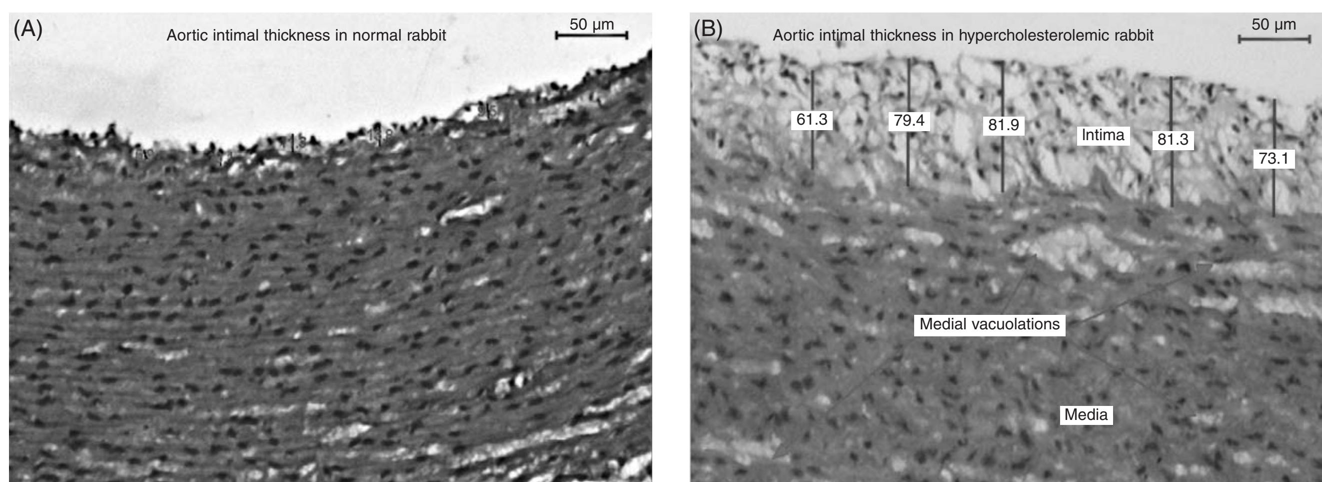

Figure 1. (A) A photomicrograph of a section in ascending aorta of a normal rabbit. Hx and E, 100×. (B) A photomicrograph of a section in ascending aorta of a hypercholesterolemic rabbit. Hx and E, 100×.

3710% of normal values, respectively (Table 2). No change in the levels of plasma VLDL-C and TG were noted. The cholesterol-enriched diet also produced a significant increase in plasma MDA level reaching to 276% of the normal level (Table 2).

Histopathological examination of normal and hypercholesterolemic rabbits' aorta (Figure 1A and B) revealed that in normal rabbits, aorta wall has a uniform thickness with no bulging in the lumen and the endothelial lining is intact without any interruption. On the other hand, hypercholesterolemia caused marked alterations in the aortic wall with the appearance of multiple large atheromatous plaques. Endothelial damage and massive degeneration with smooth muscle proliferation were detected in the plaques. The average intimal thickness of aorta was multiplied almost 19 times as compared to average normal aorta. Similar data were demonstrated in rabbits fed a cholesterol-enriched diet[22].

(II) *Effect of Alfalfa Extracts on the Cholesterol-enriched Diet*

The influence of 1 and 2% extracts I and II (locality A) as well as the ground seeds from locality B on hypercholesterolemia and atherogenicity was observed in hypercholesterolemic rabbits.

As shown in Table 2, all forms of alfalfa extracts produced significant decrease in plasma total-, HDL- and LDL-C levels, except 1% extract II. Upon comparison, 2% of extract I produced the most significant decrease in TC and LDL-C by 85.1 and 88%, respectively, of the corresponding levels in hypercholesterolemic rabbits; which is a more significant decrease than that produced by gemfibrozil (73 and 74%) upon administration concomitantly with a cholesterol enriched diet using the same animal model[20]. Also, it was obvious that all alfalfa preparations produced significant antioxidant properties (Table 2) by the significant reduction of the malondialdehyde (MDA) level.

It is worthy to mention that NO external signs of toxicity appeared on the animals received cholesterol-enriched diet containing the different forms of alfalfa extracts, e.g. no weight loss, no diarrhea, and no abnormal movements, no deaths, no outrageous violence, no noticeable sedation, no hair loss.

The beneficial effect of alfalfa preparations against hypercholesterolemia and atherosclerosis was further evaluated by histopathological examination of the aorta

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wall for the assessment of atherosclerosis in presence and absence of the alfalfa intake. Remarkable inhibition of progression of intimal thickening by hypercholesterolemia was noticed upon concomitant administration with all forms of alfalfa extracts, as shown in Figures 2 and 3. The results indicated normalization of the aortic walls with regard to appearance and thickness.

The decrease in aortic thickness from average hypercholesterolemic aorta was 91%, 90%, 89%, 89%, 91% for the groups 1% extract I, 2% extract I, 1% extract II, 2% extract II (locality A), and ground seeds (locality B), respectively.

DISCUSSION

I. *Chemical Analysis*

Trials for determining the saponin content using colorimetric methods (acetic acid/sulfuric acid[15] and vanillin/

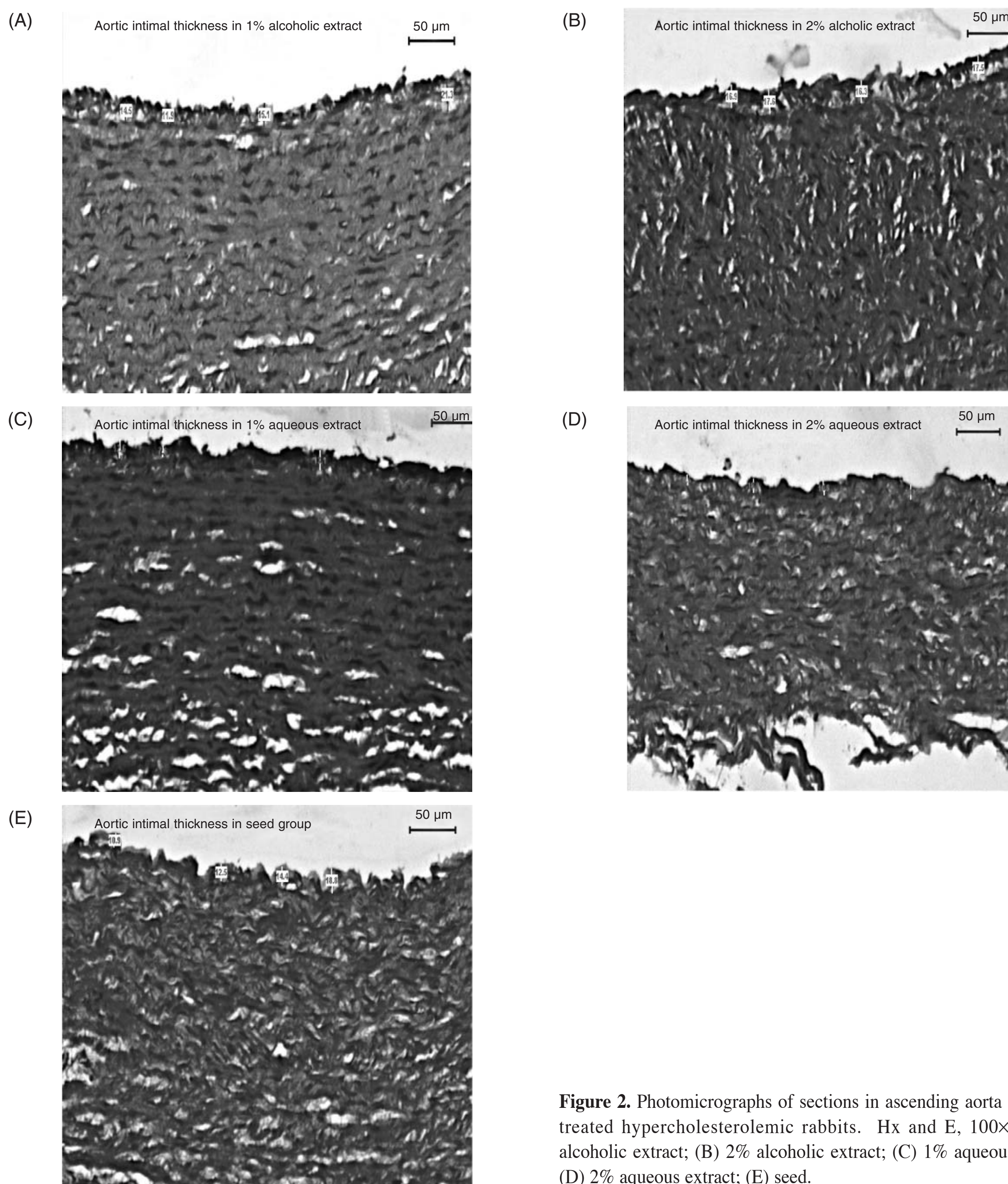

Figure 2. Photomicrographs of sections in ascending aorta of alfalfa-treated hypercholesterolemic rabbits. Hx and E, 100×. (A) 1% alcoholic extract; (B) 2% alcoholic extract; (C) 1% aqueous extract; (D) 2% aqueous extract; (E) seed.

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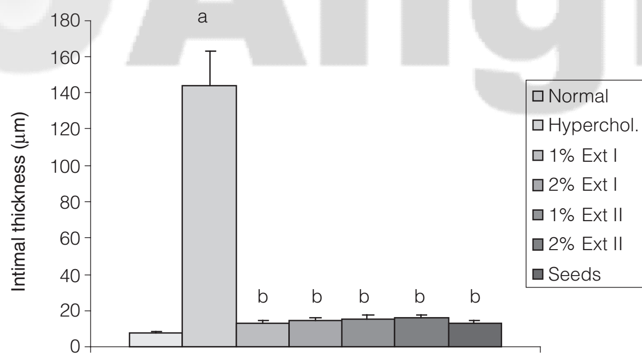

Figure 3. Intimal thickness of aorta in normal, hypercholesterolemic, 1% extract I, 2% extract I, 1% extract II, 2% extract II, and seeds groups. Values are means ± SEM. Each mean represents the average reading of 10 sections for each aorta for 7 animals (i.e. each reading is an average of 70 values). [a]Significant difference from normal at $p < 0.05$. [b]Significant difference from hypercholesterolemic control at $p < 0.05$

sulfuric acid reagents[16]) were found to be unsuccessful. Therefore, the saponin content was estimated in the different growth stages of two crops cultivated in locality A and locality B using the haemolytic index method which is one of the methods used for determining the biologically active compounds[6]. For safety, the presence or absence of canavanine and coumestrol in all the collected samples of the herb as well as their respective seed samples were tested. All the tested growth stages of the herb were free from both substances.

II. *Biological Analysis*

The remarkable plasma TC, LDL-C and HDL-C elevation observed in our study upon cholesterol feeding is in agreement with several studies. It was obvious that the dramatic increase in plasma total cholesterol originated from the remarkable increase in LDL-C rather than HDL-C. It was also noticeable that despite the dramatic increase in plasma TC upon cholesterol feeding, plasma TG was not elevated at the end of the 28-day study. Similar results were reported by Wojcicki *et al*.[23] and Sugano *et al*.[24]. A reasonable explanation is the assumption that elevated post-heparin lipolytic activity previously observed in hypercholesterolemic group[20] is sufficient to abolish the hypertriglyceridemia anticipated with cholesterol feeding.

Another significant increase observed with cholesterol feeding is the two-fold elevation in the plasma MDA level. This result indicates that hypercholesterolemia could enhance the process of lipid peroxidation.

All forms of alfalfa extracts produced significant decrease in plasma total-, HDL- and LDL-C levels, except 1% extract II, according to the following order: 2% extract I > seeds > 2% extract II > 1% extract I > 1% extract II. Also, ratiowise 2% extract I showed the best HDL-C/LDL-C ratio (0.148) as compared to other extracts which had ratios ranging between 0.039 to 0.056, which are comparable to hypercholesterolemic group (0.055). However, 2% of extract I did not show the lowest level of MDA, despite its significance.

The absence of external signs of toxicity on the animals received cholesterol-enriched diet containing the different forms of alfalfa extracts confirmed the obtained results of TLC and HPLC which showed the absence of both of coumestrol and canavanine from the tested extracts.

Histopathological examination of aorta of alfalfa-fed rabbits revealed almost normalization of the aortic wall sections with regard to appearance (Figure 2) and intimal thickness (Figure 3). There was no difference between the results of the treated groups, suggesting that the antiatherosclerotic activity of the extracts might be noticeable in even lower concentration of the active constituents.

CONCLUSIONS

Several studies[9-12, 25] have reported the hypocholesterolemic and antiatherosclerotic activities of alfalfa extracts. This study showed that:

1. The high level of serum cholesterol and MDA seems to contribute to the histopathological changes developed in the aortic wall as evidenced by the comparison of alfalfa-treated animals with the hypercholesterolemic and normal groups.
2. Alfalfa extracts exhibited highly significant antioxidant activity.
3. 2% extract I of the Egyptian crop produced the most significant decrease in TC and LDL-C.
4. All alfalfa extracts possessed antiatherosclerotic activity as observed by the almost normalization of the aortic sections upon concomitant use of alfalfa extracts with cholesterol-enriched diet.
5. The result of 30% alcoholic extract was more effective than both ground seeds and the aqueous extract of the herb. The activity cannot be attributed only to the saponin content, but to the effect of other components in addition to saponins which may be present in this extract and absent in the aqueous extract.

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