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Anti-Hyperlipidemic and Anti-Atherosclerotic Activities of Silymarins from Cultivated and Wild Plants of *Silybum marianum* L. with Different Content of Flavonolignans

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ABSTRACT

The object of this study is to evaluate the influence of silymarins from cultivated and wild *Silybum marianum* L. plants with different content of flavonolignans, especially silibinin on serum lipids levels and on the experimental atherosclerosis development in rabbits fed on high cholesterol-diet (HCD). Forty eight male six-months-old white New Zealand rabbits (1.8-2 kg) were randomly assigned into six equal groups: positive control group - fed on HCD, negative control group- fed on standard laboratory diet and four groups fed on HCD with two different doses (100 and 200 mg/kg/day) of silymarins from cultivated and wild plants. Silymarin extracts were administered via the oral route, once daily for 2 months. Feeding of rabbits on HCD supplemented with both silymarins from cultivated and wild plants at the dose of 200 mg/kg/d caused a significant decrease in levels of total cholesterol, LDL-C and triacylglycerols. On the other hand, administration of silymarin from cultivated plants at the dose of 200 mg/kg/d in the diet enhanced significantly HDL-C serum content of rabbits. Both silymarins, at the dose of 200 mg/kg/d showed a significant inhibition of atherosclerotic plaque formation. Although a clear dose-dependent relationship was observed at the applied doses, but the pharmacological effects of silymarin from wild plants with lower content of silibinin, at the dose of 100 mg/kg/d were compared to those of silymarin of cultivated ones. Our results confirmed the anti-hyperlipidemic and anti-atherosclerotic effects of silymarins from both cultivated and wild milk thistle plants. In addition, the results allowed us to suggest that other constituents rather than silibinin may be responsible for therapeutic effects of silymarin.

Keywords: *Silymarin, Flavonolignans, Serum lipoproteins, High cholesterol-diet, Atherosclerosis*

Milk thistle (*Silybum marianum* (L.) Gaertner, Asteraceae) is an herbaceous annual or biennial plant native to the Mediterranean area, but it has become naturalized in the hot, dry areas of Central Europe, North and South America, Central and East Asia and Southern Australia. It grows as wild populations in open fields of many northern and western parts of Iran [1,2]. The seeds (actually the achene's fruits) of the plant contain a group of flavonoid compounds commonly named silymarin. The dried extracts from the milk thistle seeds contain approximately 60% silymarin. Silymarin consists of four flavonolignans of silybinin (~ 50 to 60%), isosilybinin (~ 5%), silychristin (~ 20%) and silydanin (~ 10%). The main active compound is believed to be silybinin [3].

Milk thistle has been known since ancient time and recommended in traditional European and Asiatic medicine mainly for treatment of liver disorders [2]. In recent years, there have been explosions of scientific papers that deal with drugs from the fruits of milk thistle and its active substances silymarin [4]. Silymarin has clinical applications as a potential anti-hepatotoxic drug and it is currently used for supportive treatment of liver ailments [3,5]. Several studies showed that silymarin has also anti-oxidative [4-7], immunomodulatory [5,6,8,9] and anti- inflammatory [5,6,10,11] effects.

According to some clinical data, silymarin has also anti-lipoperoxidation [10,12-14] and hypocholesterolemic activity [15-20]. Significant increase in serum cholesterol especially, low density lipoprotein fraction cholesterol (LDL-C) and also

Table 1. Results of HPLC analysis of silymarin samples

| Silymarin samples | Content (g/100gDW) | | | | | |
|-------------------|--------------------|--------------|--------------|--------------|--------------|-----------------|
| | Taxifolin | Silychristin | Silydianin | Silybinin | Isosilybinin | Total Silymarin |
| SM-C | 0.59 ± 0.19 | 11.47 ± 1.83 | 2.78 ± 1.09 | 38.68 ± 2.61 | 4.26 ± 1.87 | 57.78 |
| SM-W | 3.22 ± 1.29 | 5.25 ± 2.95 | 11.18 ± 2.12 | 19.74 ± 2.86 | 6.07 ± 2.05 | 45.46 |

Abbreviations: SM-C, silymarin from cultivated plants; SM-W; silymarin sample from wild plants. Values are expressed as means ± SD.

oxidatively-modified LDL-C are proposed as risk factors involved in coronary heart diseases such as atherosclerosis. Silymarin could positively modify serum cholesterol lipoprotein profile and also inhibit lipoxygenase pathway, thus show anti-atherosclerotic activity [4,13,15,21]. The aim of the presented work was to evaluate the influence of silymarins, extracted from seeds of cultivated and wild milk thistle plants with different contents of flavonolignans on serum lipids levels and consequently atherosclerosis development in rabbits with high cholesterol diet-induced hypercholesterolemia.

MATERIALS AND METHODS

Plant Materials

Seeds of cultivated *Silybum marianum* L. (a Chinese product, Hungarian accession, Plantarum Medicinarum Hortus Botanicum Institute) were collected from the Research Station of Medicinal Plants Institute, ACECR (Karadj city, Iran). Seeds of wild plants were harvested from their natural habitats (Mazandaran province, open field of Marzan Abad, Iran) during the flowering season (from June to August 2006).

Extraction procedure from the dried fruits

About 10 g of finely-powdered fruits of different samples were extracted with n-hexane in a Soxhlet extractor for 4 hours. The adherent solvent hexane was removed from the extraction apparatus by drying and defatted fruit powders were extracted with ethyl acetate by heating. After 8 hours of extraction, ethyl acetate solution was evaporated about 40°C under reduced pressure on a rotary evaporator. Silymarins were obtained as yellow powder [22].

Silymarins from seeds of cultivated and wild plants were quantitatively and qualitatively analyzed by HPLC method (Table 1).

High cholesterol diet

High cholesterol food for rabbits was prepared as pellets by adding of 1 g cholesterol (Solvay Duphar Co., Belgium, Batch No: 6025) and 3 g corn oil to 96 g of standard laboratory food.

Animals and diets

Forty eight male six-month-old white New Zealand rabbits (1.8-2 kg) were supplied from Razi Research Institute (Iran). They were housed individually in metal cages in temperature-controlled room (24 °C) under a

12-hour light/dark cycle with free access to food and water in the Animal Research Center of Baqiatallah Medical Sciences University (Iran). The experimental protocol was approved by the Institutional Animal Ethics Committee of Baqiatallah Medical Sciences University and was conducted according to the Institutional Animal Care Iranian Guidelines for the use and care of experimental animal, drug, dose, and treatment schedule. After a 2-week adaptation period, rabbits were randomly assigned to six equal groups: positive control group - fed on HCD (1% cholesterol), negative control group- fed on standard laboratory diet and four groups fed on HCD and two different doses (100 and 200 mg/kg/day) of silymarins from cultivated and wild plants. Silymarin was administered as oral pretreatment (O.P.) once daily, one hour before feeding. The whole experiment lasted 2 months. Upon termination of the bioassay biochemical analysis of serum lipids and pathological evaluation of aortas were performed.

Biochemical analysis

After overnight fasting, the blood was taken from marginal ear veins for lipid analysis. Serum was separated by centrifuging of bloods (20 min at 2500×g). Total cholesterol (Chol) and triacylglycerols (TAG) levels in serums were measured using enzyme assay kits (Pars Azmun Co., Iran). Serum lipoproteins (HDL-C and LDL-C) were separated from serum by precipitation of lipoproteins and were determined by enzymatic tests kits (ZiestChem. Co., Iran).

Pathological analysis

After the end of the experiments (2 months), rabbits were killed by chloroform (overdose) and their aortas were separated up to diaphragm. The aortas were cleaned, opened and then treated with 70% ethanol and finally were washed with water. The aortas then stained using sodan IV and stored in 10% formalin solution [23]. After staining, atherosclerotic plaques were appeared as red spots. Photographs were taken from the aortas by digital camera, and the percentage of the atherosclerotic plaques formation was determined for each aorta by planimetry. By the using of the Internet UTHSCSA Image Tool package (version 3), the percentage of atherosclerotic plaques formation was calculated.

Statistical analysis

All values are expressed as means ± SE. Data were analyzed by one way ANOVA, and all differences were

Table 2. Effects of different silymarin treatments on serum lipoproteins and triacylglycerols levels of rabbits fed high cholesterol diet after 2 months

| Treatment groups (n=8) | Total cholesterol (mg/dL) | LDL-C (mg/dL) | HDL-C (mg/dL) | Triacylglycerols (mg/dL) |
|---------------------------|------------------------------|-----------------------------|--------------------------|-----------------------------|
| Control (negative) | 69.58±4.62 ^a | 57.69±3.21 ^a | 23.19±5.42 ^c | 98.40±14.98 ^a |
| SM-C2(200 mg/kg) | 745.95±46.20 ^b | 685.20±46.10 ^b | 36.75±3.13 ^a | 115.25±22.43 ^{ab} |
| SM-W2 (200 mg/kg) | 902.35 ±17.29 ^{bc} | 822.50±36.59 ^{bc} | 32.88±2.63 ^{ab} | 103.11±11.78 ^{ab} |
| SM-C1(100 mg/kg) | 1015.26±35.26 ^{cd} | 959.74±25.36 ^{cd} | 28.38±4.23 ^{bc} | 146.56± 9.28 ^c |
| SM-W1(100 mg/kg) | 1195.13±39.20 ^{de} | 1079.63±28.09 ^{de} | 25.88±2.68 ^c | 185.75±28.96 ^d |
| Control (positive) | 1285.88±18.28 ^e | 1198.88±15.68 ^e | 25.38±3.59 ^c | 218.63±25.00 ^e |

a,b,c,d,e

Different superscript letters show significant differences at $p < 0.05$. SM-C2 and SM-C1, silymarins from cultivated plants; SM-W1 and SM-W2, silymarins from wild plants; negative and positive controls, groups that fed on standard laboratory diet and high cholesterol diet, respectively. Values are expressed as means ± SE.

Table 3. Effect of different silymarin treatments on atherosclerotic plaque formation in aortas of rabbits fed high cholesterol diet after 2 months

| Treatment groups (n=8) | Atherosclerotic plaque formation (%) |
|---------------------------|---|
| Control (negative) | 4.27±1.71 ^a |
| SM-C2(200 mg/kg) | 6.86±3.29 ^a |
| SM-W2(200 mg/kg) | 6.29±2.74 ^a |
| SM-C1(100 mg/kg) | 16.36±4.52 ^b |
| SM-W1(100 mg/kg) | 18.32±3.12 ^b |
| Control (positive) | 21.16±5.29 ^b |

a,b Different superscript letters show significant differences at $p < 0.05$. SM-C2 and SM-C1, silymarins from cultivated plants; SM-W1 and SM-W2, silymarins from wild plants; negative and positive controls, groups that fed on standard laboratory diet and high cholesterol diet, respectively. Values are expressed as means ± SE.

inspected by Duncan's multiple test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Data presented in the Table 2 shows that feeding of rabbits for 2 months with HCD leads to a significant increase in their blood serum total cholesterol, LDL-C and with relatively lower rates in TAG levels. Lipid analyses demonstrated that treatment of HCD-fed rabbits with 200 mg of both silymarins from cultivated and wild plants daily for two months caused a significant decrease in their serum total cholesterol, LDL-C and TAG levels, as compared to those of rabbits in positive control group. Anti-hypercholesterolemic effect of silymarins from cultivated and wild plants at the dose of 200 mg/kg/day was associated with a significant increase in HDL-C levels, an effect considered to be benefit to treatment of atherosclerosis. There was no significant effect on the serum lipid levels in rabbits that were treated with both silymarins from cultivated and wild plants at the dose of 100 mg/kg/day, compared with the positive control group. These results suggested that feeding of rabbits with 100 mg of both silymarins (from cultivated or wild plants) daily for 2 months might not be enough to reveal hypocholesterolemic effects of them.

As data in Table 3 and Fig 1 show, long-term treatments of rabbits with both silymarins from cultivated and wild plants at the dose of 200 mg/kg/d

had an evident and significant effect on prevention of atherosclerotic plaque formation on their aortas.

The percentages of aortic atherosclerotic plaque formation in rabbits were treated with 200 mg silymarins from cultivated and wild plants were 6.86% and 6.29%, respectively as compared to 21.16% for positive control group. In our experiments, silymarins at lower dose (100 mg) could not have anti-atherosclerotic activity in HCD fed rabbits.

DISCUSSION

Hyperliporoteinaemias with a significant increase of serum cholesterol and its carrier LDL are known to be associated with an increased risk of heart coronary diseases [12-20]. In human, a 15% higher LDL-C can increase the risk of coronary heart diseases by about 15-45%. Moreover, formation of oxidatively-modified LDL is proposed as a critical risk factor involved in the so-known "oxidation hypothesis" of atherosclerosis [13].

Free radicals are recognized to have crucial importance in mechanisms of many pathological processes including atherosclerosis via lipid peroxidation of LDL [15]. It has been shown that flavonoids such as silymarin are large group naturally occurring antioxidants that could inhibit lipid peroxidation of LDL by scavenging free radicals [13,15,24]. Based on some clinical studies, after the administration of silymarin, the antioxidant enzyme activities reversed to near normal [15,16,25].

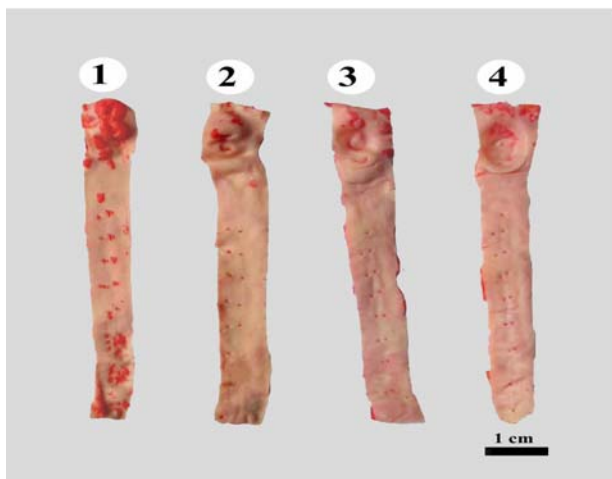


Fig 1. Presentation of atherosclerotic plaques formation in aorta sections of rabbits in different tested groups; Positive Control (1), SM-C2 (2), SM-W2 (3), and rabbits that fed on standard laboratory diet (negative control) (4). SM-C2 and SM-W2 are silymarins from cultivated and wild plants at the dose of 200mg/kg, respectively.

In accordance with results obtained by Krecman *et al.* and Skottova *et al.*, the present study showed that polyphenolic fraction of silymarin can positively modify lipoprotein profiles and antihypercholesterolemic effects of this fraction were dose-dependent [16-18]. Also, consistent with Krecman *et al.* and Skottova and Krecman, based on our results, silymarin caused a mild increase in HDL-C, a property of this component which is beneficial to treatment of atherosclerosis. Similar to Bialecka observations, in this trial we showed that silymarin has strong anti-atherosclerotic activity [21].

It has been shown recently that silymarin has beneficial effects on some risk factors of atherosclerosis owing to its hypolipidemic properties [15,20]. Recent data suggest that the inhibition of cholesterol absorption caused by silymarin could be a mechanism contributing to the positive change in plasma lipoprotein profile [15,19].

Silymarin can act as an anti-inflammatory factor through inhibition of 5-lipoxygenase [10]. Recently, atherosclerosis is more and more being recognized as a chronic inflammatory process, and some experimental findings showed that this chronic disease is due to an imbalance between synthesis of reactive oxygen species and antioxidative defective mechanisms [24]. Consequently, it is proposed that biomolecules such as flavonoides with anti-inflammatory and anti-oxidative properties could have beneficial effects on atherosclerosis [26,27]. Because of the complex mechanism(s) action of silymarin, it may be a natural multifunctional and multi-target medicinal herbal drug [4].

An interesting feature of our experiment was evaluation of anti-hyperlipidemic and anti-atherosclerotic properties of silymarin from a foreign-cultivated plant as compared to silymarin extracted from one of the Iranian wild milk thistle plants with different contents of flavonolignans. Our results confirmed the hypolipidemic and anti-atherosclerotic effects of both

silymarins. Although, the main active compound is believed to be silybinin, silymarin such as other plant extracts contains many compounds and it is not completely clear that which constituents is largely responsible for the medical benefits attributed to silymarin. There are some reports that show silybinin is not the only main active compounds of silymarin and other constituents of silymarin than silybinin may be responsible for anti-hypercholesterolemic activity of the extract [13,15].

On the other hand, the bioavailability of silymarin constituents and especially silybinin is low due to their limited solubility in water and seems to depend on the presence of other flavonoids [3,13,15]. Recent evidence suggests that silybinin is not as effective as silymarin and other constituents of silymarin may be responsible for its anti-hyperlipidemic effect or that the bioavailability of silybinin alone might be lower than that of silybinin as a compound of silymarin [3,13,17]. In accordance with observed quantitative differences between constituents of silymarin samples (Table 1), the achieved results have indicated that not only silybinin but also other silymarin constituents such as silychristin, silydianin and isosilybinin could have a role in silymarin's pharmacological activities.

CONCLUSION

The results described here clearly confirmed the anti-hyperlipidemic and anti-atherosclerotic properties of silymarin. Also, our findings on therapeutical properties of silymarins from cultivated and wild plants with different amounts of flavonolignans, especially silybinin, on lipid levels and atherosclerosis progression showed that positive pharmacological effects of silymarin is not completely due to silybinin and at least in part, it is attributed to the presence of higher amounts of other constituents such as silychristin, isosilybinin and silydianin. Further studies investigating effect of silymarins from different wild ecotypes or populations of milk thistle plants could lead to the introduction of silymarin types with more pharmaceutical ability.

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