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Original Contribution

COMPARATIVE STUDY OF THE ANTIOXIDANT PROPERTIES OF SILYMARIN AND MILK THISTLE EXTRACTS

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ABSTRACT

Purpose: Milk thistle (*Silybum marianum* (L.) Gaertn) has been used for centuries as a natural remedy for liver and gall bladder / biliary tract diseases. Silymarin is the purified and standardized extract from milk thistle seeds. The purpose of the present study is to present a comparative analysis of the total phenolic substances content and the antioxidant effect of silymarin and three different types of *Silybum marianum* extracts (i.e. ethanol extract, water extract and lyophilized extract).
Methods: Total phenolic and flavonoid content of extracts was analysed and the antioxidant activity was assessed by determining their free radical scavenging ability by DPPH method.
Results: Total phenolic content is the highest in silymarin– 134.12±3.22 mg GAE/g, followed by lyophilized water extract – 51.74±2.10 mg GAE/g. Silymarin has also been found to have the highest antioxidant activity among all extract samples (338.03 VCEAC µmol /g), followed by *S.marianum* lyophilised extract (123.05 VCEAC µmol/g).
Conclusions: A strong correlation between total phenolic and flavonoid content in the extracts and their antioxidant activity has been found.

Key words: phenolic content, flavonoid content, antioxidant activity, *Silybum marianum*

INTRODUCTION

Silymarin is a denominative term used to refer to the purified, standardized extract of milk thistle seeds. The milk thistle (*Silybum marianum* - (L.) Gaertn.) is an annual or biannual plant, found in southern parts of Europe, northern parts of Africa and South America. In Bulgaria it grows as a ruderal plant in the southern parts of the country. Over the last few years, the total area of agricultural land used for cultivated milk thistle growing has increased. Its seeds are oblong, egg-shaped, brown-coloured, about 8mm in length. *Silybum marianum* petals and leaves have been used for centuries in traditional medicine as a remedy for liver, spleen and gall bladder diseases (1). In the 1960s, Janiak and Hänsel isolated two biologically active substances from milk thistle seeds and fruits (2). Later it was found that the physiological effects of *Silybum marianum* extracts were caused by a mixture of flavolignans, known as silymarin. The silymarin complex mainly consists of four isomeric chemical compounds, namely silybin; isosilybin; silydianine and silychristin (3). The proportion of silybin is the largest **(Figure 1).**

At present, *S. marianum* extracts or silymarin are pharmaceutical components of medicinal products and nutritional supplements with hepatoprotective effects. They are considered to be safe to use if applied properly, in prescribed therapeutic doses (4). Silymarin is mainly used in human medicine but it can also be used to treat some liver conditions in pets. Experiments with low and high dose silymarin supplementation have been carried out on farm animals and poultry. They revealed its dose-dependent positive effect on productivity (5, 6). Toxic effects were not found even when applying high doses of silymarin.

Over the last few years research into the potential capacity of silymarin against cancer has been made (7). It has been suggested that

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silymarin can modulate the imbalance between cell life and apoptosis by affecting the expression of cell cycle regulators and the proteins involved in the process of apoptosis.

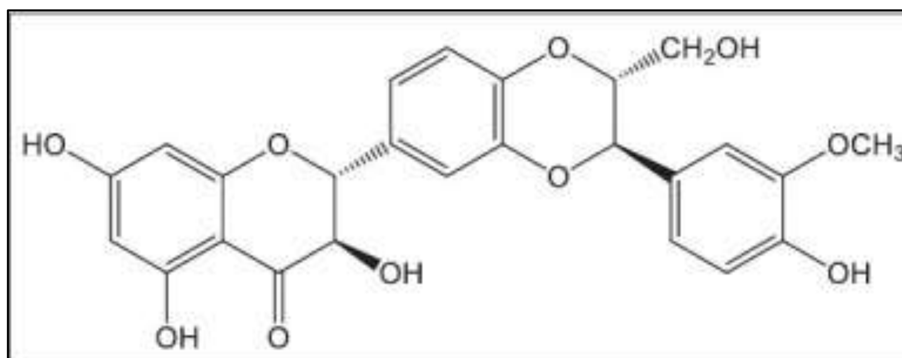


Figure 1. Chemical structure of silybin. (source: Kvasnička et al.,2003)

The liver protection mechanism of silymarin consists in direct stabilization of hepatocytes membrane permeability through lipid peroxidation inhibition and prevention of glutathione depletion (8). Shaker et al. investigated the role of ethanol and ethyl acetate extracts of *S. marianum* as a means of protection against the toxic effect of CCl_4, causing hepatic fibrosis and necrosis. A comparative study was carried out with a medicinal product containing silymarin. Laboratory experiments with Sprague–Dawley rats, in which CCl_4 liver damage was induced, revealed that the ethanol extract achieved the highest reduction of liver enzymes, while ethyl acetate extracts caused the highest increase of glutathione levels and the highest decrease of total and LDL cholesterol (9). As a whole, the beneficial physiological effect of milk thistle extracts and silymarin are associated with the antioxidant effect of the polyphenolic complexes contained in them.

The purpose of the present study is to make a comparative analysis of phenolic substances content and the antioxidant effect of silymarin and three types of milk thistle extracts.

METHODS AND MATERIALS

Materials: Milk thistle fruits (*Silybum marianum* - (L.) Gaertn), bought from a local herbs supplier in Sofia, Bulgaria. The raw material was dried and fragmented into small particles measuring1, 0-1,5 mm.

Silymarin - Milk Thistle Extract Powder, produced by Wuxi Gorunjie Technology Co., LTD.

Chemicals and reagents: DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical, ascorbic acid and gallic acid (Merck); quercetin (Sigma); aluminium chloride hexahydrate and ethanol 95% (Valerus); methanol and dichloromethane (Merck). All chemicals and materials used are analytical grade.

Silybum marianum extracts

Three different extracts were made from milk thistle fruits: aqueous extract; lyophilized aqueous extract and ethanol extract. The ethanol extract was taken at hydromodule ratio of 1:5 and 70% v/v ethanol concentration. The mixtures were treated with ultrasound three times for periods of 20 minutes (Model 7652 Ultrasonic System), after which they were left to stay at room temperature for 72 h. After removing the plant particles the extracts were filtered through a 40 µm glass filter and were then stored at 4 ºC until they were used in our analysis.

The aqueous extract (infusion) was prepared by pouring 1200 ml of boiling water on 100 g of fractioned milk thistle fruit. The infusion was left to rest for 60 min, followed by filtration. Additional hot water was then added to reach a total volume of 1200 ml. A part of the aqueous infusion was stored at 4˚C until used for analysis, while the rest was frozen at-25 ˚C and then lyophilized in a Hochvakuum-TG – 16.50 vacuum sublimation installations with an automated working mode. Until used for analysis, the samples were stored in three-ply aluminum foil vacuum packages.

Total quantity of extracted substances in liquid extracts – method certified by BSS EN 12145:2000.

Moisture content – the residual moisture in the lyophilized samples and in silymarin was measured by applying an express weight measuring method of Sartorius-thermo-control YTC 01 L moisture measuring scales.

Thin layer chromatography
Silymarin and lyophilized milk thistle extract: 0.020 g each were dissolved in 10 ml 60% ethanol. The ethanol milk thistle extract was further diluted by 60% ethanol in 1:1 ratio. Standard substances: rutin (0.002 g/10 ml methanol); quercetin and caffeic acid (0,005 g/10 ml methanol).

The samples were placed on TLC silica gel plates (Merck) - 10 µl of silymarin and standard solutions and 30µl of extract solutions were applied as 10 mm bands; or 5 µl of each sample and standard solutions were applied as spots.

Mobile phase (Eur. Ph. 7.0): anhydrous formic acid, acetone, dichlormethane(8.5:16.5:75 *v/v/v*). After drying in air, the plates were sprayed with DPPH solution (80 µg/ml) and examine immediately, or with vanillin-sulfuric acid reagent and then heated to 105-110 °C for 5 min.

Total phenolic substances content– The total quantity of phenolic substances was determined by spectrophotometric method with Folin-Ciocalteu phenol reagent and expressed as gallic acid equivalents (10, 11).

Total flavoniod content– The total quantity of flavonoids was measured by the method described by Chang et al., which we modified slightly (12, 13). In short:500 µl of each extract was mixed with 1.50 ml 95% ethanol, 0.10 ml 10% aluminum chloride solution ($AlCl_3.6H_2O$), 0.10 ml 1 M sodium acetate solution and 2.80 ml of distilled water. After 40 min of incubation time, the absorption was measured at 415 nm (UV-VIS spectrophotometre, Biochrom Libra S20). Calibration line, using quercetin as a set standard was used to measure the flavonoids concentration.

Radical scavenging ability towards DPPH
The ***radical scavenging activity*** of the examined extracts was measured according to a slightly modified method of Kim et al. (14): 2.0 mg of DPPH were dissolved in 50 ml of ethanol; 1.5 ml of the corresponding extract solutions were added to 1.5 ml of the DPPH solution. After 30 min incubation in the dark and at room temperature, absorbance was measured at 517 nm using a UV/VIS spectrometer (Libra S 22 U-Vis, Biochrom), 1.5 ml 85% ethanol plus 1.5 ml DPPH solution was used as control. Vitamin C (ascorbic acid) was used for comparison and the antioxidant capacity of the extracts was expressed as vitamin C equivalent units- VCEAC µmol/ml or VCEAC µmol/g

Statistical analysis
All experiments were repeated three times. The statistical data processing was made by using Microsoft Excel 2013 software. The data is presented as average values ± the standard deviation (SD). The results were analysed by One-way Analysis of Variance (ANOVA). The differences are considered statistically valid at $p< 0.01$.

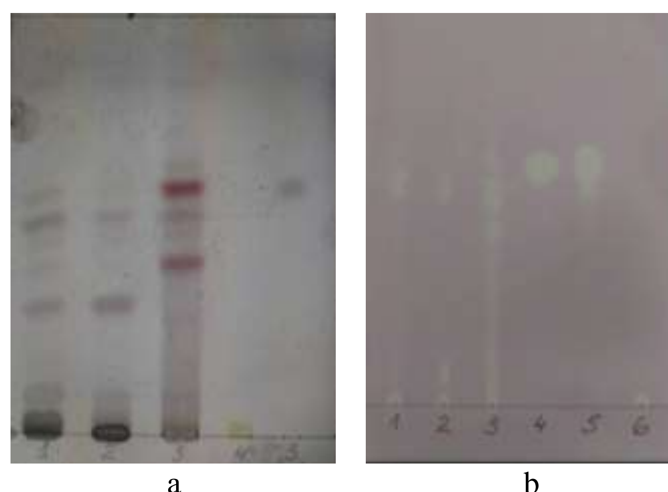
RESULTS AND DISCUSSION

The raw ethanol *Silybum marianum* extract was an easily flowing liquid, yellow in colour. The colour of the aqueous extracts was also quite similar. The quantity of extracted substances was low:0.380±0.037g/100 ml in the ethanol extract and 0.420±0.029g/100 ml in the aqueous extract. The residual moisture in silymarin was 3.15±0.06% and in the lyophilized *S.marianum* extract–4.71±0.04 %.

Chromatograms of ethanol extract, lyophilized extract and silymarin are presented in **Figure 2** and in **Table 1** are given Rf values of the separate components of extracts and silymarin after they were processed with vanillin– sulfuric acid reagent **(Figure 2a).**

Table 1. *Results of TLC analysis of the examined S. marianumextracts*

Sample No	Name	Rf value
1	*S. marianum* ethanol extract	0.08 0.30 0.38 0.50 0.56
2	*S.marianum*lyophilized aqueous extract	0.10 0.30 0.51
3	Silymarin	0.27 0.41 0.52 0.58 0.63
4	Rutin standard	0.02
5	Caffeic acid standard	0.58



a b

Figure 2. Chromatograms of *S. marianum* extracts and silymarin **- a**) after being processed with vanillin-sulfuric acid reagen; **b)** after being sprayed with DPPH (80 µg/ml)

The DPPH test presents a quick TLC screening of possible antioxidant activity in plant extracts. When a TLC plate is sprayed with DPPH solution, each antioxidant compound appears as a yellow area on the purple background (15, 16). The chromatogram on Fig. 2b shows yellow coloured areas of different Rf values, corresponding to components, possessing antioxidant activity. The images are different for sample 1 (ethanol extract); sample 2 (lyophilized aqueous extract) and sample 3 (silymarin), as the yellow areas, are most intensive in the silymarin sample. As it was expected, the three standard substances– caffeic acid (4); quercetin (5) and rutin (6) show distinct, yellow coloured single areas.

The quantity of total phenols and flavonoids varied a lot depending on the type of extract and the way it was concentrated and purified. For the liquid extracts – the quantity of phenolic substances was 0.529 mg GAE/ml in the raw ethanol extract and as low as0.211 mg GAE/ml in the aqueous extract. The tendency was quite the same for flavonoids **(Table 2).** Logically, the quantity of phenols and flavonoids was much higher in the dry extracts **(Table 3**). According to the corresponding monograph of the European Phamacopoeia the dry, purified and standardized *S. marianum* extract contains nominal quantity of silymarin within the range of 30% m/m to 65 % m/m. It is extracted from raw plant material by the use of organic solvent, after which it is concentrated and dried. The quantity of phenolic substances was over 2.5 times higher in the commercial product of Silymarin we examined, compared to the lyophilized aqueous extract. The difference was up to 10 times bigger for flavonoids. The low quantities in the aqueous and the lyophilized extracts can be explained by the low solubility of the silymarin complex in water - 0.4 mg/ml (17).

Table 2. *Total phenols and flavonoids content and antioxidant activity of aqueous and ethanol S. marianum extracts*

Type of plant extract	**Total phenolic (mg GAE/ml)**	**Flavonoids content (mg QE/ml)**	**VCEACµmol/ml**
Aqueous *S. marianum* extract	0.211±0.009	0.013±0.002	0.51 ±0.06
Ethanol *S. marianum* extract	0.529±0.029	0.054±0.001	1.01±0.02

Table 3. *Total phenols and flavonoids content and antioxidant activity of lyophilized S. marianum extract*

Type of plant extract	**Total phenolic (mg GAE/g)**	**Flavonoids content (mg QE/g)**	**VCEAC µmol/g**
Lyophilized *S. marianum* extract	51.74±2.10	3.28±0.11	123.05±12.93
Silymarin	134.12±3.22	33.14±1.71	338.03±3.23

The antioxidant capacity of the examined extracts was measured by assessing their radical scavenging capabilities for DPPH. This type of analysis is one of the most frequently applied methods for in vitro assessment of the antioxidant activity of extracted substances of biological origin. DPPH is a relatively stable free radical, dark violet in colour. When it gets in contact with hydrogen atom donors, the DPPH• radical changes into the yellow coloured DPPH•-H, which changes the violet colour of the solution and decreases the absorption at 517 nm. The radical scavenging capacity of the extracts was compared to that of Vitamin C. Silymarin again showed the highest values– 338.03 VCEAC µmol /g, while for the lyophilized extract the result was 2.75 times lower.

In the literature, there is research evidence of the existing correlation between the antioxidant activity and phenol and flavonoids content in many medicinal plants. Our current research came to similar conclusions, supported by the high correlation coefficients– 0.989 (for total phenols) and 0.926 (for flavonoids). It can be concluded that the antioxidant capacity of *S. marianum* extracts, to a large extent depends on the number of phenolic compounds contained in them.

CONCLUSIONS

The present study investigated and evaluated the content of total phenols and flavonoids in three different *S.marinum* extracts and in silymarin. We also evaluated the antioxidant activity of the extracts by assessing their radical scavenging capacity for DPPH. Silymarin proved to have the highest antioxidant activity (338.03 VCEAC µmol/g) among all extracts examined, followed by the lyophilized extract (123.05 VCEAC µmol/g). A significant correlation was found between the presence of phenolic substances and flavonoids in the samples and their antioxidant activity. The aqueous extract showed the lowest content of total phenols and flavonoids, which also corresponds to its lower antioxidant activity. The obtained results suggest that water is not a suitable solvent for the extraction of active components contained in *S. marianum* fruit, which questions the efficiency of herbal teas and infusions of this herb.

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