

The influence of the extraction method on the content of silymarin in *Silybi mariani fructus*

SOFIJA ĐORĐEVIĆ^{1,*}, TEODORA JANKOVIĆ¹, AND MILICA MIHAILOVIĆ¹

¹Institute for Medicinal Plant Research "Dr. Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia

*Corresponding author: sdjordjevic@mocbilja.rs

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This study was aimed to compare the efficiency of silymarin extraction from *Silybi mariani fructus* using different extraction methods. Maceration, percolation, extraction in a water bath, and ultrasonic-assisted extraction have been performed with 60% ethanol. Extracts were analyzed using UV/Vis and HPLC techniques. Our results showed that the highest concentration of silymarin was detected in samples extracted in a water bath with boiling solvent during 30 and 60 minutes (2.33 and 1.95%, respectively). Since extraction in a water bath is efficient and reduces a lot of time and solvent consumption, this method could present potential replacement for Pharmacopoeia-recommended Soxhlet extraction procedure.

Key words: *Silybum marianum*; silymarin; extraction method; UV spectroscopy; HPLC

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1. INTRODUCTION

Milk thistle, *Silybum marianum* (L.) Gaertn. (syn. *Carduus marianum* L.) Asteraceae, is a highly appreciated medicinal herb. There are certain data which can confirm the use of this plant since early times, for different diseases and disruptions of liver and gallbladder (hepatitis, cirrhosis and jaundice) and for the protection of the liver from the influence of alcohol and other harmful matters (Qavami et al., 2013; Blumenthal, 2003). Ancient Greek and Roman doctors were among the first to use milk thistle. The antique botanist Theophrastus named it "Pternix", Discorides named it "Sillybon", Pliny the Elder „Sillybum“ and he recommended the juice of this plant mixed with honey for bile related complaints (Qavami et al., 2013).

Silibi mariani fructus is used as the medicinal part of the plant, and is included in the European Pharmacopoeia and some national pharmacopeias (EMA, 2018a). All important monographs about medicinal plants and phytopreparations contain information about positive influence of milk thistle fruit preparations. According to EMA (2018b), the fruit of milk thistle is used as traditional herbal medicinal product for the symptomatic relief of digestive disorders, sensation of fullness and indigestion, as well as to support the liver function after serious conditions have been excluded by a doctor. According to ESCOP Monographs (2009) and WHO Monographs (2004) the fruit of milk thistle shows the stabilizing effect to the cell membranes of hepatocytes, anti-cholestatic, anti-inflammatory, antifibrotic, anti-carcinogenic effects, radical scavenging properties, effects on the regeneration of the liver and cellular metabolism. In *in vivo* experiments positive

effects in acute and chronic intoxication of the liver have been described, anti-inflammatory effects, effects on the cardiovascular system, antinephrotoxic effects, anti-carcinogenic effects, as well as other effects such as anti-ulcerogenic and laxative effects. The monograph of German Commission E (Blumenthal et al., 1998) states that the crude drug is used with dyspeptic disorders, while phyto-preparations are used in toxic liver damage and in supportive treatment of chronic inflammatory liver conditions and liver cirrhosis.

Milk thistle fruit contain the following substances: flavonolignans (1.3-3%) silybinin and isosilybinin (A and B), silycristin and silydianin that are collectively referred to as silymarin (Figure 1); flavonoids: flavones – apigenin, chrysoeriol, eriodictyol; flavonols – taxifolin, quercetin, dihydrokaempferol, kaempferol; fatty oil (20-30%): linoleic (35-55%) and oleic (24-30%) acids, palmitic (8-12%), linolenic (3-7%), behenic (3-9%) and other fatty acids; phytosterols (0.2-0.6%): β -sitosterol; and other constituents (EMA, 2018a).

Herbal preparations for the oral use are generally made as water-ethanol extracts or with other organic solvents, where residues must be eliminated by special technological procedures. The efficacy of the extraction of medicinal plants in order to obtain the extracts with the maximal content of active principles highly depends on the method of extraction and extragens. There are conventional extractions such as maceration, digestion and percolation, the extraction at increased temperature on a water bath with the air cooler, and Soxhlet extraction or hot continuous extraction. Recently, the application in the preparation of different herbal extracts have found even more complex methods of extraction such as microwave as-

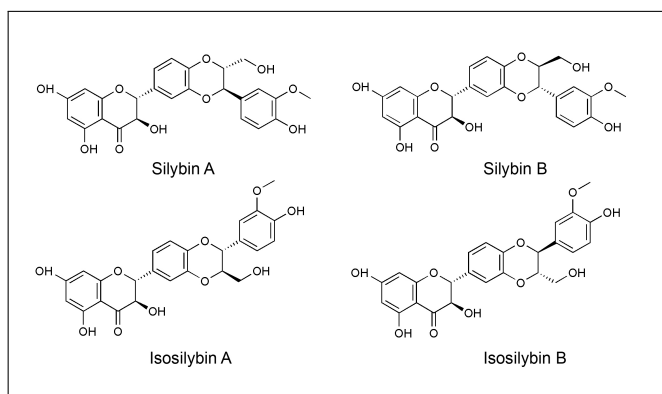


Fig. 1. Structures of the main flavonolignans from milk thistle fruit

sisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and many others (Gupta et al., 2012; Azwanida, 2015). The knowledge of solubility of active chemical compound is of high importance for the selection of suitable extracts of certain polarity.

According to the literature data, the mode of extraction has a major influence on the silymarin content (Chambers et al., 2017). Ph. Eur. 7.0. (2010) recommends Soxhlet extraction with methanol for 5 hours. Instead of this time-consuming process and in order to reduce solvent requirements, other techniques such as maceration, ultrasound-assisted extraction, or pressurized liquid extraction have been proposed (Wianowska and Winiewski, 2015; Saleh et al., 2015). Water, and various organic solvents such as methanol, ethanol, acetone and ethyl acetate were tested for their ability to recover silymarin from milk thistle (Wallace et al., 2005; Poppe and Petersen, 2016). However, water and ethanol received special attention being non-toxic and eco-friendly solvents which is important for formulation of bioactive compounds. Experimental solubility data of silymarin in various solvents showed that the mole fraction solubility of silymarin in ethanol was much higher than in water (Shakeel et al., 2014). Bilia et al. (2000) reported that 60% aqueous ethanol was more effective solvent for extracting flavonolignans than 40% ethanol. Therefore, we used 60% ethanol as extraction solvent system in relation to this eco-friendly and sustainable biotechnological platform.

Different analytical methods have been used to determine silymarin content in *Silybum marianum* extracts, the most common being UV spectrophotometry (Meghreji et al., 2010) and HPLC (Mudge et al., 2015) methods. Spectrophotometric method is routine, simple and sensitive, but HPLC technique is more reliable and specific. The aim of the present work was to give more comprehensive research about which extraction conditions were the best for the efficient extraction of silymarin from milk thistle fruit. In this study, we investigated the effects of different extraction methods (maceration, percolation, water bath extraction, and ultrasonic-assisted extraction) on the content of silymarin in milk thistle fruit. UV/Vis and HPLC techniques were used for the determination of silymarin in obtained extracts.

2. MATERIALS AND METHODS

2.1. Plant material

Fruits from cultivated milk thistle were collected from the experimental field at Institute for Medicinal Plants Research „Dr Josif Pančić“ in Pančevo, Serbia. The identification of plant material was carried out in the Laboratory for Pharmaceutical control of the Institute.

2.2. Chemicals

Silybin standard, and orthophosphoric acid were purchased from Sigma–Aldrich (Chemie GmbH, Munich, Germany). Ethanol and methanol were of analytical grade, acetonitrile (Merck, Germany) was of HPLC grade, and ultra-pure water was prepared using a Milli-Q purification system (Millipore, France). Milk thistle dry extract was purchased from European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France).

2.3. Extraction procedures

2.3.1. Percolation

Single percolation was carried out according to the Ph. Jug. IV (1984). Thirty g of grounded seeds of milk thistle were covered with 30 mL of solvent (1:1), and left for 2 hours to swell up. The saturated drug was put into the percolator and left for 24 h to macerate. The day after, the extract was easily separated and filtered.

2.3.2. Maceration

Maceration was carried out according to the Ph. Jug. IV (1984). Twenty g of the grounded seeds of milk thistle were covered with 100 mL of solvent (1:5), and in the following five days shaken few times per day. After the fifth day, the extract was filtered.

2.3.3. Ultrasound-assisted extraction

Five g of the grounded seeds were extracted with 25 mL of solvent (1:5) in an ultrasonic bath (bath power 35 W, continuous mode at frequency of 40 kHz, Maget USB 4, Bela Palanka, Serbia) at temperature of 40 °C for 15 and 30 min.

2.3.4. Extraction on a water bath

Five g of the grounded seeds was mixed with 50 mL (1:10) of the solvent in 100 mL round-bottom flask and held in a water bath with the air cooler for 30 and 60 minutes at the boiling point of solvent.

2.4. UV analysis

A Hewlett Packard 400N spectrophotometer was used for estimation of silymarin in different samples. Reference standard of silybin was dissolved in methanol to obtain final concentration of 8 µg/mL. Absorption of silybin and test solutions was detected at 288 nm, and the content of silymarin in % was expressed as silybin according to equation:

$$\text{Silymarin (\%)} = \frac{A_{\text{sample}} \times m_0}{A_0 \times m_{\text{sample}}} \times 100$$

where A_{sample} is absorption value of sample, A_0 is absorption value of silybin, m_0 is mass of silybin, and m_{sample} is mass of the sample.

2.5. HPLC analysis

Total silymarin content was assessed according to the procedure described in Ph. Eur. 7.0. (2010), with slight modifications. Analyses were carried out on Agilent 1200 RR HPLC instrument (Agilent, Waldbronn, Germany), equipped with DAD detector, using reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm × 4.6 mm i.d., 5 µm particle size), and the column temperature was maintained at 30 °C. The mobile phase was composed of (A) 1% v/v solution of orthophosphoric acid in water, and (B) acetonitrile. Gradient elution was applied according to the following scheme: 0–5 min, 80–75% A; 5–30 min, 75% A; 30–35 min, 75–65% A; 35–40 min, 65–0% A; 40–45 min, 0% A. The flow rate of 0.8 mL/min, and injection volume was 10 µL. Detection wavelength was set at 288 nm. A reference solution was prepared by exactly weighing 1 mg of milk thistle dry extract dissolving in 1 mL

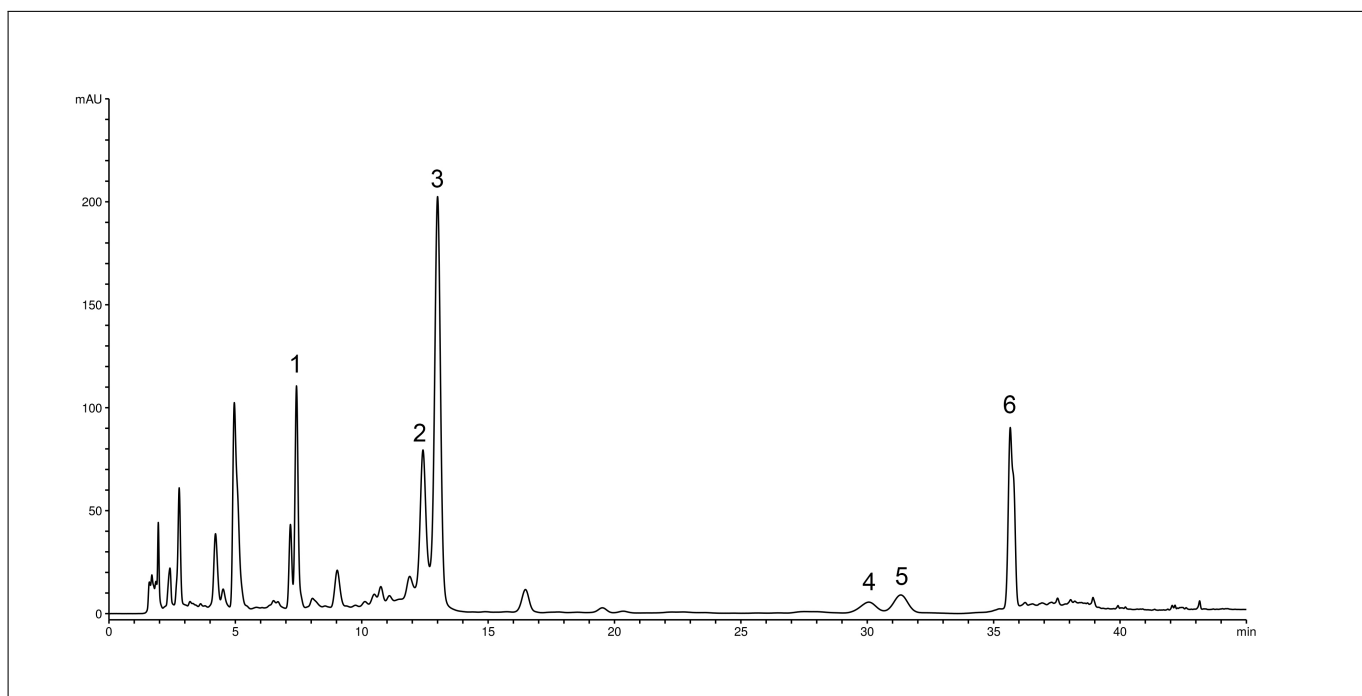


Fig. 2. HPLC chromatogram ($\lambda = 288$ nm) of 60% ethanol extract of milk thistle fruit. Peaks: 1-taxifolin; 2-silychristin; 3-silydianin; 4-silybin A; 5-silybin B; 6-isosilybin

of methanol. The amount of total silymarin in percentage (%), expressed as silybin, was calculated from the expression:

$$\text{Silymarin (\%)} = \frac{A_{\text{sample}} \times m_0}{A_0 \times m_{\text{sample}}} \times 100$$

where A_{sample} is area of flavonolignans peaks in sample, A_0 is area of silybin peak in reference solution, m_0 is mass of silybin in reference solution, and m_{sample} is mass of the sample.

3. RESULTS AND DISCUSSION

Silymarin content in various samples obtained by different extraction techniques was analyzed simultaneously by UV and HPLC methods, and results are presented in Table 1. HPLC chromatogram of extract obtained using water bath extraction is shown in Figure 2. The similarity between the employed analytical methods regarding silymarin content can be observed. According to the both HPLC and UV method, the highest amount was determined in samples extracted with boiling solvent during 30 and 60 minutes (2.33 and 1.95 %, respectively, by HPLC, and 3.60 and 3.13%, respectively, by UV). Extract obtained using maceration contained moderate amount of silymarin (0.54%), followed by samples obtained by UAE (ultrasonic-assisted extraction) during 15 and 30 min (0.25 and 0.46 %, respectively). The lowest content was determined in samples extracted by percolation technique (0.15%). Obtained results clearly showed that extraction procedure had a major influence on the silymarin content, and temperature and extraction time were the main parameters which affected the extraction efficiency of flavonolignans. According to the literature data, extraction with hot water at 100 °C yielded high level of silymarin, whereas organic solvents such as ethanol or methanol gave better results at their sub-boiling temperatures at 60 °C (Wallace et al., 2005). In our study, highest concentration of silymarin was measured after extraction in water bath at 85 °C which is a boiling temperature of 60% ethanol. Extraction in a longer time (60 minutes) slightly decreased the efficiency probably due to thermal degradation of the analytes. In the case of another conventional method, maceration, extracted amount of silymarin was slightly higher than the val-

Table 1. The content of silymarin (%) in various extracts determined by UV and HPLC methods

Extraction technique	UV	HPLC
Percolation	0.95	0.15
Maceration	2.36	0.54
UAE 15 min	0.58	0.25
UAE 30 min	0.97	0.46
Water bath 30 min	3.60	2.03
Water bath 60 min	3.13	1.94

ues obtained by UAE. When extraction was performed with UAE, considerably higher yield was achieved during longer extraction time (30 min). Saleh et al. (2015) also reported that increasing ultrasonic-assisted extraction time from 15 to 60 min increased the yield of the silymarin.

Our data regarding silymarin concentrations obtained by extraction in a water bath are in accordance with the requirements of Ph. Eur. 7.0. (2010) that prescribes a minimum of 1.5% of silymarin in milk thistle fruit. Quaglia et al. (1999) reported slightly higher values of silymarin than in our study, which could be the consequence of different extraction conditions used in the experiments.

CONCLUSION

In the present study, the efficiency of different extraction techniques in silymarin recovery from milk thistle fruit was tested. The obtained results showed that the highest concentration of silymarin was measured in sample extracted in a water bath at 85 °C for 30 min with 60% ethanol. This method is suitable for silymarin extraction due to its efficiency and reduction of time and solvent consumption, and could present potential replacement for Pharmacopoeia-recommended Soxhlet extraction procedure.

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