KUI – 18/2011
Received October 29, 2010
Accepted June 2, 2011

Isolation of Active Substances from the Seeds of the Milk Thistle Plant (*Silybum marianum*) and Determination of Antioxidant Activity

D. Gašo-Sokač,[a,b*] *S. Kovač,*[a,b] *and V. Bušić*[b]

[a]Faculty of Food Technology, J. J. Strossmayer University, Kuhačeva 20, 31 000 Osijek, Croatia
[b]Department of Chemistry, J. J. Strossmayer University, Kuhačeva 20, 31 000 Osijek, Croatia

Isolation of silymarin, a mixture of well-defined flavonolignans, from the seeds of milk thistle (*Silybum marianum*) was achieved. Silymarin was extracted from defatted seed with acetone without heating; the yield was $Y = 4.1$ %. The antioxidant activity of silymarin was determined at ambient temperature by means of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) colorimetry with a detection scheme at $\lambda = 515$ nm. The activity was evaluated by the decrease in absorbance as the result of a DPPH radical color change from purple to yellow. The results obtained showed that ascorbic acid was a substantially more powerful antioxidant than silymarin, but silymarin was a significantly stronger quencher of DPPH radical than the standard silibinin.

Keywords: *Silymarin, silibinin, DPPH radical scavenging, antioxidant activity*

Introduction

Milk thistle (*Silybum marianum,* family: Compositae) is an annual plant native to the Mediterranean area, which has now spread to other warm and dry regions.[1] The most important medicinal application of milk thistle is its use as a hepatoprotectant and as supportive treatment of chronic inflammatory liver disorders such as cirrhosis, hepatitis, and fatty infiltration due to alcohol and toxic chemicals.[2] It has also been used in the treatment of liver damage by poisonous mushrooms. The seed of milk thistle contains a relatively high fraction (approx. $w = 20$ %) of oil, which makes one-step extraction of silymarin from seeds impossible. Oil has to be removed from seeds prior to the extraction of silymarin and it is a by-product of silymarin production. This oil contains essential phospholipids and a relatively high content of vitamin E, it is therefore of interest as a natural source of vitamin E.[3]

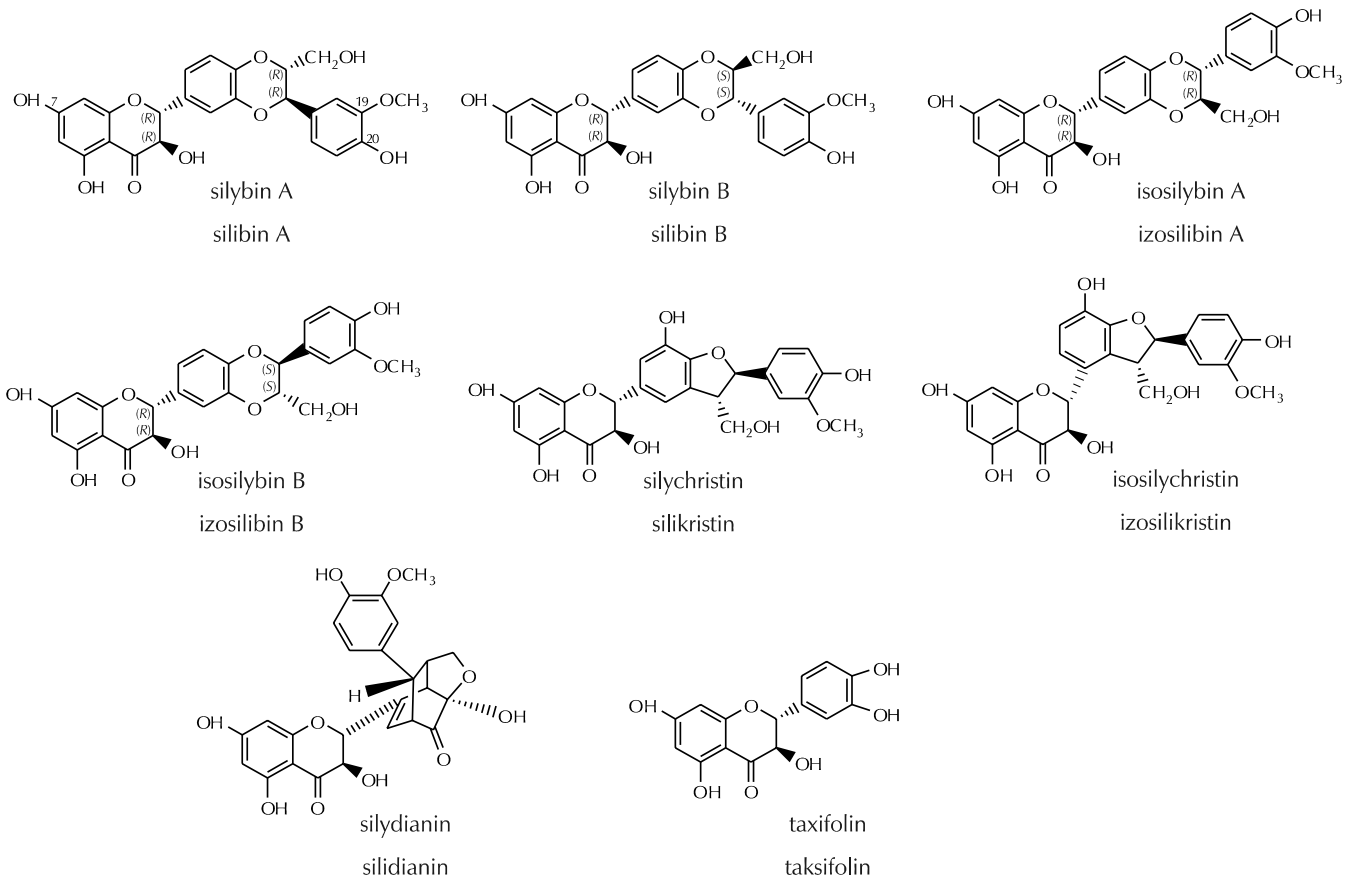
Silymarin, a crude extract, is a complex flavonolignan mixture (Fig. 1) and with that most of the clinical studies were carried out.[4,5]

The most abundant flavonolignans in silymarin are the diastereoisomers silybin A and silybin B. The diastereoisomers isosilybin A and isosilybin B are also present and represent regioisomers of silybin A and silybin B. The remaining flavonolignans are silychristin, isosilychristin, and silydianin, all of which are constitutional isomers of the aforementioned compounds.[6] A number of other flavonolignans have also been found in the seeds including desoxysilychristin, desoxysilydianin, silandrin, silybinome, silyhermin and neosilyhermin.[4] One of the important issues regarding silymarin is that it may be accepted as a safe herbal product, since no health hazards or side effects are known. Silibinin is a semipurified extract, representing approximately $\zeta_{A/B} = 1 : 1$ mass ratio of silybin A and silybin B.

The molecular mechanisms of the antioxidant activity of silybin and other silymarin components are not well known. Recent studies on activity of silybin and its derivatives against DPPH radical implies that the most important radical scavenging moiety is 20-OH group, 7-OH possesses only negligible scavenging activity and all other OH groups do not markedly participate in the overall activity (Fig. 1).[7]

Reflux extraction and Soxhlet extraction have classically been used for the extraction of botanical materials. However, these extraction processes are associated with high temperatures that could influence the thermolabile component. Therefore, it is desirable to develop a new extraction process to improve upon the inherent limitations of conventional approaches. The objective of this study was to examine the yield of extraction carried out at room temperature for prolonged time since most authors have extracted active components from milk thistle by heating of solvents (Soxhlet extraction).[8] Also this work is aimed to examine the quality of extracted material by assessing the antioxidant activity of extract (silymarin) for comparison with the antioxidant activity of well-known antioxidants such as ascorbic, gallic, tannic acid and standard silibinin using a DPPH free radical.

* Corresponding author: dr. sc. Dajana Gašo-Sokač, e-mail: dgaso@ptfos.hr



F i g. 1 – *Structures of main silymarin components*
S l i k a 1 – *Strukture glavnih komponenata silimarina*

Experimental

The milk thistle seeds originated from Fero-Leko Ltd (Požega, Croatia). The ascorbic, gallic, tannic acids were of analytical grade and purchased from Sigma Chemical Co. DPPH (2,2-diphenyl-1-pycrilhydrazyl), and standard silibinin was purchased from Sigma Chemical Co.

Isolation of oil from the seed

The milk thistle seeds were ground and the moisture content was determined by drying to a constant mass at 105 °C. The moisture fraction was 5.02 %. The ground seeds (m = 50 g) of milk thistle were defatted in Soxhlet apparatus with hexane for 3 h. After filtration and evaporation of the solvent, m = 14.53 g of pale yellow oil (yield Y = 27.54 % based on dry seed mass) was obtained.

Isolation of silymarin from the seed

The defatted seed powder (m = 37 g) was transferred into a flask fitted with a condenser and 190 mL of acetone was added and stirred for t = 72 h at room temperature. After filtration and concentration of the silymarine fraction under vacuum, the yellow residue was dissolved in methanol (100 mL) and partitioned with hexane (3 × 200 mL). Methanol extract was partitioned with diisopropyl ether, and evaporated. The remaining solid was dried to obtain m = 1.52 g (Y = 4.1 %) of silymarin.

Free radical-scavenging method

The antioxidant activity of silymarin was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical.[9] A methanol solution (0.05 mL) of a sample of various mass concentrations (γ = 0.1 – 2 mg mL^{-1}) was placed in a cuvette, and 2 mL of DPPH solution in MeOH ($c = 6 \cdot 10^{-5}$ mol L^{-1}) was added. The mixture was shaken vigorously, and then absorbance measurements commenced immediately. The decrease in absorbance at λ = 515 nm was determined continuously with data being captured at 60 s intervals with a UV-VIS spectrophotometer Analytik Jena Specord 200, until the reaction reached a plateau. Methanol was used to zero the spectrophotometer. The absorbance of the DPPH radical without the antioxidant (i. e. the control) was measured daily. All determinations were performed in triplicate.

The DPPH radical mass concentration in the reaction medium was calculated from the following equation as determined by the linear regression:

$$A_{515} = 0.02375\ \gamma_T(\text{DPPH})/(\text{mg L}^{-1}) - 0.0005$$

with r = 0.994. The fraction of DPPH inhibited by sample was calculated according to the formula:[10]

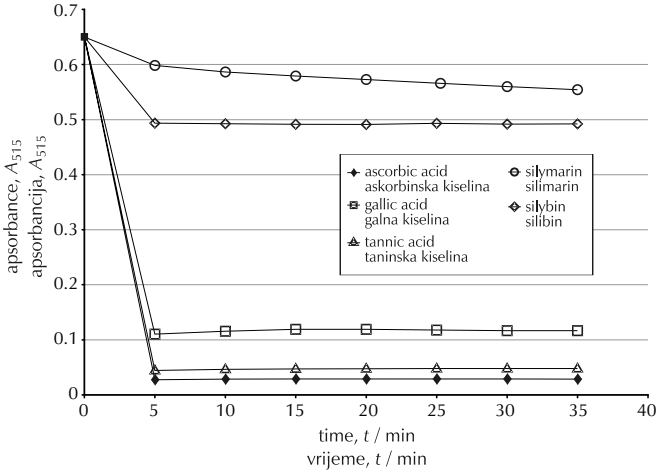
$$w = (A_{C(0)} - A_{C(t)})/A_{C(0)}$$

where $A_{C(0)}$ was the absorbance of the control at t = 0 and $A_{C(t)}$ was the absorbance of the antioxidant at t, which varied with the different mass concentrations.

Results and discussion

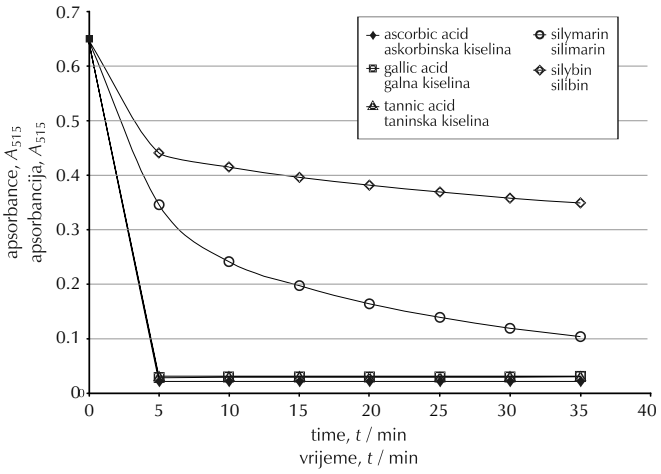
Recommended extraction procedures from literature emphasize the importance of removing the lipids from milk thistle seeds, prior to silymarin extraction. In comparing the maximum yields of the silymarin, defatted seeds yielded on average twice the quantity of compounds obtained for whole seeds under identical extraction conditions.[8,11] For extraction of oil we also used dichloromethane, the yield was similar as with hexane (Y = 27 – 28 % based on dry seed mass).

After removing the oil from the seed we extracted silymarin with acetone at room temperature. As expected, the extraction of the silymarin showed a general increase in yield with time, as equilibrium between the extracted compounds and the solvent was approached. The best yield was Y = 4.1 %, after t = 72 h. Hot extraction using Soxhlet apparatus with acetone showed similar yield. We also used petroleum ether for extraction (cold extraction for 72 h), the yield was slightly lower (Y = 3.9 %). In order to preserve the thermolabile compounds we decided to recommend cold extraction of silymarine, since less compound degradation should occur as the temperature is lowered.



F i g. 2 – *Reduction of DPPH radical with solutions of ascorbic, gallic, and tannic acid, silymarin and silibinin at concentration γ = 0.1 mg mL^{-1}*

S l i k a 2 – *Redukcija radikala DPPH otopinama askorbinske, galne i taninske kiseline, silimarina i silibinina koncentracije γ = 0,1 mg mL^{-1}*



F i g. 3 – *Reduction of DPPH radical with solutions of ascorbic, gallic, and tannic acid, silymarin and silibinin at concentration γ = 2 mg mL^{-1}*

S l i k a 3 – *Redukcija radikala DPPH otopinama askorbinske, galne i taninske kiseline, silimarina i silibinina koncentracije γ = 2 mg mL^{-1}*

After extraction of silymarin its antioxidative activity was determined. The results (Figs. 2–3) showed the decrease in absorbance of the DPPH radical due to its reduction by different antioxidants. Absorbance decreased as a result of a colour change from purple to yellow as the radical was scavenged by the antioxidant through donation of hydrogen to form the stable DPPH-H. The data showed that the DPPH radical solution was bleached with all the samples tested. However, differences could be observed through different concentrations of antioxidants used.

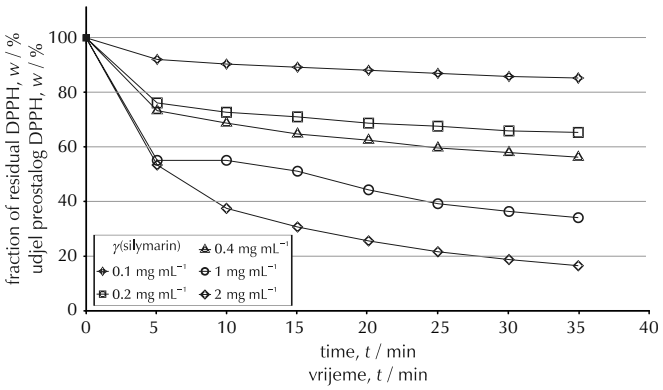
Table 1 shows that ascorbic, gallic and tannic acids are superior inhibitors of DPPH radical compared to silymarin and silibinin. These percentages (Table 1) for used acids can be considered as a full reduction of DPPH radical, because after completing the reaction the final solution always possesses some yellowish colour and therefore its absorption grade compared to colourless methanol solution cannot reach 100 %. The steady state was reached after five minutes. Silymarin had nearly the same effect for the mass concentration γ = 2 mg mL^{-1} (83 % of DPPH inhibited), however the steady state was reached after 35 min.

T a b l e 1 – *Comparison of antioxidant activity of studied compounds*

T a b l i c a 1 – *Usporedba antioksidacijske aktivnosti* istraživanih *spojeva*

Mass concentration of studied compound Masena koncentracija istraživanog spoja γ / mg mL^{-1}	Inhibition of DPPH radical Inhibicija radikala DPPH w / %				
	ascorbic acid askorbinska kiselina	gallic acid galna kiselina	tannic acid taninska kiselina	silymarin silimarin	silibinin silibinin
0.1	94.86	82.15	92.66	14.77	24.31
0.25	95.58	86.61	94.20	34.77	25.57
0.4	96.06	92.97	94.26	43.54	33.80
1	96.34	94.08	94.38	66.15	33.80
2	96.57	95.05	94.92	83.64	46.00

The absorbance of the solution decreases depending on the intrinsic antioxidant activity of the antioxidant as well as on the speed of the reaction between DPPH free radical and the antioxidant. In case of rapid kinetic behaviour, practically all samples of acid used at all concentrations reacted within a very short time, and a steady state was reached in a few minutes. On the other hand, for silymarin and silibinin slow kinetic behaviour implied longer periods before the steady state was reached at high mass concentrations.



F i g. 4 – *Time dependent plots of residual DPPH radical for various initial mass concentrations of silymarin*

S l i k a 4 – *Preostali DPPH u vremenu uz različite početne masene koncentracije silimarina*

T a b l e 2 – *IC_{50} for scavenging activity of studied compounds. Values were determined graphically from dose response curve.*

T a b l i c a 2 – *IC_{50} antioksidacijske aktivnosti ispitivanih spojeva. Vrijednosti su određene grafički.*

Compound Spoj	IC_{50} / mg mL^{-1}
ascorbic acid askorbinska kiselina	0.052
gallic acid galna kiselina	0.061
tannic acid taninska kiselina	0.054
silymarin silimarin	0.876
silibinin silibinin	2.058

Data on the time course of absorbance (reaction kinetics) enable assessment of the extent of antioxidant activity for each sample. To do so, it was necessary to convert data on the reaction kinetics into new plots displaying the fraction of residual DPPH in solution as a function of time. The fraction of residual DPPH ($DPPH_{res}$) was calculated from:

$$w_{res}(\text{DPPH}) = \gamma_t(\text{DPPH}) / \gamma_0(\text{DPPH})$$

where γ_0 and γ_t are initial mass concentration and mass concentration at t (Fig. 4).

The activity of all samples was calculated as the mass concentration of an antioxidant that reduced initial mass concentration of DPPH radical by 50 % (IC_{50}). The lower the IC_{50} the higher was the antioxidant activity. The IC_{50} values (Table 2) showed significant difference between the free radical scavenging activity of silymarin and silibinin.

Conclusion

In conclusion, the findings of the present study demonstrated that isolation of silymarin from milk thistle can be carried out without heating the solvent. The yield of the extraction was $Y = 4.1$ %. Future work in extracting silymarin from milk thistle with other organic solvents (ethanol, acetonitrile, methanol) will also focus on temperature below the normal boiling point of used solvents.

Ascorbic, gallic and tannic acid were substantially more active at all concentrations used in comparison with silymarin, they almost completely reduced DPPH radical. The higher antioxidant activity of silymarin compared to silibinin can be explained by (1) the presence of taxifolin (which has chelating characteristics and antioxidant activity similar to that of quercetin) and (2) the ability of chemically unidentified polyphenols, which comprise 30 % of silymarin,[12] to scavenge free radicals. Although silymarin has lower antioxidant activity than the used acids, the results demonstrate that silymarin, as one of the best pharmacologically characterized plant extracts, besides its biological activities, can serve as antioxidant and free radical scavenger.

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List of symbols
Popis simbola

A_{515} – absorbance at $\lambda = 515$ nm
– apsorbancija pri $\lambda = 515$ nm

c – amount of substance concentration, mol L^{-1}
– množinska koncentracija, mol L^{-1}

IC_{50} – half maximal inhibitory mass concentration, mg L^{-1}
– masena koncentracija koja uzrokuje 50 %-tnu inhibiciju, mg L^{-1}

m – mass, g
– masa, g

r^2 – correlation coefficient
– korelacijski koeficijent

t – time, min, h
– vrijeme, min, h

w – mass fraction, %
– maseni udjel, %

Y – yield, %
– iskorištenje, %

γ – mass concentration, mg mL^{-1}
– masena koncentracija, mg mL^{-1}

ζ – mass ratio
– maseni omjer

λ – wavelength, nm
– valna duljina, nm

SAŽETAK

Izolacija aktivnih tvari iz sjemena biljke sikavice (*Silybum marianum*) i određivanje njihove antioksidacijske aktivnosti

D. Gašo-Sokač,[a,b*] *S. Kovač*[a,b] *i V. Bušić*[b]

Opisana je izolacija silimarina, smjese dobro definiranih flavonolignana, iz sjemena biljke sikavice (*Silybum marianum*). Silimarin je ekstrahiran acetonom iz deoleinizirana sjemena bez zagrijavanja. Iskorištenje je iznosilo $Y = 4{,}1$ %. Antioksidacijska aktivnost silimarina određena je na sobnoj temperaturi kolorimetrijski 2,2-difenil-1-pikrilhidrazilom (DPPH) pri $\lambda = 515$ nm. Aktivnost je određena na temelju smanjenja apsorbancije, kao rezultat promjene boje DPPH iz purpurne u žutu. Dobiveni rezultati pokazuju da je askorbinska kiselina znatno jači antioksidans od ispitivana silimarina, ali je silimarin znatno jači hvatač radikala DPPH od standarda silibinina.

[a] *Prehrambeno-tehnološki fakultet Osijek, Sveučilište J. J. Strossmayera, Kuhačeva 20, 31 000 Osijek, Hrvatska*
[b] *Odjel za kemiju, Sveučilište J. J. Strossmayera, Kuhačeva 20, 31 000 Osijek, Hrvatska*

Prispjelo 29. listopada 2010.
Prihvaćeno 2. lipnja 2011.