EVALUATION OF BIOLOGICAL ACTIVE COMPOUNDS FOUND IN Silybi mariani fructus

Daniela IONESCU, Simona SPÎNU, Alina ORȚAN, Ionuț MORARU, Gina FÎNTÎNERU, Radu Claudiu FIERĂSCU, Irina FIERĂSCU, Manuel DRUGULESCU

1University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd., 011464, Bucharest, Romania
2Hofigal Export Import S.A., 2 Intrarea Serelor Street, 042124, Bucharest, Romania
3University of Bucharest, Faculty of Physics, Doctoral School of Biophysics and Medical Physics, 405 Atomiștilor Street, 077125, Măgurele, Ilfov, Romania
4S.C. Laboratoarele Medica S.A., 11 Frasinului Street, Otopeni, Romania
5The National Institute for Research & Development in Chemistry and Petrochemistry – ICECHIM Bucharest, 202 Splaiul Independenței, 060021, Bucharest, Romania
6SC BIOTECH PROD SRL, 45 Prelungirea Ghencea Street, Bragadiru, Ilfov, Romania

Corresponding author email: alina_ortan@hotmail.com

Abstract

Milk thistle (Silybum marianum L.) is a herbaceous plant, annual or biennial, cultivated, sometimes as sub-spontaneous plant, which is found from the Mediterranean Region to Central Asia. This plant contains biologically active compounds such as flavonoids (quercetin, taxifoline and dehydrokermepherol), lipids (linoleic acid, oleic acid and palmitic acid), sterols and mucilages, sugars (arabinose, rhamnose, xylose, glucose), amines and saponins (cholesterol, campesterol and stigmasterol). This paper describes methods for obtaining extracts, in order to obtain active biological compounds from Silybi mariani fructus, extracts characterised through modern analytical techniques, as well as the evaluation of their antioxidant activity.

Key words: Silybi mariani fructus; fatty oil; silymarin; fatty acids; antioxidant activity.

INTRODUCTION

Milk thistle (Silybum marianum L.) is a herbaceous plant, annual or biennial, cultivated, sometimes as sub-spontaneous plant, which is found from the Mediterranean Region to Central Asia. In Romania, it is cultivated in the south part of the country (Ialomita, Teleorman, Constanta, Olt, Dolj). Fruits (Silybi mariani fructus) should be harvested in the morning, in the sunny time, after the fall of the leaves, by cutting the calatides. This plant contains biologically active compounds such as flavonoids (quercetin, taxifoline and dehydrokermepherol), lipids (linoleic acid, oleic acid and palmitic acid), sterols and mucilages, sugars (arabinose, rhamnose, xylose, glucose), amines and saponins (cholesterol, campesterol and stigmasterol). Milk thistle has been used since ancient times as a medicine in the treatment of liver diseases (Barbarino et al., 1981; Luper, 1998; Nitin et al., 2007; Pepping, 1999). Currently, milk thistle is one of the most important medicinal herbs used in hepatotherapy, and the active component – silymarin - is already a traditional remedy, especially in Central Europe, being widely prescribed by phytotherapists in the treatment of liver diseases (Ahmed-Belkacem et al., 2010; Cornelli et al., 2007; Kim et al., 2003; Mhamdi et al., 2016; Pradhan and Girish, 2006; Thakur, 2002). The German Commission E recommends it for the treatment of toxin-induced liver diseases, liver cirrhosis and supportive therapy for chronic inflammation of the liver (Murphy et al., 2000).

Silymarin, with strong hepatoprotective activity, is a polyphenolic flavonoid, a mixture of 3 flavonolignans - silybin, silidianeine and silicristine - of which the most active is silybin.
The action of silybin and its related substances provides both the protection of the membrane of the hepatic cells and the repair of injured cells (Ball and Kowdley, 2005; Barrett, 2007; Ozturk et al., 2012; Oufi et al., 2012; Pradhan and Girish, 2006; Valenzuela and Garrido, 1994).

The remarkable hepatoprotective action of the extract from the milk thistle seeds through several experimental models with hepatotoxic substances has been evidenced by inhibition mechanisms of hepatotoxin related to receptors of the membrane of hepatocyte, reduction of glutathione oxidation and increasing its level in the liver and intestines, improvement the activity of antioxidant enzyme, stimulation of the ribosomal RNA polymerase activity and protein synthesis, resulting an enhanced hepatocyte regeneration (Bosisio et al., 1992; Morazzoni and Bombardelli, 1994; Muriel and Mourelle, 1990).

Silybin and related substances have a double action: protect the integrity of the non-damaged cell membranes and stimulate the formation of new hepatic cells (Pepping, 1999; Morazzoni and Bombardelli, 1994). Silybin, experimented on mice as an antidote to phalloidin intoxication (toxin of Amanita phalloides fungus), has shown to be a very good hepatoprotective agent, not only in vitro but also in vivo, preventing hepatocyte poisoning by blocking receptors of the cell membrane level and inhibiting other toxic peptides, such as α-amanitin, prevented from reaching the cell nucleus (Cornelli et al., 2007; Desplaces et al., 1975; Muriel and Mourelle, 1990; Valenzuela and Garrido, 1994).

Popular medicine uses root, leaves and fruits in the form of tea, powder or tincture to treat epidemic hepatitis, pleurisy and spleen diseases (Pârvu, 2006).

This paper describes methods for obtaining extracts, in order to obtain active biological compounds from Silybi mariani fructus, which will be characterised through modern analytical techniques.

**MATERIALS AND METHODS**

**Plant material**
The plant material was harvested from plants during the fruiting period, from *Silybum marianum* L. plants grown in the culture system at AROMA PLANT SRL, Furculesti, Teleorman County, Romania (Figure 1).

![Figure 1. Silybum marianum L.](image)

From the harvested fruits, the following types of extracts were obtained:

a. For determination of the fatty oil: approximate 50 g of chopped fruits is degreased for 3 h with petroleum ether in the Soxhlet apparatus (encoded sample A).

b. For the determination of silymarin: 5 g of chopped fruits (degreased) is refluxed with light oil; after 8 h is dried at room temperature; extraction is carried out with 100 ml of methanol on a water bath for 5 hours; 5 ml of this solution is diluted with the same solvent (encoded sample B1); the same procedure for non-degreased fruits (sample B2).

c. For the determination of fatty acids: 0.3 g of chopped fruits is mixed with a solution obtained from 5 ml petroleum ether and 50 ml hydrochloric acid in methanol 0.5 M. This mixture is refluxed for 1 h at 65°C. After cooling the sample, the mixture is washed with distilled water and 10 ml of iso-octane in a separation funnel, until pH is neutral; the iso-octane layer is removed, and the extract is dried over sodium sulphate and filtered (encoded sample C).

d. For determination of antioxidant activity: were obtained an alcoholic extract, an hydroalcoholic extract and a water extract from 5 g of upper chopped fruits. The vegetal material was mixed in ethanol (50 ml) for the
alcoholic extract, at 70°C for 2 hours (encoded sample D1); for the hydroalcoholic extract, the vegetal material was mixed with 1:1 water-ethanol (25 ml: 25 ml) at 78°C for 2 hours (encoded sample D2); for the water extract, the vegetal material was mixed with bidistilled water (50 ml) at 90°C for 2 hours. All the reagents were analytic grade, purchased from Merck KGaA, Germany.

Methods

For anatomic analysis of the fruit, samples were fixed in ethyl alcohol 70%. Cutting the plants, the fixation and colouring the preparations was made in accordance with the methodology of the achievement of plant anatomy preparations (Pârvu, 2006; Metcalfe and Chalk, 1979).

The sections were made on a hand microtome and sectioned with the anatomical razor. Fruit observation was made with a stereomicroscope Optika SZM1 at different magnification and the terminology used in the Brouwer and Stählin seed treaty was used (Brouwer and Stählin, 1973).

For quantification of the biologic active compounds were used a high-performance liquid chromatograph (HPLC) and a gas chromatograph coupled with a mass spectrometer (GC-MS).

For HPLC analyses was used a DIONEX system equipped with a Diode Array detector (200-600 nm) and a gradient performing pump (P580), with a column with 12.5 cm length, 4 mm diameter. Stationary phase was octadecylsilyl silica gel for chromatography (5 μm), mobile phase A phosphoric acid: methanol: water (0.5:35:65 V/V/V) and mobile phase B phosphoric acid: methanol: water (0.5:50:50 V/V/V); flow rate 1 ml/minute; injection 10 μl; retention time 30 minutes.

For GC-MS analyses was used a FOCUS GC equipment coupled with DSQII Thermo mass spectrometer (Focus GS - MS/DSQII) with a 30 m length, 0.25 mm diameter capillary column. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. Samples were introduced via split mode (100) in an auto sampler with the injection port at a temperature of 270°C. The column temperature was initially held at 160°C for 5 min then increased from 160°C to 200°C (from minute 6 to 10) and then from 200°C to 250°C. The GC/MS interface temperature was set at 270°C.

Evaluation of antioxidant activity

The antioxidant activity was performed using DPPH assay. The DPPH assay involves mixing 0.5ml of the sample with 1ml of 0.02 mg/ml DPPH solution (Sigma Aldrich, USA). After an incubation of 30 minutes, solutions were tested by reading the absorbance at 517 nm on the UV-VIS spectrophotometer SPECORD 210 Plus.

The antioxidant activity (AA%) percentage was calculated using the formula (Fierascu et al., 2015):

$$AA\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$  \hspace{1cm} (1)

where:

$A_{control}$ is the absorbance of the DPPH solution without sample;

$A_{sample}$ is the absorbance of the extract mixed with 0.02 mg/ml DPPH solution.

Determination of fatty oil - Fatty oil content (FO) is calculated using the formula, and the result is expressed as a percentage (*** ANM, 2010).

$$FO\% = \frac{A \times \frac{100}{m} \times \frac{100}{100-U}}{1}$$  \hspace{1cm} (2)

where:

$A$ is the quantity of oil obtained by distillation, in grams;

$m$ represents the mass of the sample, in grams;

$U$ represents the loss on drying.

RESULTS AND DISCUSSIONS

Following the anatomical analysis, the seed is observed at the longitudinal section of the achene (general aspect - Figure 2a and longitudinal section - Figure 2b) and seminal endosperm (Figure 2c). Applying the method described above, a content of 30.2% fatty oil was determined from sample A (calculated according equation 2).
From sample B1 (degreesed) and B2 (non-degreased) through HPLC analysis, the content of silymarin, expressed in silybin is 2.65% and 1.94% (Figures 3 and 4).

Sample C (Silybi mariani fructus oil) was characterised by GC-MS. The results obtained are presented in Figure 5, while the identified components are summarized in Table 1.

In sample C (Silybi mariani fructus oil) were identified 10 components, from which the most important are linoleic acid (Omega 6) - 36.1% and linolenic acid (Omega 3) - 4%.

The results of the DPPH assay performed on the samples D1, D2 and D3, calculated according to eq. (1) revealed a better antioxidant activity for D1 (91.88% for D1, 54.81% for D2, and, respectively, 33.32% for D3.

CONCLUSIONS

The results obtained support the use of Silybum marianum L. as a very good hepatoprotective agent, its action being mainly due to the high content in silymarin and fatty oils. The results also show a high content of fatty acids as

Table 1. Compounds identified by GC-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound name</th>
<th>RT (min.)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Palmitic C16:0</td>
<td>8.89</td>
<td>8.13</td>
</tr>
<tr>
<td>2</td>
<td>Stearic C18:0</td>
<td>13.08</td>
<td>5.38</td>
</tr>
<tr>
<td>3</td>
<td>Oleic C18:1</td>
<td>13.44</td>
<td>32.38</td>
</tr>
<tr>
<td>4</td>
<td>Cis-Vaccenic C18:1</td>
<td>13.57</td>
<td>0.51</td>
</tr>
<tr>
<td>5</td>
<td>Linoleic C18:2</td>
<td>14.38</td>
<td>43.97</td>
</tr>
<tr>
<td>6</td>
<td>Linolenic C18:3</td>
<td>15.67</td>
<td>4.19</td>
</tr>
<tr>
<td>7</td>
<td>Arachic C20:0</td>
<td>17.56</td>
<td>2.83</td>
</tr>
<tr>
<td>8</td>
<td>11-Eicosenoic C20:1</td>
<td>18.02</td>
<td>0.68</td>
</tr>
<tr>
<td>9</td>
<td>Behenic C22:0</td>
<td>24.63</td>
<td>1.59</td>
</tr>
<tr>
<td>10</td>
<td>Lignoceric C24:0</td>
<td>33.81</td>
<td>0.35</td>
</tr>
</tbody>
</table>
linoleic acid, that could be used in therapeutic purposes. 
Also, the results obtained for the antioxidant assay proposes the use of the ethanolic extract (showing a very good antioxidant activity) for the formulation of antioxidant pharmaceuticals and dietary supplements.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support obtained through the project SusMAPWaste, SMIS104323, Contract No. 89/09.09.2016, from the Operational Program Competitiveness 2014-2020, project co financed from the European Regional Development Fund.

REFERENCES


