**Silymarin content in Silybum marianum fruits at different maturity stages**

Ahlam Elwekeel¹, Ahlam Elfishawy² and Sameh AbouZid¹*

¹Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62111, Egypt.
²Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt.

Accepted 31 May, 2013

Silymarin is one of the most investigated plant extracts with known mechanism of action. It is used for oral treatment of toxic liver damage. Silybin, isosilybin, silychristin and silydianin are the main flavonolignan components of silymarin isolated from the fruits of *Silybum marianum*. A reversed-phase high-performance liquid chromatography (HPLC) method for determination of flavonolignans was developed. The method depended on an isocratic solvent system comprising acetonitrile and water containing 0.5% (v/v) phosphoric acid. The method was used for analysis of the flavonolignans content in the fruits of *S. marianum* at different maturity stages. A correlation between fruits color, maturity stage and silymarin content is demonstrated.

**Key words:** *Silybum marianum*, silymarin, flavonolignans, fruits, maturity, high performance liquid chromatography (HPLC).

**INTRODUCTION**

Milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] is an annual herb, native to the Mediterranean and North African regions (Boulos, 2000). The plant reaches to heights of 10 feet. It has a stem of 20 to 150 cm high, erect, ridged and branched in the upper part. Each stem ends with solitary composite flower heads, about 2 inches in diameter, consisting of purple disc florets. The flower heads of milk thistle differ from other thistles by the presence of leathery bracts that are also tipped with stiff spines. The fruits are hard skinned achenes, 6 to 8 mm long flat, smooth and shiny dark brown in color.

*S. marianum* fruits contain an isomeric mixture of flavonolignans collectively known as silymarin (Morazzoni and Bombardelli, 1995). The principal components of silymarin are silybin, isosilybin, silychristin and silydianin (Figure 1). Diastereoisomers exist for these compounds (silybin A, silybin B, isosilybin A, isosilybin B, silychristin A and silychristin B). Basically, flavonolignan nucleus consists of the dihydroflavanol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. Silymarin is one of the most investigated plant extracts with known mechanism of action. It is used for oral treatment of toxic liver damage (Flora et al., 1998). Silymarin is also used to reduce the risk for developing cancer (Deep et al., 2008). Differential antiproliferative effects of individual flavonolignans on human prostate carcinoma cells were reported (Davis-Searle et al., 2005).

Isosilybin B was the most potent suppressor of cell growth relative to other flavonolignans. Isosilybin B, and to a lesser extent isosilybin A, seem to be the most potent in various prostate cancer chemopreventive and antiproliferative activity assays (Sy-Cordero et al., 2010).

*Corresponding author, E-mail: sameh.zaid@pharm.bsu.edu.eg. Tel: +2 (082)2317958. Fax: +2 (082)2317958.*
The hepatoprotective action of individual flavonolignans and one flavonoid that comprise silymarin was evaluated. The most potent compounds were taxifolin, isosilybin A, silybin A, silybin B and silibinin, a mixture of silybin A and silybin B (Polyak et al., 2010). Silymarin is known to possess other biological activities such as anti-inflammatory (Breschi et al., 2002), immunostimulant (Alidoost et al., 2006), antidiabetic (Maghrani et al., 2004) and hyperprolactinemic (Capasso et al., 2009) effects.

Silymarin content and composition in S. marianum is affected by many factors such as sowing depth, raw spacing, fertilizers, harvesting and post harvesting treatment (Omer et al., 1993; Karkanis et al., 2011). There are large differences in the silymarin content and composition between plant parts and in the fruits during maturity (Martin et al., 2006). The highest concentrations of silymarin are in the fruit heads and mature fruits (10.7 and 14.7 g/kg, respectively). The content in the flowers was much lower (0.65 g/kg). There is no previous study on silymarin content in the fruits of S. marianum at different stages of maturity.

Extract obtained from the fruits of S. marianum is available worldwide in the pharmaceutical market as antihepatotoxic drug under a variety of brand names. Extract is standardized so that the silymarin content is 70 to 80% of the extract weight. There is a need to have a selective and accurate analytical method for qualitative and quantitative determination of silymarin flavonolignan components during standardization of the extract. This is expressed as silymarin percentage and it corresponds to

Figure 1. Chemical structures of flavonolignans components of silymarin.
the sum of silybins, isosilybins, silychristins and silydianin concentrations. It is important that the analytical method characterizes and quantifies each component in silymarin.

In this report, individual flavonolignans content in *S. marianum* fruits at different maturity stages was evaluated and correlated to their site of synthesis in this plant.

### MATERIALS AND METHODS

#### Plant

*S. marianum* fruits were collected in May, 2007, from wild plant growing in Beni-Suef governorate, Egypt. Beni-Suef has a dry climate and is classified as having a hot desert climate (May climate data: 35°C average high and 18°C average low). The fruits were taxonomically identified by Dr. Abdelhalim Mohamed, Flora & Phyto-taxonomy Researches Department, Horticultural Research Institute, Agricultural Research Centre, Ministry of Agriculture, Egypt. A voucher specimen was deposited at the herbarium of Faculty of Pharmacy, Beni-Suef University. The collection was done every 10 days to obtain three developmental stages according to color (creamy white, light brown, dark brown).

#### Flavonolignan content in different maturity stages of fruits

Five grams of finely powdered fruits of each developmental stage were separately defatted with petroleum ether by a soxhlet apparatus for 4 h. The solvent was discarded, the residue was dried in air then extracted with methanol for 5 h. The methanolic extract was evaporated to dryness on a rotary evaporator. The dried extract was dissolved in methanol and introduced to 10 ml volumetric flask. The volume was completed to the mark. An aliquot of 20 µl was injected into HPLC to quantify flavonolignans content. The HPLC system consisted of Shimadzu liquid chromatograph equipped with LC-10 AD pump, SPD-10A UV detector, Inertsil ODS-3 (5 µm, 4.6 × 250 mm) column for analytical purposes. Flavonolignans were quantified at 288 nm using peak versus concentration. The regression equation was computed and calibration curves were constructed (areas under peaks corresponding to each concentration of flavonolignans were separately injected into HPLC. Three replicates were used for each injection. The mean values or areas under peaks corresponding to each concentration of flavonolignans were calculated and calibration curves were constructed (areas under peak versus concentration). The regression equation was computed for each individual flavonolignan. A linear relationship was obtained by plotting the recorded areas under peak versus concentration of authentic flavonolignan.

#### Statistical analysis of the data

The data are the mean of triplicate measurements. The results are expressed as mean ± standard error (SE). Statistical significance was determined by Student’s *t*-test with *P* < 0.01 considered significant.

#### RESULTS AND DISCUSSION

The color of *S. marinaum* fruits (achenes) changes during maturation from creamy white to light brown to dark brown color when it is ripe and fully mature. A reversed-phase HPLC method was developed to determine the content of individual flavonolignans in the fruits at different developmental stages. The result of analysis of silymarin flavonolignans content is shown in Table 1. A correlation of seed maturity/color to silymarin content was found. The fully mature fruits contain the highest silymarin content, of which silybin represents about 40%. At this stage of maturity, silymarin represents 76% of the extract using the reported extraction method. This result is in agreement with that reported by Kroll et al. (2007) in which silymarin represents 65 to 80% of the extract. The present study shows that silymarin content in the dried ripe and fully mature fruits of *S. marianum* collected in Beni-Suef, upper Egypt is 3.5%. It is well-known that silymarin content varies between 1.5 to 3.5% in the fruits, with 3 to 6% considered of high quality (Greenlee et al., 2007).

Micro-dissection of *S. marianum* fruits allowed four parts to be distinguished: pericarp, seed integument, albumen and embryo (Cappelletti and Caniato, 1984). The pericarp includes epidermis, subepidermic layer and

<table>
<thead>
<tr>
<th>Maturity stage</th>
<th>Silychristin (mg/g)</th>
<th>Silydianin (mg/g)</th>
<th>Silybin (mg/g)</th>
<th>Isosilybin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>3.4±0.6*</td>
<td>12.6±1.4</td>
<td>2.6±0.3</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Stage 2</td>
<td>10.1±2.6</td>
<td>39.3±9.4</td>
<td>44.9±12.4</td>
<td>24.7±8.2</td>
</tr>
<tr>
<td>Stage 3</td>
<td>56.5±12.3</td>
<td>207.2±33.2</td>
<td>311.0±45.2</td>
<td>106.8±32.6</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SE.*

---

Table 1. Flavonolignans content (mg/g) in of *Silybum marianum* fruits at different stages.
membraniform layer. Some of the cells of the subepidermic layer are filled with dark brown substance responsible for the spotted appearance of the fruits. The seed integument consists of epidermis of the integument, few cell layers containing calcium oxalate crystals. The albumen consists of one layer of cells with protein as a storage material. The embryo includes two large cotyledons with fat as storage material. Little research has been performed on silymarin localization within the milk thistle fruits.

Flavonolignans were localized in the outer portion of the fruits which includes all the cell layers from the pericarp epidermis to the albumen (Cappelletti et al., 1983). Using a histochemical method (Cappelletti and Caniato, 1984), which stains silybin, silydianin and silychristin as well as taxifolin, these flavonolignans were found to be localized in the following cell layers: subepidermic cell layer and membraniform layer of the pericarp and all the layers making up the seed integument. Moreover, it was hypothesized that flavonolignans synthesized from taxifolin and coniferyl alcohol which is a lignin precursor, may be associated with wall materials. Results obtained from this study are a further evidence for localization of flavonolignan in the outer portion of the fruits.

Ultra violet (UV)-visible spectrophotometry was proposed for the quantitative determination of flavonolignans (Famacopea Ufficiale Italiana (1985)). The method was time consuming and showed a non-satisfactory repeatability. Capillary zone electrophoresis was used for separation and determination of silymarin components (Kvasnička et al., 2003). The method showed shorter analysis time and better resolution of silydianin and silychristin compared to HPLC. High performance capillary electrophoresis (HPCE) was used for determination of silymarin in the extract of *S. marianum* using borate buffer solution at pH 9. At this pH, the flavonolignans having many phenolic groups in their structure were negatively charged (Quaglia et al., 1999). Under these conditions, isosilybin co-eluted together with silybin.

Adding 12 mM dimethyl β-cyclodextrins solution to the running buffer, the separation of silybin from isosilybin was obtained. HPLC was proposed as a method for determination of silymarin (Quaglia et al., 1999). Two reversed stationary phases, RP-18 and RP-8, were compared for resolution of all considered flavonolignans. The RP-18 stationary phase showed good separation among silybin and isosilybin, while silydianin and silychristin were not baseline resolved. The increase in water concentration in the mobile phase allowed the separation of the two diastereomers of silybin. RP-8 stationary phase, a more polar phase, improved the resolution of peaks related to all flavonolignans but did not allow the resolution of the two silybin diastereomers. The good separation of all compounds allowed the purity control of each peak. However, time consumption, the need for pre-purification step and availability of pure reference compounds are the main disadvantages. Differences between methods for quantitative determination of flavonolignan content in silymarin is presented in Table 2. In our experiments, RP-18 stationary phase was able to separate taxifolin, silydianin, silychristin, silybins and isosilybins mixture. The two peaks of silybin A and silybin B were separated; however, they were not base-line resolved. Our results is contraindicated to other silymarin HPLC analysis methods (Quaglia et al., 1999) where the RP-18 column allowed a good separation among taxifolin, silybin and isosilybin, while the peaks related to silydianin and silychristin were not base line-resolved even after increasing the water percentage in the mobile phase.

### Conclusion

This study correlated maturity stage to flavonolignan content in *S. marianum* fruits. These results may provide chemical evidence for localization of flavonolignans within the pericarp of *S. marianum* fruits and their absence in the fat rich cotyledons. Therefore, when silymarin has to be extracted, only the easily mechanically detachable outer
part should be utilized.

ACKNOWLEDGMENT

Thanks to Prof. Ahmed Attiah Saida, Department of Pharmacognosy, Faculty Pharmacy, Cairo University, for providing authentic sample of silymarin.

REFERENCES


