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SCIENTIFIC PAPER

582.475:665.3:547.972.2/.3:581.95

EXAMINATION OF LOCALIZATION OF SILYMARIN AND FATTY OIL IN *Silybum marianum* (L.) Gaertn. FRUIT

Physical characteristics and localization of flavonoids and fatty oil in Silybum marianum (L.) Gaertn. fruit of different origin were examined. Physical characteristics of the fruit were determined by its origin, and samples cultivated on plantations were, concerning their colour, shine, shape and size, very similar to those they originated from. By examination of the fruit size, we wanted to give our contribution to the choice of adequate equipment for picking, cleaning and packing of the fruit. Using a suitable mechanical procedure, the separation of the fruit into its inner part (endosperm) and outer cover (testa) was carried out. The Soxhlet extraction was carried out using petroleum ether (40–70°C) and methanol, The silymarin content (calculated as silibinin) was spectrophotometrically determined in the whole fruit (1.94–2.45%) and its respective parts: endosperm (0.19–0.33%) and testa (4.75–6.01%). By evaporation of petroleum ether extracts under the lowered pressure we determined the oil content in the whole fruit (19.38–24.08%), endosperm (27.85–34.19%) and testa (2.45–4.34%). We also established that more than 94% of silymarin was located in the testa more than 88% of fatty oil was localized in the fruit endosperm. The obtained results indicated that it was better to use testa, than whole fruit, when extracting silymarin.

Key words: Milk–thistle, fruit, localization of flavonoids, silymarin.

Silybum marianum (L.) Gaertn. (*Asteraceae*) is a medical plant, widely used in a traditional European medicine [1]. The fruit of this plant is used in the form of liquid extracts for treatment of cholelithiasis, hepato– and cholangiopathy, due to their cholagogue and protective effect on the liver. The efficiency of the extracts from the milk–thistle fruit in a treatment of liver diseases, actuated their examination in a chemical and pharmacological sense. Nowadays, standardized mixture of flavonoids, known as silymarin or silymarin complex, is used in the production of pharmaceutical preparations – hepatoprotectives. Silymarin, in the form of standardized liquid or dry extracts, is used in the production of mono– and polycomponent phytopharmacs in combination with other medical plants with a similar effect [2].

Silymarin (synonim: silibinin) represents a mixture of three isomers: silibinin, silidianin and silicristin, of the molecule formula $C_{25}H_{22}O_{10}$ and of the molecule weight 482.4 (Figure 1). Silymarin (silibinin) belongs to the class of natural substances with a benzodioxane grupation in a molecule, which originates in coupling of taxifolin with coniferyl alcohol. Because of this type of the connection within the molecule, this group of compounds was named flavonolignans. Biochemical studies of silymarin demonstrated that both silymarin and silibinin have antioxidative [3–6] and anticarcinogenic effects [7–10]. Due to their phenolic nature [11] they are capable of reacting with free radicals, including oxygenic (O°) and hydroxylic (HO°) radicals [12]. Some researchers examined anti–inflammatory and anti–alergic activity of silymarin and silibinin in their papers [10,13,14]. It appeared that anti–inflamatory activity was particularly manifested in the complexes of silymarin with phospholipides [15].

Besides their protective effect on cellular membranes and an antioxidative effect, it appears that active ingredients of *S. marianum* (L.) Gaertn. fruit also stimulate synthesis of DNA, that is, dividing and regeneration of liver cells, which represents the second important mechanism of their action [16,17]. Due to anti–inflammatory activity of silymarin and particularly its action as a "free radicals captor", the application of silymarin in dermatological therapy and cosmetics is enabled [7,18,19].

The first papers on isolation and identification of silymarin active components were published by Wagner et al. [20–22]. The greatest contribution to the confirmation of the silibinin structure, as the major component of silymarin complex, was given be Pelter and Hänsel [23–25]. The first analytical methods used for analysis of silymarin were based on spectrophotometrical determinations [22] and one of these methods, determination with 2,4–dihydrophenylhydrazine, was described in German Pharmacopoeia in a monography on *S. marianum* (L.) Gaertn. fruit [26]. Using TLC it was found that silymarin represents mixture of three isomers: silibinin, silicristin, and silidianin [27]. The separation and a separate determination of the three flavonolignans and flavanonol taxifolin were carried

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Paper accepted: December 01, 2006

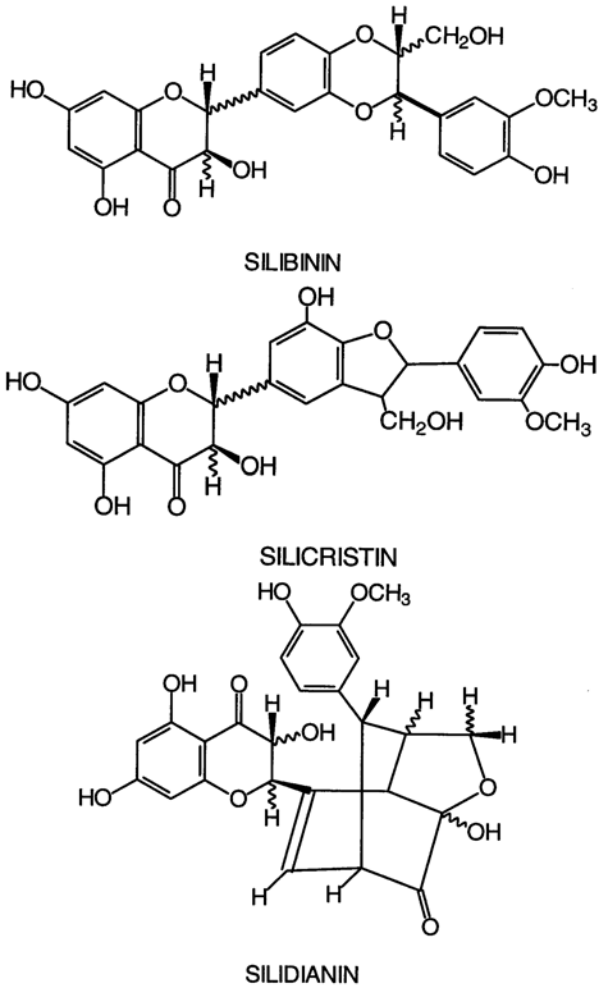

Figure 1. Chemical structures of the most important ingredients of S. marianum (L.) Gaertn. fruit

out using reverse–phase HPLC with the mixture of methanol–water–acetic acid 40:60:5 as a mobile phase and 1–naphthol as an internal standard [28,29] or with isocratic elution by CH_3CN:H_2O over Separon SGX C18 [30]. Although *S. marianum* (L.) Gaertn. fruit was well examined concerning its content, composition and effect of its active components, in modern pharma- ceutical industry enough attention has not yet been drawn to localization of flavonolignans in respective parts of the fruit. This is very important when known that in most of the procedures for obtaining silymarin, a special difficulty is isolation of a great quantity of fatty oil present in the fruit (20–30%). For that reason, the goal of this paper was to, using a spectrophotometrical method, examine the content of silymarin in the extracts of endosperm, testa and the whole *S. marianum* (L.) Gaertn. fruit of different origin, in order to localize fatty oil and flavonolignans and contribute to the improvement of the existing technological procedure for obtaining silymarin.

EXPERIMENTAL

Plant material

Selected ripe milk–thistle fruits were used for the investigation: samples 1 and 4 were obtained from "Indena", Italy and were used as a sowing material in our own experimental parcels (samples 2 and 5), while the sample 3 was obtained from the producer Hajnak Ilona (surrounding of Subotica, Vojvodina). The fruit was depurated and freed of pappus, then dried at room temperature to reach the moisture content bellow 8%. From the whole fruits, the inner parts of the fruit–endosperm and the outer shell–testa were obtained by mechanical separation.

Until they were analysed, the samples were kept in paper bags, at room temperature, protected from light, and subsequently, they were ground in the electric mill (n=1200 min^{-1}, the diameter of the propeller = 60 mm) and passed through a sieve with holes of 1 mm.

Examination of physical properties of the fruit

Colour, shine, odour, shape and size of the mass of 1000 fruits and specific (volume) mass were examined. These parameters are important in order to select suitable equipment for picking, cleaning and peeling of the fruit, as well as to find out if the samples are fresh and healthy. The results shown represent the mean value of three measurements.

Procedure of extraction

The ground plant material (5 and 10 g of the whole fruit and respective parts of the fruit, respectively) was extracted with petroleum ether (40–70°C) in a Soxhlet extractor at a drug-to-solvent ratio of 1:30 (m/v) in 5 hours. The fatty oil content was determined by evaporation of the petroleum ether extract and drying of the oily residue under vacuum at 40°C to the constant weight. The extraction of the very same sample was continued with methanol, for ten hours at the drug-to-solvent ratio of 1:30 (m/v). Extracting solvents used, petroleum ether and methanol, were of p.a. quality.

Determination of silymarin

The methanolic extract was evaporated under vacuum, to a small volume, and quantitatively transferred to a 50 mL measuring vessel, to which the extracting solvent was added up to the mark (the solution examined). 1 mL of the solution examined and 2 mL of 2,4–dinitrophenylhydrazine–sulphuric acid solution (DNPH) were pipeted into a 10 mL measuring vessel and thermostated for 50 minutes at 50–55°C. After cooling, the measuring vessel was reloaded up to the mark with a methanolic solution of potassium hydroxide (c=10%, m/v). Subsequently, 1 mL of the solution examined is transferred to a centrifuge cuvete,

and after additing methanol (20 mL), methanolic solutions were centrifugated at 3000 min^{-1}. Methanolic solutions were transferred to 50 mL measuring vessels and the procedure of centrifuging with new 20 mL of methanol was repeated. Another methanolic extract was blended together with the first one and methanol was added to the measuring vessel up to the mark. The solution for comparison was prepared using 1 mL of methanol instead of the solution examined, and it was treated in the same manner as the solution examined. Absorptions of metanolic solution samples were measured at 490 nm (Perkin–Elmer Lambda 15 UV/VIS spectrophotometer) using the standard solution as a blank probe. The silymarin content (calculated as silibinin) was calculated by the following formula:

$$X = (A/585) \times (25 \times 10^3 / G \times d)$$

where X is the content of silymarin, calculated as silibinin (%); d is the cuvete thickness [=1 cm]; A is the absorption of examined solution at 490 nm; 585 is the specific coefficient of absorption $A^{1\%}_{1cm}$; and G is the mass of the sample [g].

The preparation of 2,4–dinitrophenylhydrazine–sulphuric acid solution: 250–255 mg of 2,4–DNPH (previously dried in vacuum at room temperature for eight hours) was transferred to a 25 mL measuring vessel, wherein 0,5 mL of concentrated sulphuric acid was added, and dissolved with temperate heating. Approximately 15 mL of methanol was added and, after cooling to the room temperature, methanol was added up to the mark. The fresh solution was always prepared.

RESULTS AND DISCUSSION

The physical properties of milk–thistle fruits of different origin are shown in Table 1. Colour, shine and odour of the fruits are the parameters which can differ, depending on the seniority of the fruit, position during umbelling, moisture content, etc. The colour indicates the conditions of maturation and storage. The fruits exposed to light have a different nuance of colour, because the pigments determing the colour are unstable and dependent on the influence of exterior factors. Fresh fruits are shiny, and the odour gives information if the fruit is healthy or the decay has occurred due to different biochemical processes.

According of the form and size *S. marianum* (L.) Gaertn. fruits are three-dimensional, length, width and thickness being different among themselves. The examined milk–thistle fruits are of a peaked shape, with white pappus along its ledge. With their length of 6–7 mm, *S. marianum* (L.) Gaertn. fruits belong to the group of corpulent achenes. The mass of 1000 fruits, the colour and the size are similar to the related fruits (the samples 1 and 2, that is the samples 4 and 5). The sample 3 is different from the related fruits in colour, size, mass of 1000 fruits and volume mass, due to different origin and growing conditions.

Milk–thistle fruit has a structureless surface (without structure on pericarp), and the samples examined are shiny (they retract light) and odourless, which is the proof of the unchanged quality during storage. According to absolute mass, achenes of *S. marianum* (L.) Gaertn. belong to the group of corpulent, with the absolute mass of 1000 fruits greater than 20 g. The volume mass was determined by measuring the mass of the fruits preceding the volume of 1 dm^3. Each sample was mechanically devided into exterior part – testa and interior part – endosperm. Mass fractions of endosperm and testa in ripe milk-thistle fruit are shown in Figure 2.

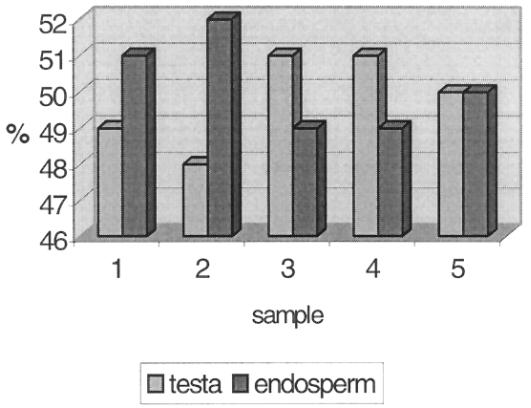

Figure 2. Mass share of endosperm and testa in ripe milk–thistle fruit
- selected milk–thistle fruit, Italy: samples 1 and 4
- grown on our own experimental parcels: samples 2 and 5
- sample 3 from a producer (Subotica, Vojvodina)

Table 1. Physical properties of S. marianum (L.) Gaertn. fruit

Sam-ple	Colour	Shine	Odour	Shape	Size (mm)	Mass of 1000 fruits (g)	Volume mass (g/dm^3)
1.	greyish–brown	shiny	odourless	obliquely obovoid	7.50x3.21x2.06	24.7	557.8
2.	greyish–brown	shiny	odourless	obliquely obovoid	7.54x3.21x2.18	24.4	531.3
3.	greyish–brown, black	shiny, matt	odourless	obliquely obovoid	7.34x3.31x2.14	25.4	582.4
4.	brownish–black	shiny	odourless	obliquely obovoid	6.99x3.04x1.72	21.0	602.4
5.	brownish–black	shiny	odourless	obliquely obovoid	6.92x3.24x1.88	21.4	577.2

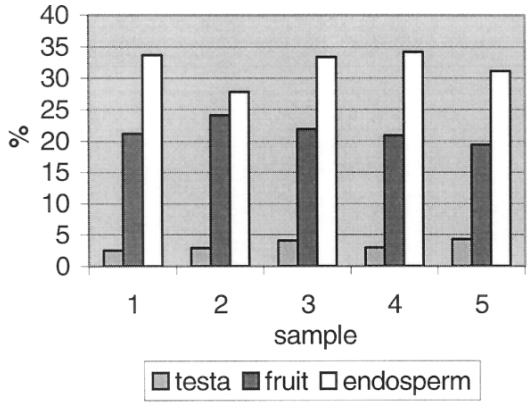

Figure 3. Content of fatty oil in endosperm, testa and ripe S. marianum (L.) Gaertn. fruit of different origin
- selected milk–thistle fruit, Italy: samples 1 and 4
- grown on our own experimental parcels: samples 2 and 5
- sample 3 from a producer (Subotica, Vojvodina)

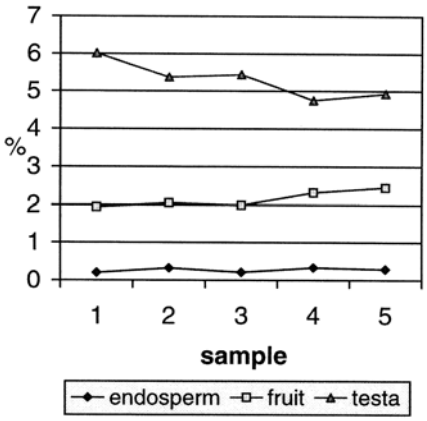

Figure 4. Content of silymarin in endosperm, testa and ripe S. marianum (L.) Gaertn. fruit of different origin
- selected milk–thistle fruit, Italy: samples 1 and 4
- grown on our own experimental parcels: samples 2 and 5
- sample 3 from a producer in the vicinity of Subotica, Vojvodina

The contents of silymarin and fatty oil in endosperm, testa and ripe fruit, are shown in Figures 3 and 4. From the mass fraction of endosperm and testa and the corresponding contents of silymarin and fatty oil the mass fraction of silymarin and oil in the single parts of the fruit were calculated (table 2).

The content of fatty oil in *S. marianum* (L.) Gaertn. fruit showed that the samples examined were rich in fatty matters (19–24%). Such a high content of fatty oil complicates the procedure of obtaining silymarin, and for that reason, the fruit should be deffated by using a suitable solvent which considerably interlopes the process of extraction of active components. The greatest part of fatty oil (88–94%) was localized in endosperm of the fruit.

On the other hand, the examined samples of the ripe fruit had a satisfactory content of silymarin (>1%), which corresponded to the quality requirements for ripe fruit. Endosperm contained 0.19–0.33% and testa

Table 2. Mass fractions of fatty oil and silymarin in the milk–thistle fruit

Sample	Fatty oil, %		Silymarin, %	
	testa	endosperm	testa	endosperm
1	6.4	93.6	97.0	3.0
2	8.9	91.1	94.2	5.8
3	11.3	88.7	96.5	3.5
4	8.2	91.8	93.7	6.3
5	12.3	87.7	94.7	5.3

4.75–6.01% of silymarin. With regard to the mass fraction of endosperm and testa in the whole fruit, it was found that 94–97% of the total silymarin content was situated in the testa, and only 3–6% in endosperm.

CONCLUSION

The obtained results showed that the physical properties of different samples were determined by the origin of *S. marianum* L Gaertn. fruit the samples obtained from our own experimental plantations, very similar to those they originate from. Examining their physical properties, we wanted to give our contribution to choosing suitable equipment for picking, cleaning and packing of the fruit. The examinations carried out also showed that flavonolignans, as pharmacologically important raw material, are mostly situated in the outer cover of the fruit, while endosperm of the fruit contains fatty oil. Such a localization of flavonolignans can contribute to the improvement of the existing technological procedure for obtaining silymarin and economical exploitation of the fruit. The use of outer cover of the fruit only, for obtaining silymarin reduces consumption of the solvents necessary for deffating of the fruit, while endosperm, due to its content of fatty oil and proteins, can be used in food industry.

ACKNOWLEDGEMENTS

This work is a part of the project TR–6708B, which has been financed by the Ministry of Science of the Republic of Serbia.

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