*Pak. J. Sci. Ind. Res. 2003* ***46****(4) 307-310*

# Commercial Extraction and Resolution of Silymarin Isomers

***L Khan*, N Shafi, S Farooq, S N Gilani, T Mahmood and N Ahmad***

*Medicinal Botanic Centre, PCSIR Laboratories Complex, Peshawar-25120, Pakistan*

(Received 25 October 2000; accepted 31 October 2002)

A simple and economical procedure for the large-scale extraction of silymarin from the dried seeds of *Silybum marianum* has been developed. After harvest the cellular and acellular constituents of the seeds are biodegradable. Using the fresh seeds, a viable procedure has been perfected for the extraction of silymarin (yield 1.47 %). The product appeared as a mixture and was resolved into the isomers by centrifugal chromatography. The identity of the product and its isomers were confirmed through comparison with standards using mp, UV, IR, $^{1}$H NMR and Mass spectrometric data and 2,4, dinitrophenylhydrazone assay.

***Key words*****:** *Silybum marianum* seeds, Silymarin, Silybin, Silydianin, Silychristin.

## Introduction

*Silybum marianum* a medicinal herb has been widely used in the European traditional medicine (Eichler and Hahn 1949). Extracts prepared from the roots and seeds have been used in the treatment of liver diseases, disorders of bile duct and spleen. (Sonnenbichler *et al* 1998) Nowadays, silymarin, the purified compound of the seeds and its major isomer silybin are used in the manufacture of therapeutic products administered against liver diseases, jaundice, and gallstones (Wagner *et al* 1968; Wagner 1973). Despite the abundance of the indigenous species, the seeds have not been studied as a source for silymarin or its isomers silybin, silydianin and silychristin. The present studies are undertaken for the development of a commercially viable process for the extraction of silymarin from the seed powder and preparation of the isomers.

## Materials and Methods

*Materials*. Ripe fruits of *Silybum marianum* L. were collected from the experimental garden of PCSIR Laboratories Peshawar. Silica gel 60 PF 254+366 was used for resolution of the isomers. Solvents and chemicals were BDH Laboratory analytical grade reagents. Melting point was determined using Griffin MFB S90 010T Electrothermal apparatus. Resolution of silymarin isomers was attempted using Harrison Research Model Chromatotron.

*Extraction of silymarin*. Dried capitulums of *Silybummarianum* were thrashed, seeds were separated and powdered material (10 kg) was extracted three times with hexane (25 l) through solvent recycling at room temperature for 10 h. Concentration and removal of hexane under reduced pressure yielded 2.2 kg oil.

*Author for correspondence

The defatted material (7.7 kg) was then extracted four times with ethanol (20 l) by percolation and recycling at room temperature for 12 h. The combined alcoholic extract (15 l) was filtered, concentrated to one litre under reduced pressure and gradually added to water (5 l) under intensive stirring. The product salted out from the aqueous extract with sodium chloride (400 ml, 25%). The precipitated material was filtered, washed with water, dried in an electric vacuum oven and powdered (Fig 1). The yield of the crude material was 204g.

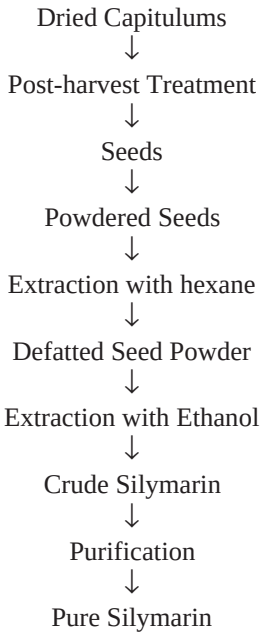



**Fig 1.** Flow Chart for the Extraction and Purification of Silymarin

The crude product was dissolved in absolute ethanol (1 l) with stirring and shaking. The solution was filtered and then cooled

to 10°C. The resultant turbid solution was filtered and the filtrate dried under reduced pressure, residue obtained was dissolved in acetone (400 ml), the final yield of silymarin was 1.47%.

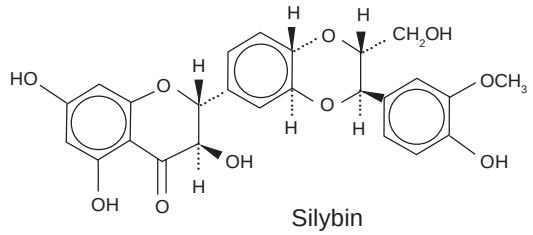
*Analysis Techniques*. The UV spectra in methanol were recorded on a Hitachi Model U-2000 Instruments. Infrared spectra were recorded using a Pye-Unicam SP 3-100 model. $^{1}H$,$^{13}C$-NMR spectra were recorded with the help of Bruker 400 MHz spectrophotometer using tetramethylsilane (TMS) as internal standard. Deuterated methanol and acetone were used as solvent. Mass spectra was obtained at the HEJ Research Institute of Chemistry, University of Karachi, Pakistan. Melting point of the product was 170-175°C, UV: 288, 326 nm, IR (KBr disc): 3380, 3450, 1752, 1615, 1507, 1262, 1137 $cm^{-1}$. Assay of the product was performed according to Wagner *et al* (1968, 1974). TLC examination of the product against reference silymarin, carried out in a solvent system hexane, chloroform, methanol (4: 3: 1.8) showed the presence of three components.

*Resolution of silymarin*. Fractionation of silymarin components was carried out using Harrison Research Mode 7924T Chromatotron. Sorbent layer of 1 mm thickness was prepared from silica gel 60 PF 254+366. The layer was equilibrated with the elution system comprising of hexane, chloroform and methanol (3: 2.5: 0.75) Purified silymarin (100 mg) was dissolved in methanol (0.5 ml) and the solution applied without loss to sorbent layer as a compact ring. Washing of the sample was performed at a flow rate 2 - 4 ml/min, whilst revolution/min was maintained at 750. Separation of the components was monitored under UV light. Ten fractions, 10 ml each, were collected and compared by TLC against standard silybin, silydianin and silychristin. The yield of each compound recovered was 55%, 34% and 11 % respectively.

*Spectroscopy*. *Compound:* 1 (yield 40 mg, m.p. 180 °C dec) UV (Methanol) λ max. 288 nm $^{13}C$-NMR ($CD_3OD$, DMSO-$d_6$) δ196.6, 165.9, 162.5, 161.6, 146.8, 146.2, 142.9, 142.5, 129.3, 126.7, 120.9, 120.1, 115.8, 114.7, 111.0, 99.8, 95.5, 94.5, 82.0, 77.6, 75.3, 71.0, 59.8, 55.2. MS m/z: 482 $M^+$, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 180, 162, 138 and 124.

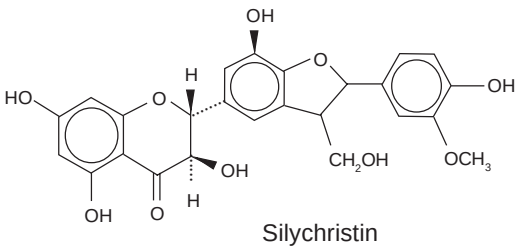
*Compound.* 2 (yield 25 mg, m.p.-191°C) $^{1}H$-NMR (acetone-$d_6$, TMS, 100MHz) δ2.97 (1H, m, $H_\gamma$), 3.27 (1H, d, $H_\alpha$), 3.40 (1H, m, $H_\beta$), 3.70 (1H, q', $H_{3'}$), 3.87 (3H, s, $OCH_3$), 3.88 (1H, d, $H_6$), 4.32 (1H, q, $H_D$), 4.63 (1H, d, $H_3$), 4.98 (1H, q, $H_2$), 6.06 (2H, s, $H_6$, $H_8$), 6.27 (1H, d, $H_{2'}$), 6.73-7.00 (3H, m, $H_{2''}$, $H_{5''}$, $H_{6''}$), 11.70 (1H, s, $H_5$-OH). $^{13}C$-NMR ($CD_3OD$, DMSO-$d_6$) δ201.80, 196.40, 166.80, 163.30, 161.90, 147.00, 144.90, 139.40, 132.90, 124.00, 120.20, 114.80, 112.40, 100.20, 96.60, 96.10, 95.00, 81.60, 72.70, 70.80, 55.40, 53.30, 46.60, 46.00, 44.00. MS m/z: 482 $M^+$, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 302,180.

*Compound.* 3 (yield 8 mg, m.p. 174-176°C) UV (Methanol) λ max. 288nm. $^{1}H$-NMR (acetone-$d_6$, TMS, 100MHz) δ3.61 (1H, m, $H_\beta$), 3.84 (H, S, $OCH_3$), 3.86 (1H, m, $H_7$), 3.92 (1H,



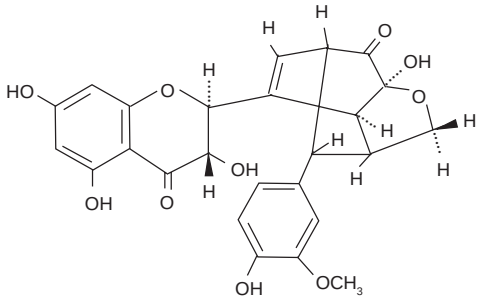
Silybin

Structural formula of silybin



Silychristin

Structural formula of silychristin



Silydianin

Structural formula of silydianin

Scheme 1. Structure of the active constituents of Silymarin.

dd, $H_7$), 4.64 (1H, d, $H_2$), 5.05 (1H, d, $H_3$), 5.59 (1H, d, $H_\alpha$), 5.96 (1H, d, $H_6$), 6.00 (1H, d, $H_8$), 6.83 (1H, d, $H_{5''}$), 6.93 (1H, dd, $H_{6''}$), 7.00 (1H, d, $H_{2'}$ or $H_{6'}$), 7.03 (1H, d, $H_{2'}$ or $H_{6'}$), 7.10 (1H, d, $H_{2''}$). $^{13}$C-NMR ($CD_3OD$, DMSO-$d_6$) δ198.20, 168.70, 165.20, 164.40, 149.10, 147.50, 142.10, 134.70, 131.50, 130.10, 119.80, 117.00, 116.60, 116.20, 110.70, 101.80, 97.40, 96.30, 89.10, 85.20, 73.70, 64.80, 56.40, 55.40. MS m/z: 482 $M^+$, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 302, 180, 162, 137.

## Results and Discussion

The isolation of phytopharmaceuticals in pure form from plants is often carried out with multistage extraction and purification procedures (Meilroy 1951; Trease and Evans 1983). Normally neutral solvents are used for separation because they tend to hydrolyze. After extraction from plants, resolution of the complex mixture required the application of a number of other techniques due to the co-extracted proteins, fats, oils, carbohydrates, pigments and other constituents. The undesired components are usually precipitated from the extract by the addition of specific reagents.

On account of the therapeutic and economic importance of silymarin, specific procedures have been reported on the industrial extraction of the plant but the information is mainly confined to patent literature. In the present work for the selective extraction of the pigments, fats, oil and resinous substances from the seeds powder, hexane was used. During extraction heating of the seed powder was avoided on account of decomposition of the labile compounds. In addition, concentration and purification of the hexane extract performed under reduced pressure at 30°C, yielded 22% oil. Evaluation of which on TLC showed presence of oleic acid, sitosterol, stigmasterol and cholesterol. The results were consistent with earlier findings of Hammouda *et al* (1994).

Commercial scale purification of phytochemical often presents several problems. When it appears that a plant derived product may contain several adhering constituents, the choice of obtaining the desired constituent becomes very significant. For selective extraction of silymarin from the seed powder extraction at room temperature (25°C) and concentration, purification of the extracted constituents under reduced pressure at 35°C, prevented the decomposition of the coextracted components and adhering impurities. Initial separation of macro impurities was achieved at 15°C by solubilisation and sedimentation. It was thought that if particles of varying size were allowed to suspend in alcohol, then the force of gravity would appear to be the decisive factor for their sedimentation. As a result the large and denser molecules quickly settled on the bottom and were removed by filtration. Microfine particles appeared completely dispersed in the solvent phase. Lowering the temperature to 10°C coagulated and facilitated their removal from the solvent phase. With the removal of adhering impurities purification of silymarin appeared very simple. The yield of pure silymarin was 1.47% and was close to the reported values. (Kurkin *et al* 1996). Assaying the material as 2,4-dinitrophenylhydrazone of silybin confirmed the presence of 98 percent flavonoids. As compared to patent procedures for extraction of silymarin (Wagner *et al* 1968; Wagner 1973; Wagner *et al* 1974; Kurkin *et al* 1996) the method described was simple efficient and feasible.

During centrifugal separation of the isomers, the selection of eluent was made on the basis of Rf values, which were kept below 0.5. Under the chosen experimental conditions (Rf, flow rate, r.p.m and temp.) the components were easily separated. Physicochemical characteristics, m.p, TLC, UV, IR, $^1$H-NMR, $^{13}$C-NMR, and Mass spectrometric data of component 1,2 and 3 were identical with those reported in literature for *silybin, silydianin* and *silychristin* (Pelter and Hansel *et al* 1968; Abraham *et al* 1970; Wagner *et al* 1971; Szilagi *et al* 1981). The yields of the isomers were consistent with the reported values (Kurkin *et al* 1996). Compared to the reported resolution procedures, (Hostettmann *et al* 1986) the technique applied for separation of the silymarin isomers was convenient.

The methodology applied for the commercial extraction of silymarin and its isomers is simple, efficient and designed for minimum equipment. The products are of therapeutic and commercial significance and have been used in the preparation of hepatoprotective drugs marketed under various trade names.

## References

Abraham J D, Takagi S, Rosenstein R D, Shiono R, Wagner H, Horhammer L, Seligmann O 1970 The structure of Silydianin, an isomer of Silymarin (Silybin) by X-ray analysis. *Tetrahedron Letters* **31** 2675-2678.

Eichler O, Hahn M 1949 Protection against liver damaging poisons by plant extract. *Arch Exper Path* **206** 674-684.

Hammouda F M, Ismail S I, Hassan N M, Zaki A K 1994 Comparative studies of oil from *Silybum marianum* cultivated in Egypt using GLC. *Qatar Uni Sci J* **14** 154-157.

Hostettmann K, Hostettmann M, Marston 1986 A Preparative Chromatography Techniques. In: *Natural Product Isolation* pp 5-18.

Kurkin V A, Zapesochnaya G G, Adeeva E V, Tsybulko N S 1996 Determination of silybin and total flavonolignans in *Silybum marianum* (L). Gaertn fruits. *Rastit Resur*. (Russ) **32**(3) 80-87.

Meilroy R J 1951 *The Plant Glycosides*. Edward Arnold and Co. London pp 5-13.

Pelter A, Hansel R 1968 The structure of silybin (*Silybum* substance $E_6$), the first flavonolignan. *Tetrahedron Letters* **25** 2911-2916.

Sonnenbichler J, Sonnenbichler I, Scalkera, F 1998 Influence of the flavonolignan silybinin of milk thistle on hepatocytes and kidney cells. *ACS Symp Ser (Phytomedicines of Europe*) **691** 263-277.

Szilagi I, Tetenyi P, Antus S, Seligmann O, Chari V M, Seitz M, Wagner H 1981 Structure of silandrin and silymonin, two new flavonolignans from a white blooming *Silybum marianum* variety. *Planta Medica* **43** 121-127.

Trease G E, Evans W C 1983 *A Text Book of Pharmacognosy*. Bailliere and Tindale 12th ed pp 17-73.

Wagner H 1973 Flavonolignane, ein neuer Verbindungstyp aus *Silybum marianum*. *Dtsch Apoth Ztg* **114** 93-94.

Wagner H, Diesel P, Seitz M 1974 Zur Chemie und Analytik von Silymarin aus *Silybum marianum* Gaertn. *Arzneim Forsch* **24** 466-471.

Wagner H, Horhammer L, Munster R 1968 Zur Chemie des Silymarins (Silybin), des Wirkprinzips der Fruchte von *Silybum marianum* (L) Gaertn. (*Carduus marianus* L.) *Arzneim-Forsch* **18** 688-696.

Wagner H, Seligmann O, Horhammer L, Seitz M, Sonnenbichler J 1971 Zur struktur Von silychristin, Einem Zweiten Silymarin-isomeren aus *Silybum marianum*. *Tetrahedron Letters* **22** 1895-1899.