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Silymarin, Natural Flavonolignans from Milk Thistle

Sameh AbouZid

Faculty of Pharmacy, University of Beni-Suef,
Egypt

1. Introduction

Plants are a valuable source of pharmaceuticals, food ingredients, agrochemicals, insecticides, flavors and pigments. These compounds are called secondary metabolites. These are compounds with a restricted occurrence in taxonomic groups that are not essential for an organism to live but play a role in the interaction of the organism with its environment, ensuring the survival of the organism in its ecosystem (Verpoorte and Alfermann, 2000).

Milk thistle or St. Mary's thistle [*Silybum marianum* (L.) Gaertn. (Syn. *Cardus marianum*) Asteraceae] is an annual or biennial herb. The plant is native to the Mediterranean and North African regions (Boulos, 2000). It grows wild throughout Europe, North Africa, Americas and Australia (Hamid et al., 1983). The plant reaches to heights 10 feet. It has a stem of 20-150 cm high, erect, ridged and branched in the upper part. A distinguishing characteristic of milk thistle is the white patches found along the veins of the dark green leaves (Fig. 1). The broad leaves are deeply lobed, 50 cm long and 25 cm wide. The leaf margins are yellow and tipped with woody spines (3-12 mm long). The leaves are alternate and clasping to the stem. 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 (Fig. 2) are hard skimmed achenes, 6-8 mm long flat, smooth and shiny dark brown in color. The fruits yield 1.5-3% of an isomeric mixture of flavonolignans collectively known as silymarin (Morazzoni and Bombardelli, 1995). Silymarin accumulates mainly in the external cover of the fruits of *S. marianum* (Madrid and Corchete, 2010).

2. Chemistry of flavonolignans

The principal components of silymarin are silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B and silydianin (Fig. 3). The first six compounds exist as equimolar mixtures as trans diastereoisomers. These diastereomers have very similar ^1H and ^{13}C NMR spectra and have no characteristic signals for facile identification of the individual isomers (Lee and Liu, 2003). A number of other chemically related compounds have been found in the fruits including dehydrosilybin, desoxysilychristin, desoxysilydianin, silandrin, silybinome, silyhermin and neosilymermin. The common feature of these



Fig. 1. Milk thistle with white patches along the veins of dark green leaves.



Fig. 2. *Silybum marianum* fruits.

compounds is a flavonolignan skeleton ($C_{25}H_{22}O_{10}$, mol wt 482). Basically, flavonolignan nucleus consists of the dihydroflavanol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. The oxeran ring is responsible for the biological activity of silymarin, and opening of this ring results in loss of activity. Only silybins and isosilybins contain the 1,4-dioxane ring system in their structure. Silybin and isosilybin have the same trans conformation of C-2, C-3 and C-7', C-8'. Silybin is considered the major and most active component in silymarin (Ligeret et al., 2008; Kim et al., 2009). The chemical structure of

silybin has been identified in 1975 using a degradative method (Lee and Liu, 2003). The first trials to synthesize silybin suffered from the problem of giving a product which is a mixture of regioisomers, silybin and isosilybin (57:43). Regioselective synthesis of diastereomeric silybin in 63% overall yield was achieved by synthesizing a key intermediate which was coupled with 2,4,6-trimethoxyacetophenone to form a chalcone intermediate. Epoxidation, deprotection and acidic cyclization were followed (Tanaka et al., 1985).

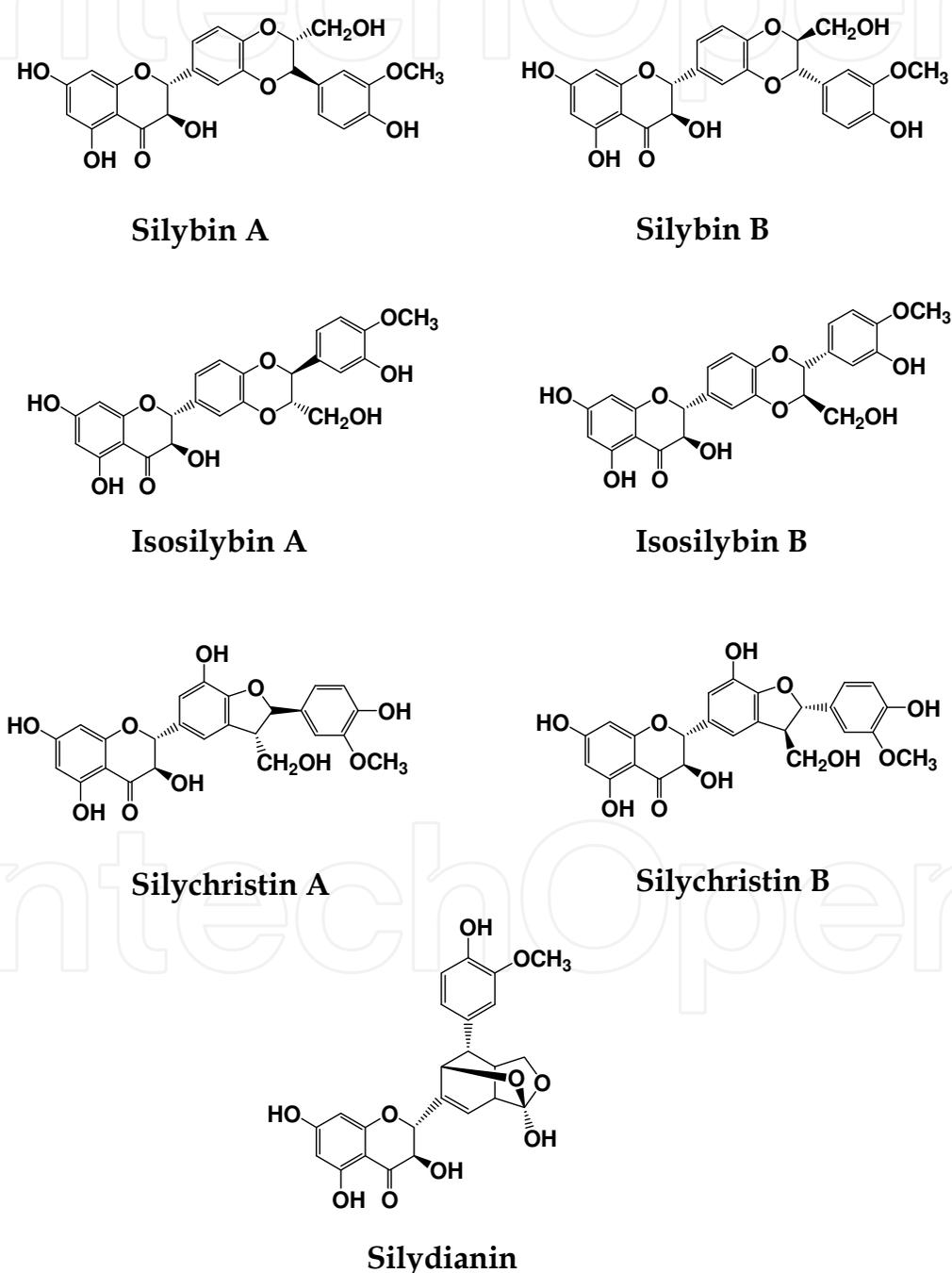


Fig. 3. Chemical structures of silymarin components.

3. Analysis of flavonolignans

Extract obtained from the fruits of *S. marianum* is available worldwide in the pharmaceutical market as antihepatotoxic drug under a variety of brand names. There are many products that contain silymarin either as a single component or in a mixture with other active constituents. The extract contains about 80% wt/wt of flavonolignans. Due to its poor water solubility and thus low bioavailability, silymarin is complexed with phosphatidylcholin, β -cyclodextrin or even given as glycosides, which have better water solubility and higher activity. A method for extraction of silymarin from plants on an industrial level has been reported (Madaus et al. 1983). In this method large part of the fruit oil is removed by cold pressing, the compressed mass is broken up, the pressed residue is extracted with ethyl acetate and the ethyl acetate extract is evaporated and processed. 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 the sum of silybins, isosilybins, and silychristins and silydianin concentrations. It is important that the analytical method characterizes and quantifies each component in silymarin.

3.1 Thin layer chromatography analysis

Flavonolignans were analyzed by Thin Layer Chromatography (TLC) (Wagner et al., 2009). Chloroform-acetone-formic acid (75:16.5:8.5) was used as a solvent system and detection was done using natural products-polyethylene glycol reagent. Silymarin is characterized in UV-365 nm by two intense green-blue fluorescent zones of silybin/isosilybin ($R_f = 0.6$), silychristin ($R_f = 0.35$) and an orange zone of taxifolin ($R_f = 0.4$).

3.2 UV-visible spectrophotometry analysis

UV-visible spectrophotometry was proposed for the quantitative determination of flavonolignans (Famacoepa Ufficiale Italiana, 1985). This spectrophotometric method is time consuming, shows a non-satisfactory repeatability and measures total and not individual flavonolignans. A fast, simple and sensitive spectrophotometric method for determination of silymarin in pure form and in pharmaceutical formulations was reported. This method was based on oxidation with potassium permanganate at pH 7. The reaction was followed spectrophotometrically by measuring the decrease in the absorbance at 530 nm (Rahman et al., 2004).

3.3 High performance liquid chromatography analysis

High Performance Liquid Chromatography (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 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. Among the advantages of this method are precision, sensitivity, ability to measure individual constituents in a mixture, the good

separation of all compounds allowed the purity control of each peak, plotting of UV spectra, useful for the peak identification and a more correct quantification. However, time consumption, the need for pre-purification step and availability of pure reference compounds are the main disadvantages of HPLC. Analysis of silymarin components by HPLC on RP-18 in our laboratory only showed separation of the two diastereomers of silybin. However, the two peaks were not base-line resolved (Fig. 4).

3.4 Capillary electrophoresis analysis

Capillary zone electrophoresis has been proposed as a method for separation and determination of silymarin components (Kvasnička et al., 2003). Repeatability, accuracy, linearity and limit of detection were evaluated. The method was comparable to HPLC results. Shorter analysis time and better resolution of silydianin and silychristin from sample constituents were the main advantages of this method. 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). In 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.

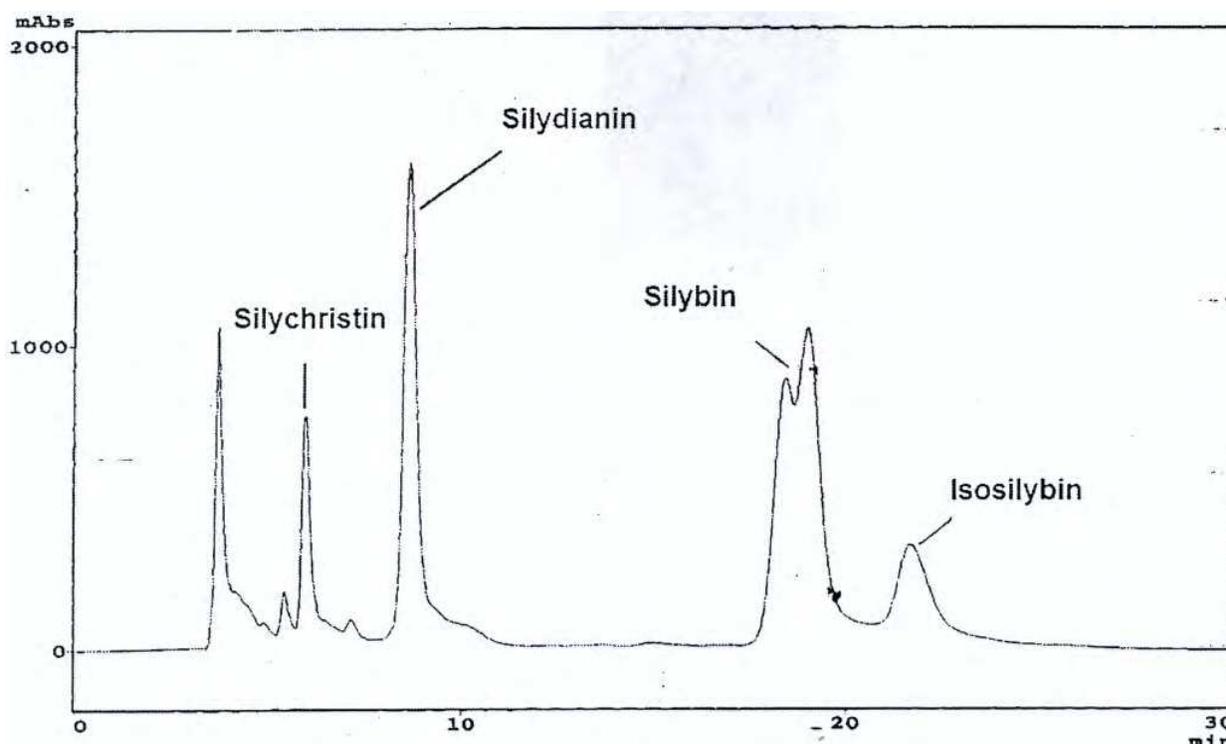


Fig. 5. Analysis of silymarin components by HPLC on RP18 (analysis was carried out in author laboratory).

3.5 Ultra performance liquid chromatography analysis

Ultra-Performance Liquid Chromatography (UPLC) offer many advantages over traditional HPLC for separation and quantification of multicomponent analytes such as silymarin

components. Among these advantages are short analysis time, maintaining the resolution and increasing peak capacity and sensitivity. Complete separation of the seven major active flavonolignans of silymarin by UPLC RP18 column was reported (Wang et al., 2010). In this study, the use of electrospray ionization tandem mass spectrometry allowed to obtain detailed analysis of fragmentation and distinguish between the seven flavonolignans for online identification. Advantages and disadvantages of different methods for quantitative analysis of flavonolignan components in silymarin are summarized in table 1.

Method	Advantages	Disadvantages
Spectrophotometric	<ul style="list-style-type: none"> • Fast and simple • Sensitive 	<ul style="list-style-type: none"> • Individual flavonolignans are not quantified
HPLC	<ul style="list-style-type: none"> • Precise and sensitive • Individual flavonolignans are quantified • Peak identification • Purity control 	<ul style="list-style-type: none"> • Not all flavonolignans are separated from each other • Time consuming • Needs pre-purification step • Pure reference compounds are needed
HPCE	<ul style="list-style-type: none"> • Shorter analysis • Less solvent consumption • Individual flavonolignans are quantified including diastereomers 	<ul style="list-style-type: none"> • Needs pre-purification step
UPLC	<ul style="list-style-type: none"> • Short analysis time • Less solvent consumption • Increased resolution • Increased peak capacity and sensitivity 	<ul style="list-style-type: none"> • Expensive • Needs pre-purification step • Needs calibration curve

Table 1. Advantages and disadvantages of different methods for quantitative analysis of flavonolignan components in silymarin.

4. Biosynthesis of flavonolignans in *Silybum marianum*

Flavonolignans are formed by combination of flavonoid and lignan structures. This occurs by oxidative coupling processes between a flavonoid and a phenylpropanoid, usually coniferyl alcohol (Dewich, 2002). Oxidative coupling occurs between free radical generated from the flavanol taxifolin and the free radical generated from coniferyl alcohol. This would lead to an adduct formation. This adduct could cyclize by attachment of the phenol nucleophile on to the quinone methide generated from coniferyl alcohol (Figure 5). The product in this case would be silybin. The fact that silybin exists in *S. marianum* in a mixture of two diastereomers reveals that the radical coupling reaction is not stereospecific. This is also true for isosilybin and silychristin. The latter flavonolignan originate from a mesomer of the taxifolin-derived free radical. Silydianin has a more complex structure and is formed by intramolecular cyclization of the coupling product. This is followed by hemiketal formation.

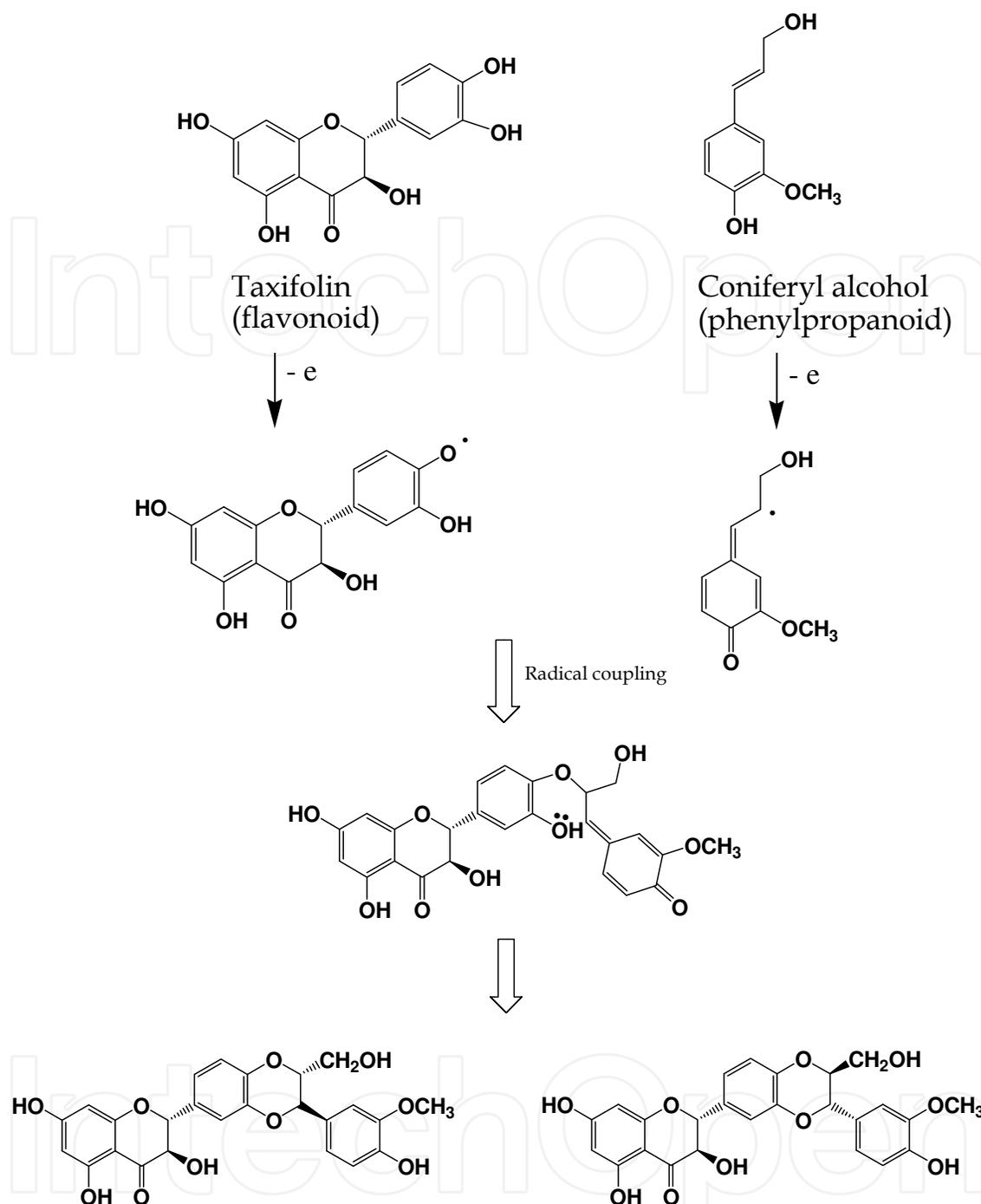


Fig. 5. Proposed biosynthetic pathway to silybin in *Silybum marianum*.

5. Biological activity of flavonolignans

Silymarin has been used for centuries to treat liver, spleen and gall bladder disorders (Shaker et al., 2010). It is known to possess hepatoprotective, antioxidant (Morazzoni and Bombardelli, 1995), anticancer (Zi et al., 1997), anti-inflammatory (De La Puerta, 1996) and anti-diabetic (Maghrani et al., 2004) properties. As a hepatoprotective agent, silymarin is used for oral treatment of toxic liver damage and for the therapy of chronic inflammatory liver diseases (Flora et al., 1998).

5.1 Hepatoprotective activity

Silymarin is one of the most investigated plant extracts with known mechanisms of action for oral treatment of toxic liver damage (Hiroshi et al., 1984). Silymarin is used as a protective treatment in acute and chronic liver diseases (Flora et al., 1998). Silymarin supports the liver cells through multifactor action including binding to cell membrane to suppress toxin penetration into the hepatic cells, increasing superoxide dismutase activity (Feher and Vereckei, 1991), increasing glutathione tissue level (Pietrangelo et al., 1995), inhibition of lipid peroxidation (Bosisio et al., 1992; Carini et al., 1992) and enhancing hepatocyte protein synthesis (Takahara et al., 1986). The hepatoprotective activity of silymarin can be explained based on antioxidant properties due to the phenolic nature of flavonolignans. It also acts through stimulating liver cells regeneration and cell membrane stabilization to prevent hepatotoxic agents from entering hepatocytes (Fraschini et al., 2002). Recently it has been shown that flavonolignans inhibit leucotriene production; this inhibition explains their anti-inflammatory and antifibrotic activity (Dehmlow et al., 1996).

5.2 Anticancer activity of silymarin

Silymarin is also beneficial for reducing the chances for developing certain cancers (Deep et al., 2007; Zhao et al., 1999). The molecular targets of silymarin for cancer prevention have been studied (Ramamany and Agrawal, 2008). Silymarin interfere with the expressions of cell cycle regulators and proteins involved in apoptosis to modulate the imbalance between cell survival and apoptosis. Sy-Cordero et al., 2010, isolated four key flavonolignan diastereoisomers (silybin A, silybin B, isosilybin A and isosilybin B) from *S. marianum* in gram scale. These compounds and other two related analogues, present in extremely minute quantities, were evaluated for antiproliferative/cytotoxic activity against human prostate cancer cell lines. Isosilybin B showed the most potent activity (Deep et al., 2007; Deep et al., 2008a; Deep et al., 2008b). The isolation of six isomers afforded a preliminary analysis of structure-activity relationship toward prostate cancer prevention. The results suggested that an *ortho* relationship for the hydroxyl and methoxy substituents in silybin A, silybin B, isosilybin A and isosilybin B was more favorable than the *meta* relationship for the same substituents in the minor flavonolignans. Silymarin suppressed UVA-induced oxidative stress that can induce skin damage (Svobodová et al., 2007). Therefore, topical application of silymarin can be a useful strategy for protecting against skin cancer.

5.3 Anti-inflammatory activity

Silymarin seems to possess anti-inflammatory properties by acting through different mechanisms such as its antioxidant action, membrane-stabilizing effect and inhibition of the production or release of inflammatory mediators such as arachidonic acid metabolites (Breschi et al., 2002). Gastric anti-ulcer activity of silymarin has been reported (Alarcon et al., 1992). This action was attributed to the inhibition of enzymatic peroxidation in the lipoxygenase pathway and free radical scavenging activity (Bauman et al., 1980). Silymarin exhibited significant anti-inflammatory and antiarthritic activities in the papaya latex induced model of inflammation and mycobacterial adjuvant induced arthritis in rats (Gupta et al., 2000). This action is mediated through inhibition of 5-lipoxygenase.

5.4 Effect on asthma

Activity of silymarin was examined against bronchial anaphylaxis and against post-anaphylactic, propranolol- or platelet activating factor-induced hyperreactivity in guinea-pigs (Breschi et al., 2002). Silymarin pretreatment reduced the bronchospasm induced by antigen-challenge in sensitized animals. This protective effect was due to indirect mechanism that reduces airway responsiveness to histamine, and consequently the immediate anaphylactic response. Therefore, silymarin can be used as protective agent in the management of asthmatic disorders.

5.5 Immunostimulatory activity

Several studies have reported the immunostimulatory actions of silymarin (Wilarusmee et al., 2002). The effect of treatment with silymarin was studied on glutathione level and proliferation of peripheral blood mononuclear cells of β -thalassemia major patients (Alidoost et al., 2006). In vitro treatment with 10 g/ml silymarin restored glutathione levels and enhanced cellular proliferation. This was explained by its antioxidant activity.

5.6 Treatment of obsessive-compulsive disorder

Many patients cannot tolerate the side effects of pharmaceutical agents available for treatment of obsessive-compulsive disorder, do not respond properly to the treatment or the medications lose their effectiveness after a period of treatment. An 8-week pilot double-blind randomized clinical trial on 35 adult patients was conducted to compare the efficacy of the extract of *S. marianum* with fluoxetine in the treatment of obsessive-compulsive disorder (Sayyah et al., 2010). The results showed that the extract of *S. marianum* has positive effects on obsession and compulsion starting from the fifth week. There were no any serious side effects accompanying *S. marianum* extract administration.

5.7 Hyperprolactinemic effect

S. marianum fruits have been traditionally used by nursing mothers for stimulating milk production (Newall et al., 1996). It was demonstrated that milk thistle increases lactation (Carotenuto and Di Pierro, 2005). The mechanism that led to the increase in lactation has been studied by measuring the concentration of circulating prolactin in female rats treated with silymarin (Capasso et al., 2009). It was shown that silymarin is able to produce a significant increase in circulating prolactin levels after oral administration. The levels of prolactin remains elevated for up to 66 days after silymarin discontinuation. Fig. 6 shows a summary of the wide range of biological activities attributed to silymarin.

5.8 Toxicity of silymarin

An average daily dose of silymarin (420 mg/day for 41 months) was found to be non-toxic, relative to placebo, in clinical trials (Tamayo and Diamond, 2007). Drug-drug interaction and liver toxicity by interference with co-drugs by induction or inhibition of cytochrome-P450 is a major concern for the use of silymarin (Izzo and Ernst, 2009). Studies were performed to investigate the potential for hepatotoxicity, cytochrome-P450 isoenzymes induction and inhibition on dry extract from *S. marianum*, as contained in HEPAR-PASC® film-coated tablets (Doehmer et al., 2011). The results indicated that interference or

hepatotoxicity of the dry extract from *S. marianum* at the recommended maximum daily dose equivalent to 210 mg silybin is unlikely and is to be considered safe.

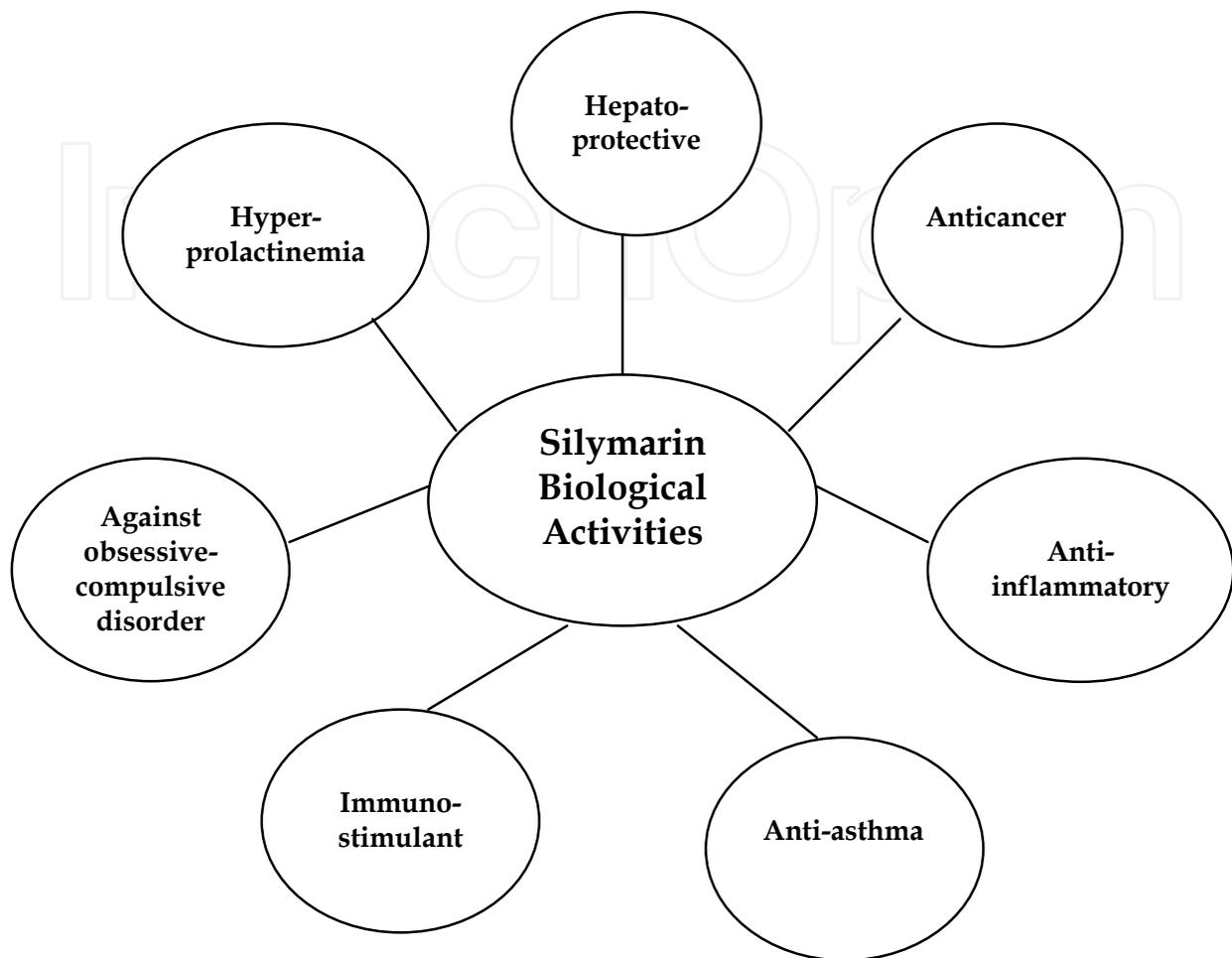


Fig. 6. Biological activities of silymarin.

6. Tissue culture studies

Plant tissue culture can be a potential source for important secondary metabolites (Misawa, 1994). This is based mainly on using plant cultures in a similar manner to microbial fermentation for factory-type production of pharmaceuticals and food additives. This technology has some advantages over conventional agricultural methods: production is independent of variation in crop quality or failure, yield of secondary metabolites would be constant and geared to demand, there is no difficulty in applying good manufacturing practice to the early stages of production, production would be possible anywhere under strictly controlled conditions, independent of political problems, free from risk of contamination with pesticides, herbicides or fertilizers and new methods of production can be patented (AbouZid et al., 2008). Cell suspension culture and hairy root culture were established from *S. marianum*. The former is established from callus tissue that developed on injured plant surface as a result of wounding or exogenous hormones (Fig. 7). The latter represent an approach to increase the yield of flavonolignans using morphologically differentiated/organized cultures.

6.1 Cell culture

In vitro cultured cells of *S. marianum* may offer an alternative and renewable source for this valuable natural product. However, the yield of silymarin was very low or sometimes not detectable in undifferentiated cultured cells (Becker and Schroll, 1977). In order to obtain silymarin in concentrations high enough for commercial manufacturing, many approaches have been made to stimulate the productivity of silymarin in cultured cells of *S. marianum*. These approaches comprise changes in the media composition (Cacho et al., 1999), treatment with elicitors such as yeast extract and methyl jasmonate (Sánchez-Sampedro et al., 2005a), addition of precursor (Tůmová et al., 2006) and morphological differentiation. Such approaches for improving silymarin production by manipulating plant cell cultures may also help in studying signal transduction pathways, cloning biosynthetic genes, studying metabolic flux and regulation of silymarin production (Zhao et al., 2005).

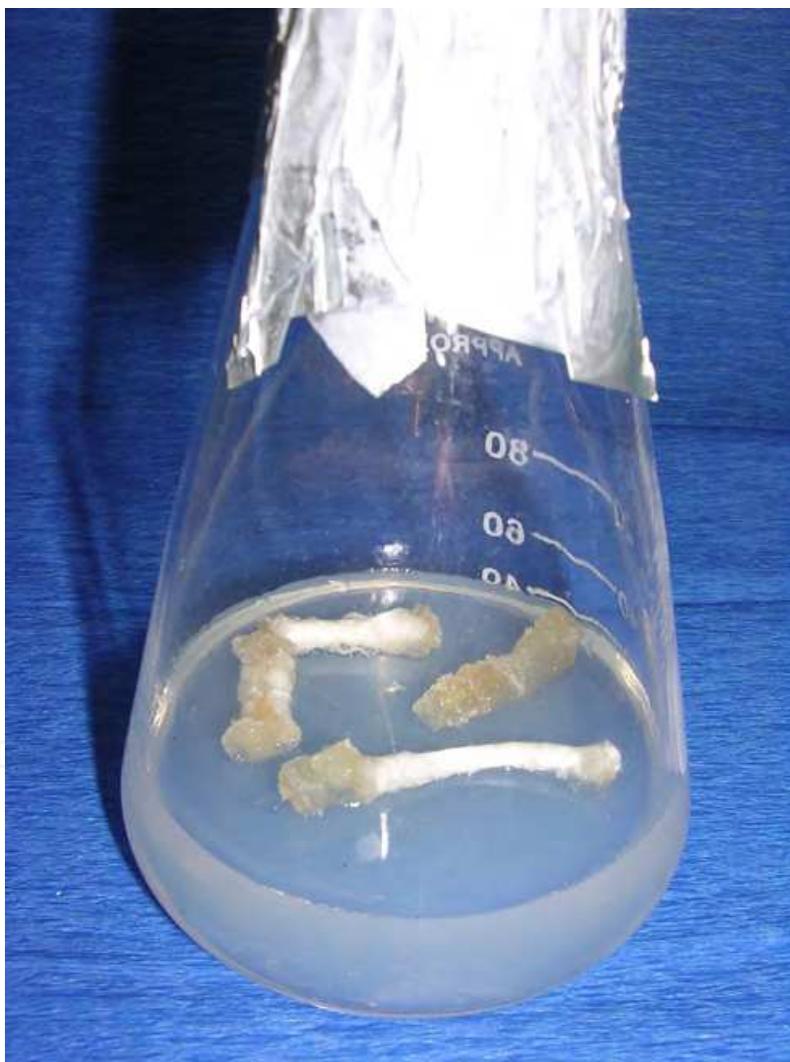


Fig. 7. Callus of *Silybum marianum* developed on explants.

Becker and Schroll, (1977) cultured cotyledon explants on MS media using different growth hormones for establishment of cell suspension culture. Typical flavonolignans of *S. marianum* were not detected. This was possible after feeding coniferyl alcohol and taxifolin

to cell suspension cultures (Schrall and Becker 1977). Feeding the culture medium with precursor of coniferyl alcohol offered enhancement of silydianin production but other components of silymarin were not influenced (Tůmová et al., 2006). Cacho et al. (1999) reported that callus and cell cultures of *S. marianum* could produce silymarin but to a lesser extent than that accumulates in the fruits. They also reported that elimination of calcium ion positively affected silymarin production. This point was further confirmed by Sánchez-Sampedro et al. (2005a), who also reported that silymarin accumulation was not altered by treatment of cultures with the calcium ionophore A23187. These results suggest that inhibition of external and internal calcium fluxes play a significant role in flavonolignans metabolism in *S. marianum* cell cultures. Sánchez-Sampedro et al. (2005b) reported that yeast extract and methyl jasmonate elicited the production of silymarin. Elicitation is one of the most effective approaches to enhance the yield of secondary metabolites in *in vitro* cultures (Namdeo, 2007). It has been shown that elicitors can affect level of secondary metabolites in medicinal plants by modulating the rates of biosynthesis, accumulation, and/or vacuolar transit, turnover and degradation (Barz et al., 1990). Jasmonic acid and its methyl ester are known to be involved in the plant defense response through altering the gene expression. The mechanism by which jasmonate induces gene expression was studied in *Catharanthus roseus* (van der Fits and Memelink, 2000). In this plant species induction occurs through an ORCA3 transcription factor with a conserved jasmonate-response domain. The use of methyl jasmonate as an elicitor has an advantage of being only one compound of well-defined chemical structure. The effect of elicitation with picloram, jasmonic acid and light on silymarin production was reported (Hasanloo et al., 2008). The greatest silymarin content (0.41 mg/g DW) was obtained with 3 mg/l picloram and 2 mg/l jasmonic acid in the dark after 28 days. The sequence of the signaling processes leading to stimulation of flavonolignan production by methyl jasmonate is not well-known. Madrid and Corchete, 2010, studied the possible involvement of a phospholipase D-mediated lipid signaling in the elicitation of flavonolignans. It was reported that methyl jasmonate increased the activity of phospholipase D. Mastoparan, a phospholipase D activity stimulator, caused a substantial increase in silymarin production. Phosphatidic acid, a product of phospholipase D activity, promoted silymarin accumulation. N-butanol which inhibits phospholipase D activity prevented silymarin elicitation by methyl jasmonate or mastoparan.

6.2 Root culture

Production of flavonolignans from root cultures (Fig. 8) of *S. marianum* was reported before (Alikaridis et al., 2000). Silybin (1.79×10^{-3} % DW) and silychristin (0.81×10^{-3} % DW) were the major flavonolignans produced by the established root cultures. In the referred study hairy root cultures of *S. marianum* were established. Hairy root cultures are the roots obtained by genetic transformation of plant tissues with the pathogenic soil bacterium *Agrobacterium rhizogenes*. These roots can then be cultured on hormone-free media and have three main advantages: genetic and biochemical stability, cultivation without addition of growth regulators and ability to give high final biomasses from low inocula.

Salicylic acid was effective in increasing the flavonolignan content 2.42 times in hairy root cultures of *S. marianum* higher than control cultures (Khalili et al., 2009). Yeast extract stimulated flavonolignan production in hairy root cultures two-fold higher than the control cultures. Moreover, it was reported that yeast extract treatment induced the activity of

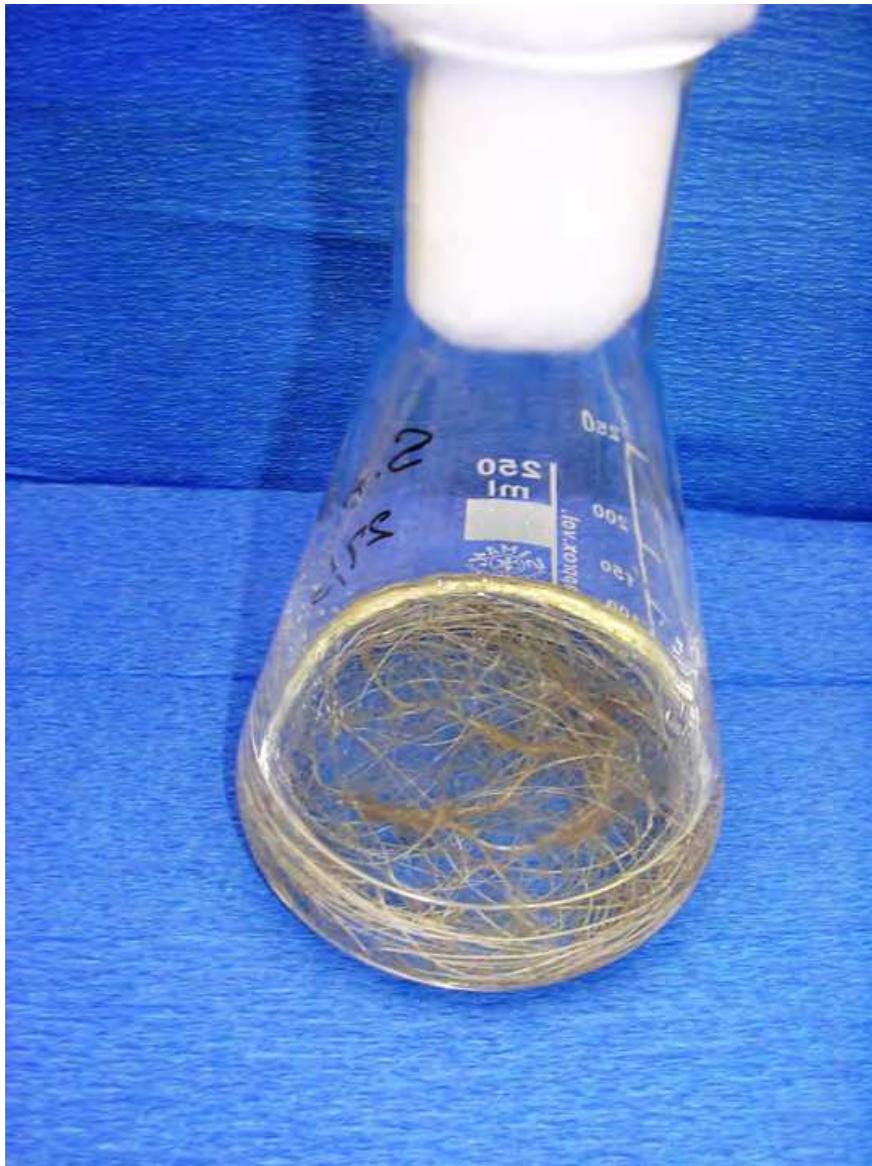


Fig. 8. Root culture of *Silybum marianum* growing in Murashige and Skoog medium.

lipoxygenase to allow for the production of jasmonate. It was concluded that jasmonate signaling is an integral part of the yeast extract signal transduction for the production of flavonolignans (Hasanloo et al., 2009).

7. Future directions

Plant tissue culture studies have contributed to our understanding of biosynthesis and regulation of silymarin in *S. marianum*. Using elicitation technology may offer an effective approach to improve silymarin production for industrial purpose. However, the possible signaling pathway that may be involved in accumulation of silymarin is still unknown. Understanding the basic components of this pathway is mandatory before these biotechnological methods can replace field crops as the basic source of pharmaceutical raw material. Establishment of plant tissue culture systems able to produce these biologically valuable compounds in high yield will facilitate such studies.

8. Conclusion

Milk thistle is an annual or biennial herb native to the Mediterranean and North African regions. The fruits of the plant contain an isomeric mixture of flavonolignans collectively known as silymarin. Basically, flavonolignan nucleus consists of the dihydroflavanol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. Little is known about the coupling of coniferyl alcohol to taxifolin. Silymarin is widely used as a hepatoprotective agent for oral treatment of toxic liver damage and for the therapy of chronic inflammatory liver diseases. The hepatoprotective activity of silymarin is based on antioxidant properties, stimulating liver cells regeneration and cell membrane stabilization to prevent hepatotoxic agents from entering hepatocytes. It has been shown that flavonolignans exhibit wide range of biological activity including anticancer, anti-inflammatory, hyperprolactinemic properties. Various methods have been developed for analysis of the content and composition of main silymarin components in plant material and pharmaceuticals. Among these methods are thin layer chromatography, spectrophotometric, high performance liquid chromatography, capillary zone electrophoresis and ultra performance liquid chromatography. *In vitro* cultured cells of *S. marianum* may offer an alternative and renewable source for this valuable natural product. Flavonolignans production in cell and root cultures of *S. marianum* has been reported. Many approaches have been used to increase the yield of flavonolignans in *S. marianum* tissue culture including change in media composition, addition of precursors and elicitation.

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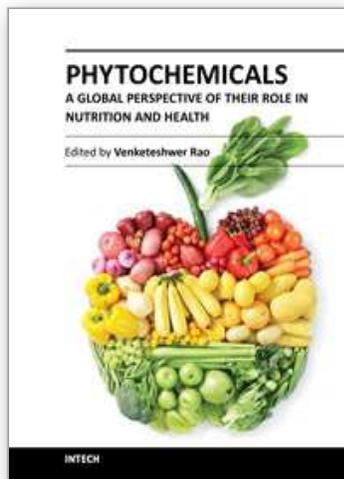
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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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