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The study of flavonolignan association patterns in fruits of diverging *Silybum marianum* (L.) Gaertn. chemotypes provides new insights into the silymarin biosynthetic pathway

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ABSTRACT

Silymarin is the phytochemical with medicinal properties extracted from *Silybum marianum* (L.) Gaertn. fruits. Yet, little information is available about silymarin biosynthesis. Moreover, the generally accepted pathway, formulated thus far, is not in agreement with actual experimental measurements on flavonolignan contents. The present work analyses flavonolignan and taxifolin content in 201 *S. marianum* samples taking into consideration a wide phenotypic variability. Two stable chemotypes were identified: one characterized by both high silychristin and silybin content (chemotype A) and another by a high silydianin content (chemotype B). Through the correlation analysis of samples divided according to chemotype, it was possible to construct a simplified silymarin biosynthetic pathway that is sufficiently versatile in explaining experimental results responding to the actually unresolved questions about this process. The proposed pathway highlights that three separate and equally sized metabolite pools exist, namely: diastereoisomers A (silybin A plus isosilybin A), diastereoisomers B (silybin B plus isosilybin B) and silychristin. In both A and B diastereoisomers pools, isosilybin A and isosilybin B always represent a given amount of the metabolite flux through the specific metabolite pool suggesting the possible involvement of dirigent protein-like enzymes. We suggest that chemotype B possesses a complete silymarin biosynthetic pathway in which silydianin biosynthesis is enzymatically controlled. On the contrary, chemotype A is probably a natural mutant unable to biosynthesize silydianin. The present simplified pathway for silymarin biosynthesis will constitute an important tool for the further understanding of the reactions that drive flavonolignan biosynthesis in *S. marianum*.

1. Introduction

Silybum marianum (L.) Gaertn. or common name milk thistle, is an annual or biannual species of the Asteraceae family. The species is native to southern Europe, Asia Minor and northern Africa and it is naturalized in North and South America, Australia and New Zealand (Carrier et al., 2002; Groves and Kaye, 1989; Morazzoni and Bombardelli, 1995; Martin et al., 2000). *S. marianum* has been utilized as a medicinal plant for more than 2000 years and was first reported by Theophrastus in the 4th century B.C. (Morazzoni and Bombardelli, 1995). At present *S. marianum* is grown as a medicinal plant in Europe and Asia (Andrzejewska et al., 2015) and it is among the top selling herbal products in the US, in Italy and in other countries (Smith et al., 2016; ISMEA report, 2013).

The medicinal properties of milk thistle are determined by its ability to accumulate bioactive flavonolignan complexes referred as

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silymarin in the fruit (Wagner et al., 1974). Interestingly, the different flavonolignans have been reported to have different biological activities (Polyak et al., 2010) and drugs based on purified silymarin have been available for almost 50 years (Albrecht et al., 1992).

Silymarin has a hepato-protective effect, as well as antioxidant, anti-inflammatory and antifibrotic properties. Additionally, silymarin is suggested to inhibits cell growth, DNA synthesis and other mitogenic signals in human prostate, breast and cervical carcinoma cells. New perspectives relating to the use of silymarin reside in the prevention or reduction of chemotherapy, as well as of radiotherapy-induced toxicity (Ladas and Kelly, 2003; Abenavoli et al., 2010). Recently, silybin, one of the silymarin constituents, was included in the list of molecules useful in a broad-spectrum integrative approach for cancer prevention and treatment (Block et al., 2015).

Efforts have been undertaken to obtain flavonolignan production through chemical synthesis and plant cell cultures (McDonald et al., 2015; Poppe and Petersen, 2016). Despite that, silymarin is extracted from *S. marianum* fruit, thereby taking advantage of the natural silymarin biosynthetic pathway. In spite of the medicinal and economic importance of silymarin, little information is available about the pathway that leads to flavonolignan biosynthesis (Poppe and Petersen, 2016; Torres and Corchete, 2016).

Silymarin consists of an isomeric mixture of flavonolignans that includes both positional isomers and diastereoisomers (Fig. 1). The main silymarin constituents in purple-flowering *S. marianum* genotypes are: silybin (diastereoisomers A and B; SILA, SILB), isosilybin (diastereoisomers A and B; ISOA, ISOB), silychristin (SILYC), and the structurally most complex flavonolignan silydianin (SILYD; Lee et al., 2007). In addition, the minor constituents silychristin B and isosilychristin (ISOSILYC) have also been identified (Kaloga, 1981; Smith et al., 2005). The precursors for the biosynthesis of these flavonolignans are, respectively, taxifolin (TAXIF), for the flavonoid part, and coniferyl alcohol for the lignan moiety of each molecule (Pelter and Hänsel, 1968).

Our current understanding of silymarin biosynthesis is based on the pathway illustrated in Fig. 2. In this pathway, the two precursors, following one-electron oxidation, are able to perform a random radical coupling, thereby producing silymarin flavonolignans. The different isomers are the result of the positioning of radical groups that are present on the two precursors and of the post-coupling reactions that take place within the formed quinone methide intermediate (QMi; Dewick, 2002). To date there is no consensus about which radicals participate the process (Dewick, 2002; Althagafy et al., 2013; AbouZid et al., 2017). According to the current pathway, the diastereoisomer couples, SILA, SILB and ISOA, ISOB are described as equimolar mixtures reflecting the lack of stereospecificity of the original radical coupling (Dewick, 2002; Nyiredy et al., 2008). Interestingly, experimental data do not support the 1:1 ratio between the constituents of each diastereoisomers couple (Carrier et al., 2002; Martin et al., 2006; Martinelli et al., 2016; AbouZid et al., 2017). Moreover, the random radical coupling theory does not explain why in certain genotypes SILYD is virtually absent (Martin et al., 2006; Martinelli et al., 2016). The results obtained by Becker and Schrall (1977), and confirmed by Sánchez-Sampedro et al. (2007), indicate that *S. marianum* extracellular peroxidases are reputed to be responsible for taxifolin and coniferyl alcohol oxidation and, therefore, for the radical coupling between the two precursors. Despite that, it is not known if peroxidases are the only enzymes involved in the final step of flavonolignan biosynthesis (Sánchez-Sampedro et al., 2007). Recently, Poppe and Petersen (2016) studying different *S. marianum* fruit samples characterized by different flavonolignan profiles

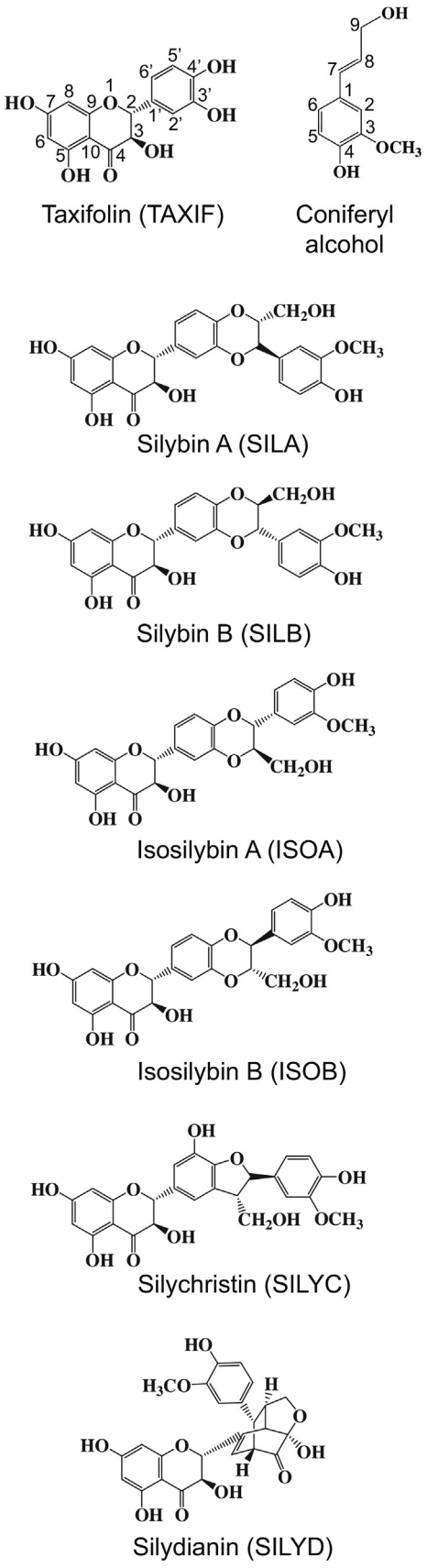

Fig. 1. Chemical structure of taxifolin, coniferyl alcohol and of main flavonolignans present in purple-flowering *S. marianum* genotypes. Numbers indicate the labeling of atoms in both taxifolin and coniferyl alcohol. The abbreviations utilized for each molecule are reported in brackets.