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Probable Biosynthetic Pathways of Silymarin Precursors

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ABSTRACT

Background: Silymarin is composed of a mixture of flavonolignans derived from secondary plant metabolism. These constituents are present in substantial amounts in milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] seeds. Silymarin has antioxidant properties that impact it with protective effects. Because of its chemoprotective effect against liver disease, silymarin is considered a complementary and alternative hepatoprotective medicine.
Methods and Results: Coniferyl alcohol and taxifolin, the two precursors for silymarin biosynthesis, are derived from phenylpropane and flavonoid units, respectively. Coniferyl alcohol is synthesized *via* the monolignol biosynthetic pathway, whereas taxifolin is synthesized via the flavonoid pathway. Multiple variables, including related substrates, production, and activating enzymes require consideration to study the biosynthetic pathway of silymarin.
Conclusions: This review is helpful as it summarizes the probable biosynthetic pathways of silymarin and multiple related activating enzymes and substrates found in various plants. A further understanding of silymarin is expected to increase its industrial use value.

Key Words: *Silybum marianum* (L.) Gaertn, Coniferyl Alcohol, Silymarin, Taxifolin

INTRODUCTION

Unlike primary metabolites, secondary metabolites are not necessary for the life cycle of plants but are synthesized in higher plants (Wink, 1988; Rhodes, 1994). Secondary metabolites are produced by interactions with the surrounding environment and other organisms, which provides efficient defense strategies against abiotic and biotic stress (Waterman, 1992).

Phytoalexins, which produce antimicrobial secondary metabolites, accumulate upon microbial exposure in plants (Rakwal *et al*., 1996). Isoflavonoids recognize phytoalexins and categorized flavonoids, for example, and exert a feeding deterrent activity against the larvae of scarabs and are also resistant to soil-borne fungal pathogens (Sutherland *et al*., 1980; Lozovaya *et al*., 2004).

Given the defense strategy for abiotic stress, biosynthetic flavonoids have been associated with tolerance to abiotic stress (salt, drought, and chilling stress) in other studies (Mahajan *et al*., 2014; Meng *et al*., 2015; Song *et al*., 2016; Wang *et al*., 2016). In secondary metabolites with antioxidant activity, flavonoids extracted from food, such as red grape and black tea, demon-

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strate an antioxidant capacity and are effective in scavenging free radicals and retarding lipid oxidants (Leung *et al*., 2001; María *et al*., 2004; Köksal *et al*., 2009; Hidalgo *et al*. 2010).

Silymarin is a mixture of constituents, including silybin, silydianin, and silychristin, a group of flavonolignans derived from secondary plants metabolites, and is well known for its antioxidant and therapeutic effects against liver disease (Flora *et al*., 1998; Köksal *et al*., 2009; Nancy *et al*., 2014).

Silymarin is highly accumulated in the seeds of the milk thistle [(*Silybum marianum* L.) Gaertn. (Asteraceae)] plant, which is native to the Mediterranean area and contains Cirsium plants, which belong to the same family as Compositae (Ma *et al*., 2016; Nam *et al*., 2018; Rodriguez *et al*. 2018; Kim *et al*. 2020; Aziz *et al*., 2021). In addition, regulatory gene expression analysis related to the biosynthesis of silymarin in milk thistle and *Cirsium japonicum* has been conducted (Roy *et al*., 2018; Drouet *et al*., 2020).

Various biosynthetic pathways and precursors exist for silymarin biosynthesis, and multiple enzymes are activated to catalyze these pathways. The probable biosynthetic pathways, and the list of phased precursors and enzymes in this pathways can be required to study related to biosynthesis in plants that retain silymarin constituents, such as milk thistle.

There are two major biosynthetic pathways for silymarin, amalgamating coniferyl alcohol and taxifolin, the precursors for silymarin biosynthesis (Yang *et al*., 2020). Coniferyl alcohol is synthesized via monolignol-specific pathways derived from the phenylpropanoid pathway (Vanholme *et al*., 2010). In the phenylpropanoid pathway, one molecule of *p*-coumaroyl CoA can be a precursor for the flavonoid pathway by condensing with three molecules of malonyl CoA (Dao *et al*., 2011). Thus, the biosynthesis of taxifolin is grouped in the flavonoid pathway from phenylpropanoids.

MATERIALS AND METHOD

In this review, the probable silymarin biosynthetic pathways and the related multiple activating enzymes and substrates that have been identified in various plants are presented to understand the biosynthesis mechanism.

RESULTS AND DISCUSSION

1. Silymarin contents

Silymarin is a pharmacologically active structural complex extracted from the seeds of milk thistle and is present in a range of 1.5% to 3% of the seed weight (Flora et a., 1998**;** Martin *et al*., 2006; Abenavoli *et al*., 2010).

Structural complexes are composed of flavonolignan isomers, and the major isomers include silybin (silibinin), isosilybin (isosilibinin), silychristin, isosilychristin, and silydianin (Deep *et al*., 2008; Valková *et al*., 2021). Of the isomers, the principal active compound is silybin, representing approximately 50% - 60% (Saller *et al*., 2001). Silybin, isosilybin, and silychristin form two diastereoisomers, namely A and B (Smith *et al*., 2005). Silymarin is a flavonolignans amalgamated with phenylpropane and flavonoid units (Althagafy *et al*., 2013; Bijak, 2017).

Phenylpropane and flavonoid units are structurally related to coniferyl alcohol and taxifolin, respectively. The structures of silymarin components are shown in Fig. 1.

2. Biosytnetic pathway of coniferyl alchool

One of the silymarin precursors, coniferyl alcohol, is synthesized via the monolignol biosynthetic pathway, which plays a major role in producing source materials for lignin biosynthesis (Wang *et al*., 2019).

The overall biosynthetic pathway of coniferyl alcohol is presented in Fig. 2. Monolignol belongs to one of the phenylpropanoid classes, and its pathway starts with the phenylpropanoid biochemical pathway (Vogt, 2010).

The phenylpropanoid pathway begins with the use of the aromatic amino acids phenylalanine and tyrosine, which are the end products of the shikimic acid pathway (Tzin and Galili, 2010; Santos-Sánchez *et al*., 2019).

L-Phenylalanine is catalyzed into *trans*-cinnamic acid by the deamination of phenylalanine ammonia-lyase (Koukol and Conn, 1961). Cinnamate 4-hydroxylase, which belongs to the CYP73A family of cytochrome P450 monooxygenases, catalyzes the *p*-hydroxylation of *trans*-cinnamic acid to yield *p*-coumaric acid (Hahlbrock and Scheel, 1989; Duan *et al*., 2004; Zhang *et al*., 2020). In several studies, tyrosine ammonia-lyase has also been shown to convert L-tyrosine to *p*-coumaric acid by deamination (Beaudoin-Eagan and Thorpe, 1985; Rosler *et al*., 1997; Nishiyama *et al*., 2010). This part, from aromatic amino acids to *p*-coumaric acid, is the general phenylpropanoid pathway.

The activity of *p*-coumarate 3-hydroxylase (C3H), which is a cytochrome P450 monooxygenase belonging to the CYP98 family, and caffeic acid 3-*O*-methyltransferase can transform *p*-coumaric acid into caffeic acid and ferulic acid, respectively (Inoue *et al*., 1998; Franke *et al*., 2002). 4-Coumaric acid:

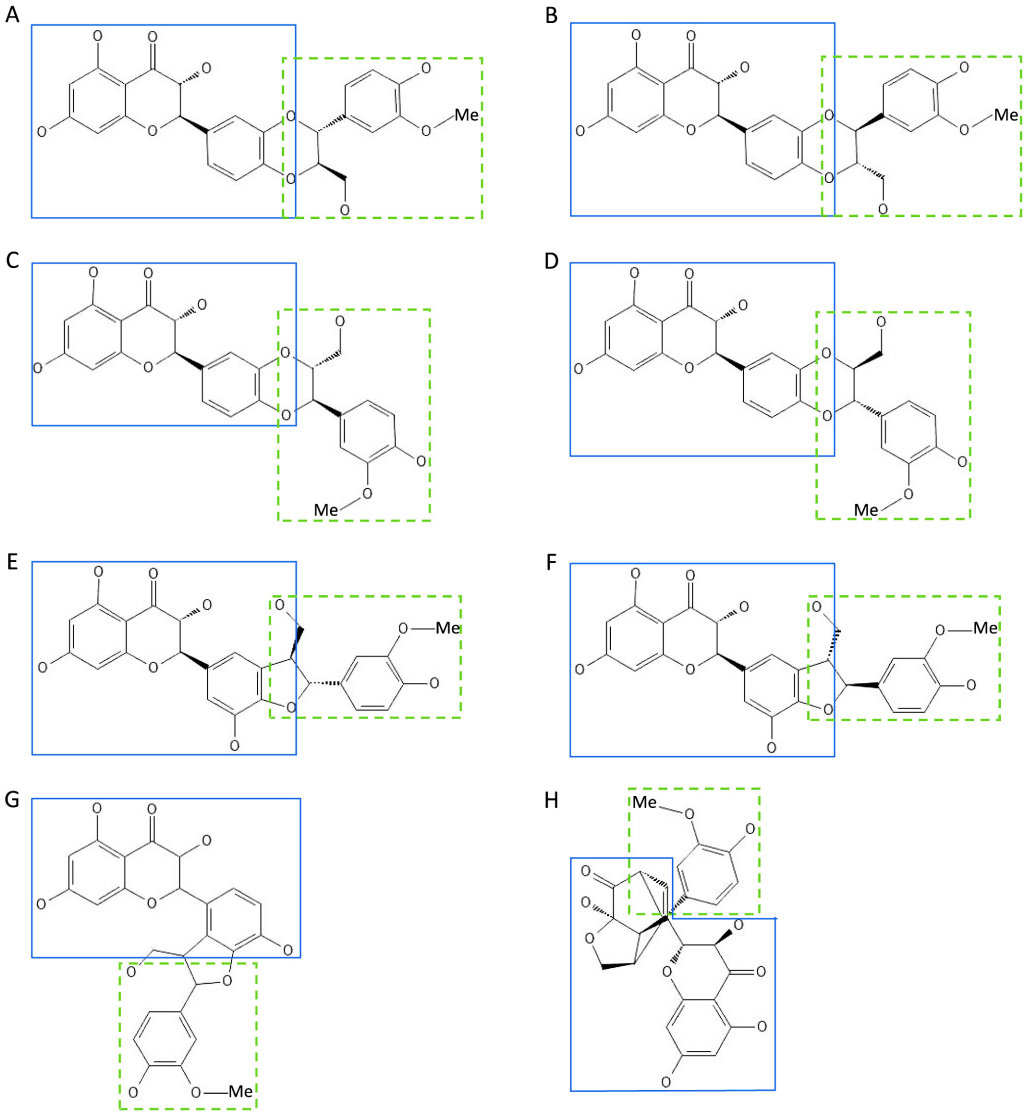

Fig. 1. Chemical structure of various silymarin components. (A) silybin A, (B) silybin B, (C) isosilybin A, (D) isosilybin B, (E) silychristin A, (F) silychristin B, (G) isosilychristin, and (H) silydianin. Representative examples of silymarin containing flavonid (retangle by straight lines) and phenylpropane moiety (retangle by dotted lines).

Coenzyme A ligase can convert hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, and ferulic acid) to hydroxycinnamoyl-CoA thioesters (*p*-coumaroyl, caffeoyl, and feruloyl-CoA) by ligation of coenzyme A (CoA) (Chen *et al*., 2013).

p-Coumaroyl-CoA is treated as a precursor for the production of secondary plant metabolites, including flavonoids, and is catalyzed to caffeoyl-CoA by the two enzymes *p*-coumaroylester 3′-hydroxylases, which are cytochrome P450s belonging to the CYP98A3 family, and hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyl transferase with shikimate/quinate, leading to *p*-coumaroylquinic/*p*-coumaroylshikimic acid and caffeoylquinic/caffeoylshikimic acid (Hoffmann *et al*., 2004; Boudet, 2007; Mahesh *et al*., 2007).

Caffeoyl CoA 3-*O*-methyltransferase activates caffeoyl-CoA to produce feruloyl-CoA (Inoue *et al*., 1998). The production of hydroxycinnamaldehydes (*p*-coumaryl, caffeyl, and coniferyl-aldehyde) from hydroxycinnamoyl-CoA thioesters is achieved via activation of cinnamoyl-CoA reductase (Lauvergeat *et al*., 2001; Vanholme *et al*., 2019). Cinnamyl alcohol dehydrogenase converts hydroxycinnamaldehydes to hydroxycinnamyl alcohols (*p*-coumaryl, caffeyl, and coniferyl alcohol) (Liu *et al*., 2018). Caffeic acid 3-*O*-methyltransferase has high activity for caffeyl-

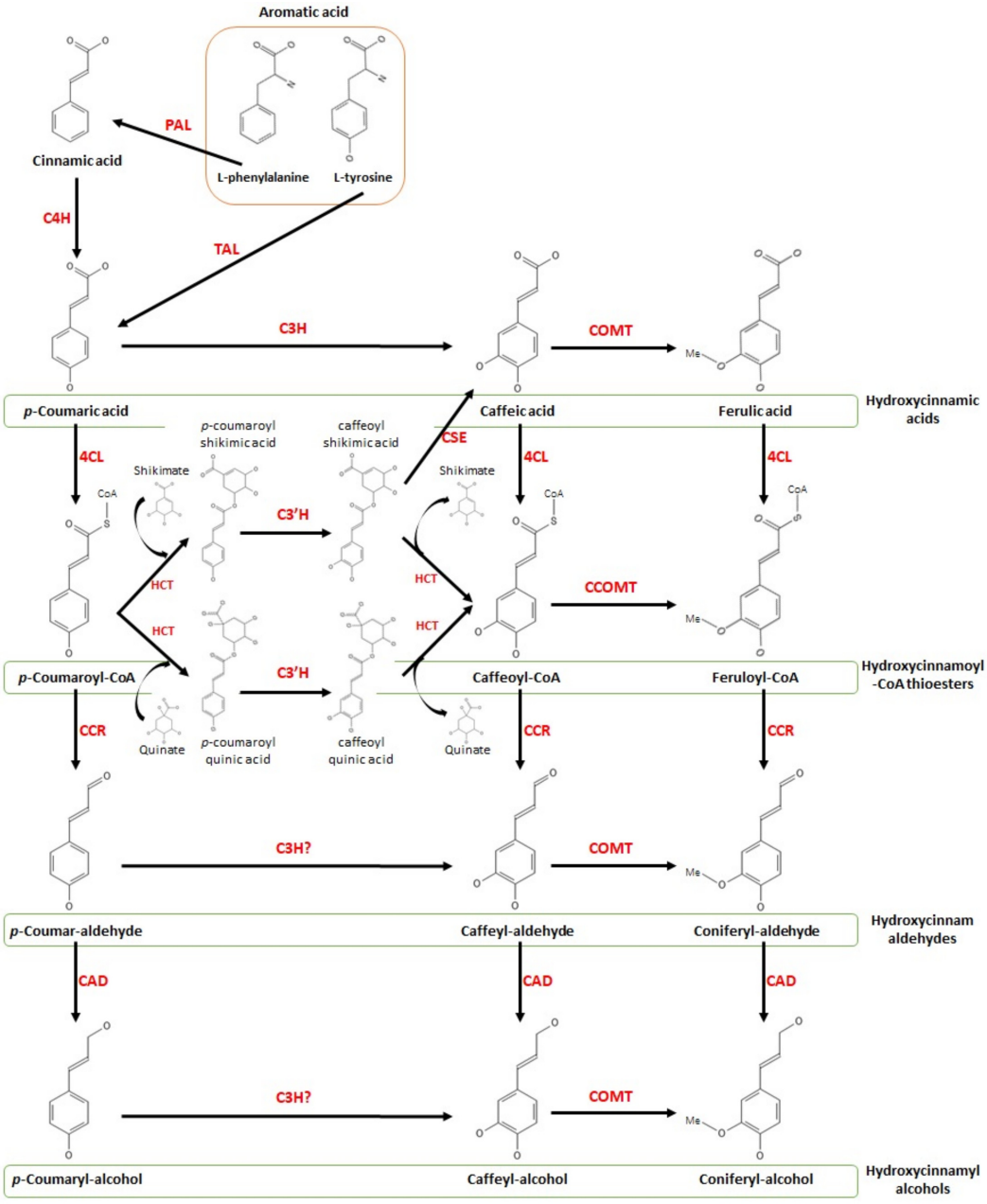

Fig. 2. This schematic view present coniferyl alcohol biosynthetic pathway. The aromatic acids (L-phenylalanine and L-tyrosine) are converted to hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, and ferulic acid), hydroxycinnamoyl-CoA thioesters (*p*-coumaroyl, caffeoyl, and feruloyl-CoA), hydroxycinnamaldehydes (*p*-coumaryl, caffeyl, and coniferyl-aldehyde), and hydroxycinnamyl alcohols (*p*-coumaryl, caffeyl, and coniferyl alcohol) by several enzymes. The enzymes involved in this pathway are phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), cinnamate 4-hydroxylase (C4H), *p*-coumarate 3-hydroxylase (C3H), *p*-coumaroylester 3′-hydroxylase (C3′H), caffeic acid 3-O-methyltransferase (COMT), caffeoyl-CoA 3-O-methyltransferase (CCOMT), 4-coumaric acid:coenzyme A ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), caffeoyl shikimate esterase (CSE), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD).

aldehyde and caffeyl-alcohol to catalyze the conversion of coniferyl-aldehyde and coniferyl-alcohol, respectively (Parvathi *et al*., 2001).

3. Biosysteic pathway of taxifolin

In the biosynthesis of silymarin, the phenylpropane unit (coniferyl alcohol) and flavonoid unit (taxifolin) synthesis require

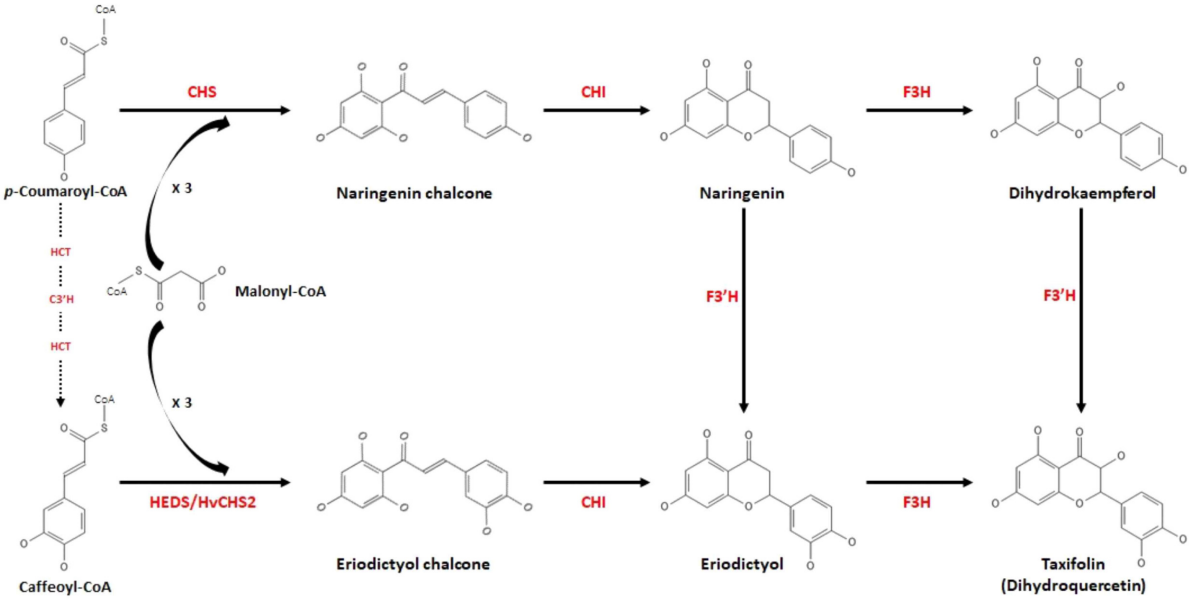

Fig. 3. This schematic view present taxifolin biosynthetic pathway. The *p*-coumaroyl-CoA and caffeoyl-CoA are converted to taxifolin by several enzymes. The enzymes involved in this pathway are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), and homoeriodictyol/eriodictyol synthase (HEDS/HvCHS2).

p-coumaryl CoA, which activates both synthesis pathways (Torres and Corchete, 2016). The taxifolin biosynthetic pathway is shown in Fig. 3.

Chalcone synthase, which is a key enzyme in the flavonoid biosynthesis pathway and a member of the plant polyketide synthase family, uses one *p*-coumaroyl CoA as a substrate and forms naringenin chalcone by a condensation reaction with three malonyl-CoA (Flores-Sanchez, 2008; Dao *et al*., 2011).

Chalcone isomerase, also known as chalcone-flavanone isomerase and belonging to the class of intramolecular lyases, catalyzes the conversion of naringenin chalcone into its corresponding flavanone, naringenin (Sun *et al*., 2019).

Flavonoid 3′-hydroxylase (F3′H) and flavanone 3-hydroxylase (F3H) are oxoglutarate-dependent dioxygenases and cytochrome P450 hydroxylases, respectively (Dixon and Steele, 1999; Winkel-Shirley, 2001). The sequential reaction of F3′H and F3H allows the catalysis of naringenin to eriodictyol and taxifolin (dihydroquercetin) (Hammerbacher *et al*., 2019). In addition, the other sequential reaction of F3H and F3′H catalyze the conversion of naringenin into dihydrokaempferol and taxifolin (Brugliera *et al*., 1999).

In other routes to taxifolin biosynthesis, homoeriodictyol/eriodictyol synthase uses one caffeoyl-CoA as a substrate to form eriodictyol chalcone as the reaction of the chalcone synthase catalyst, and the activity of chalcone isomerase and F3H catalyzes eriodictyol chalcone into taxifolin (Christensen *et al*., 1998; Flores-Sanchez and Verpoorte, 2008; Morita *et al*., 2010; Meinert *et al*., 2021).

4. Amalgamation of coniferyl alcohol and taxifolin

Coniferyl alcohol and taxifolin are amalgamated via oxidative coupling for silymarin biosynthesis (flavonolignan). The oxidative coupling reaction is mediated by the formation of free radicals and is catalyzed by a peroxidase enzyme called a radical generator (AbouZid and Ahmed, 2013).

In addition, laccases (benzenediol: oxygen oxidoreductases) mediate oxidative coupling for dimerization and production of silybin (Setti *et al*., 1999; Gažák *et al*., 2008; Gavezzotti *et al*., 2014). A study by Lv *et al.* (2017) reported that silybin components of silymarin were catalyzed by ascorbate peroxidase 1 (APX1), one of the candidates for peroxidase, and APX1 showed a distinct peroxidase activity and the capacity to synthesize silybin.

Schrall and Becker (1977) reported that horseradish-peroxidase and a cell-free extract of milk thistle suspension cultures could synthesize silybin starting from coniferyl alcohol and taxifolin. In some studies, peroxidases of APX1 and horseradish-peroxidase have been used to demonstrate a green process for silybin and isosilybin production (Yang *et al*., 2020).

5. Others

The contents and components of silymarin biosynthesis are influenced by some factors. The study of Martin *et al.* (2006), use various milk thistle cultivars to present the factors influencing on contents and components of silymarin. This study show that each plant parts have different total contents and components of silymarin. The part of root have only silychristin B and silybin B components, and the two components are major in flowers. But, the contents have very low levels. In the seeds and seed heads, silychristin A, silydianin, and silybin B are the dominant components, and it have the highest total silymarin content. In addition to the difference of contents and components in each plant parts, the study show difference of contents and components depending on growth stage between the cultivars.

In the study of Liava *et al.* (2022), it present effects of fertilization regimes on growth, fruit, and silymarin yield in two cultivars of milk thistle. Sheep manure and calcium ammonium nitrate are used to exhibit the difference of plants growth depending on fertilization regimes. The use of manure and calcium ammonium nitrate fertilizer increase plant rosette

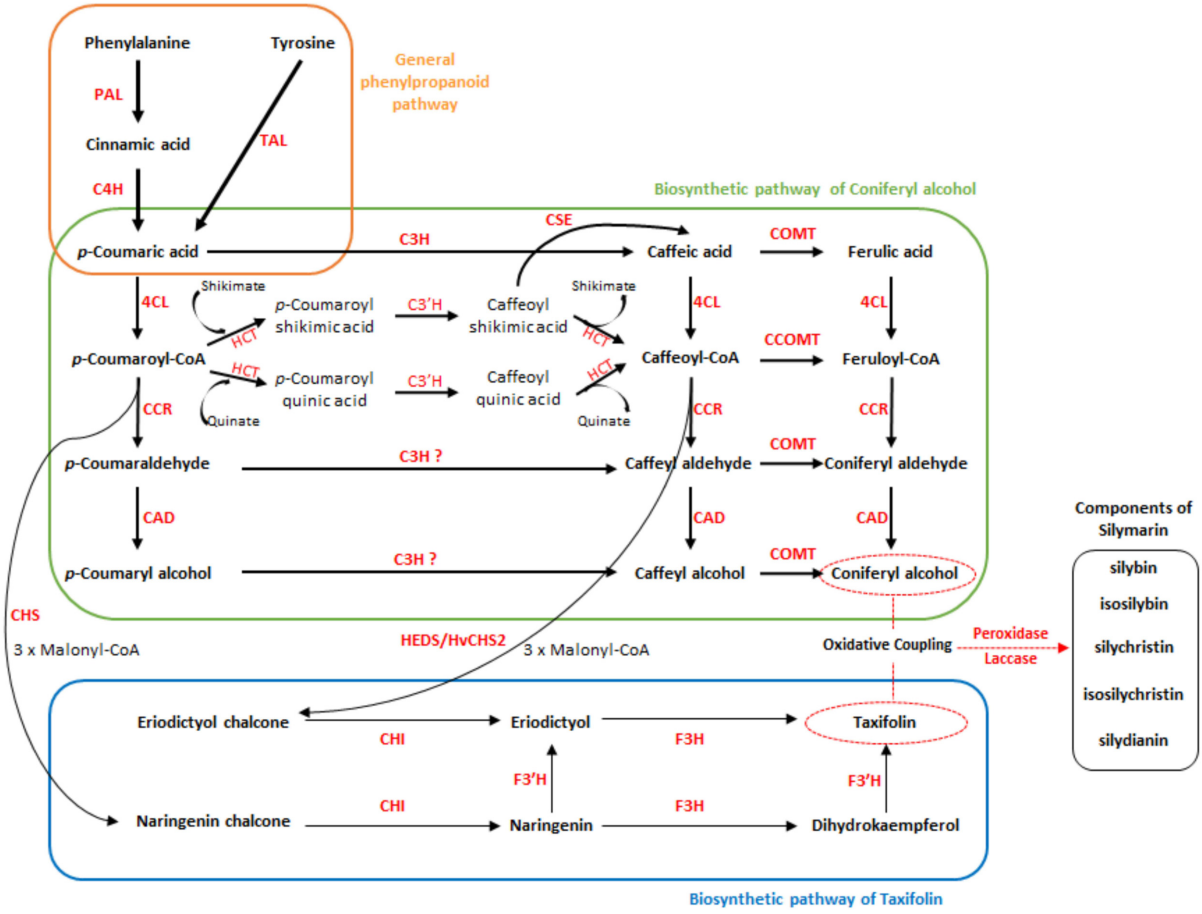

Fig. 4. This schematic view present overall biosynthetic pathway of silymarin. In general phenylpropanoid pathway, the phenylalanine and tyrosine are converted to *p*-coumaric acid, and the involved enzymes are phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), cinnamate 4-hydroxylase (C4H). In biosynthetic pathway of coniferyl alcohol, the *p*-coumaric acid is converted to coniferyl alcohol, and the involved enzymes are *p*-coumarate 3-hydroxylase (C3H), *p*-coumaroylester 3′-hydroxylase (C3′H), caffeic acid 3-O-methyltransferase (COMT), caffeoyl-CoA 3-O-methyltransferase (CCOMT), 4-coumaric acid:coenzyme A ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), caffeoyl shikimate esterase (CSE), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD). The *p*-coumaroyl-CoA and caffeoyl-CoA are converted to naringenin chalcone and eriodictyol chalcone by chalcone synthase (CHS) and homoeriodictyol/eriodictyol synthase (HEDS/HvCHS2), respectively. In biosynthetic pathway of taxifolin, the eriodictyol chalcone and naringenin chalcone are converted to taxifolin, and the involved enzymes are chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H). Coniferyl alcohol and taxifolin are amalgamated via oxidative coupling to formation for component of silymarin (silybin, isosilybin, silychristin, isosilychristin, and silydianin) by peroxidase and laccase.

diameter, biomass, fruit yield, and silymarin content. In this study, it is noticed that the plants growth with silymarin content can be increased based on treatment of fertilization regimes, and that the difference of silymarin content as well as silymarin composition between the two cultivars.

According to this two studies, we need to consider suitable milk thistle cultivars selection to produce silymarin as commercial viewpoint and crop management guidelines. Futhermore, the studies of Lv *et al.* (2017) and Torres *et al.* (2016) reported genes expression for silymarin biosynthetic pathway. It is regarded that molecular study for genetics is requiered using the understanding for segmented biosynthetic pathway and milk thistle cultivars which have difference of the contents and components of silymarin.

Metabolic engineering of biosynthetic pathways to produce high-value secondary metabolites as pharmaceuticals and food additives, have industrial importance using plant cell cultures, shoot cultures, root cultures, and transgenic hairy root cultures acquired through biotechnological means (Rao *et al.,* 2002). These plant tissue cultures can be potential alternative sources for the secondary metabolites production.

The studies of Alikaridis *et al.* (2000) and Rahnama *et al.* (2008) show that production of silymarin and the components produced by the hairy root and root cultures of milk thistle. In the studies of Elwekeel *et al.* (2012a) and El Sherif *et al.* (2013), these show that silymarin accumulated through the root and shoot cultures of milk thistle can be improved by various elicitors. In addition to enhancement of silymarin production through addition of elicitors, the Elwekeel *et al.* (2012b) study present that the cultured cells of milk thistle have comparable cytotoxic, antioxidant, and hepato-protective effects to that of the fruits.

The study of plant tissue cultures for producing silymarin may be necessary to pharmaceutical industries. Thus, the overall understanding about silymarin biosynthetic pathways will be helpful to define efficient ways of silymarin production to utilize the various elicitors and tissue cultures.

The overall biosynthetic pathway for the silymarin components is shown in Fig. 4. The processing of the precursors (coniferyl alcohol and taxifolin) for biosynthetic pathways cannot be simply explained by one route, and the activating enzymes involved vary. In particular, the biosynthetic pathway of the precursor coniferyl alcohol, which is included in monolignol-specific pathways, has multiple routes than biosynthetic pathway of taxifolin.

In a study by Ha *et al.* (2016), caffeoyl shikimate esterase activity converted caffeoyl shikimic acid to caffeic acid in *Medicago truncatula*. Furthermore, the study by Liu *et al.* (2018) reported that cinnamyl alcohol dehydrogenases, cloned and characterized from *Asarum sieboldii* Miq., displayed efficient catalytic activity and substrate preference with the capability of converting aldehydes (*p*-coumaryl, coniferyl, and sinapyl aldehydes) to their corresponding alcohols. Regarding substrate preference, Franke *et al.* (2002) also reported that C3H, which is encoded by the *REF8* gene isolated from Arabidopsis, had different levels of substrate activity for *p*-coumaric acid, *p*-coumaryl-aldehyde, and *p*-coumaryl-alcohol.

Thus, enzymes used in biosynthetic pathways in plants have substrate preferences that are activated differently on each substrate, and all plant species may not use identical enzymes for specific biosynthetic pathways. The study of biosynthetic pathways for specific secondary metabolites requires the recognition of the overall pathways with related factors (substrates, production, and activating enzymes), and an efficient activity pathway may be required in individual plants considering these factors.

Therefore, the overall biosynthetic pathway presented in this review can be utilized to study the biosynthesis of silymarin components.

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