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Phytoconstituents and pharmacological activities of *Silybum marianum* (Milk Thistle): A critical review

Quang-Ung Le, Horng-Liang Lay, Ming- Chang Wu and Rakesh Kumar Joshi

Abstract
Silybum marianum (SM) has attracted substantial attention because it is been used as a gold medicinal herb in traditional folk medicine for treatment of liver diseases and it possesses other wondrous health benefits. To support its functional attributes, various investigations have been carried out to find out its antioxidant, anti-inflammatory, anti-antiviral, anti-diabetic, anti-amnesia, cardio-protection, hepatic protective activities, the efficacy in the treatment of obsessive-compulsive disorder, sepsis and burn prevent and application on veterinary under publications of certain types of articles including "*original research* and *short communications*". Some publications either reiterate previously reports or create slight mistakes in performing methods, the problem associated quality performance to claim exactly biological activities found in the literature. Furthermore, correct expressions and original citations of the relevant models are provided to interpret and clarify. The authors hope that this work will be helpful to give insight knowledge for readers, researchers, reviewers, and editors who are interested in the related field of the SM studies.

Keywords: Milk thistle, Silybum marianum, Flavonolignan, Silymarin

1. Introduction
Silybum marianum (also known as *Silybum marianum* (L.) Gaertn belongs to the Asteraceae family and is commonly known as milk thistle in English. It grows in Europe, Asia, and Northern Africa. It is regarded as an ancient medicinal weed of ethno-pharmacological significance to treat a range of liver diseases [1-4].
Considering the medicinal significance of the plant, it was of interest to review the pharmacological reports on the plant through data base searches (PubMed, Scopus, Google Scholar).

2. Economics
S. marinum is sometimes cultivated as an ornamental, a minor vegetable or as a medicinal herb. The flowers provide a useful source of pollen for bees in early summer [5]. *S. marianum* is an important food source in many countries, whether native or introduced. It is grown as a commercial crop in several countries, including Iran and Pakistan. Is has also been considered, at least in Sardinia, as a possible biomass crop for the production of bio-energy. Until the 1930s it was widely cultivated as an oil-seed plant in Russia [6]. Its average sale is about US$ 8 billion/annum and its demand varies from 18 to 20 tons per year [7]. It extract is now marketed as silymarin and silybinin capsules and tablets with an improved bioavailabity under the trate names like Livergol, Silipder and Legalon, Indena [8].

3. Suspension and callus culture reports
In 2000, Alikaridis evaluated flavonolignan content in root cultures of the SM. Interestingly, the authors found that Silybin, isosilybin, silychristin and silydianin were detected in untransformed root culture, but only isosilybin was found in the "hairy" root cultures [9].
Increasing flavonolignan production basing on different medium conditions was also showed in some reports of Sampedro. Treatment of cultures with the calcium ionophore A23187 did not change silymarin accumulation. Interestingly, this compound increased to 200% when treatment of cultures with specific Ca^{2+} chelator or EGTA [10]. In another study, Sampedro reported that methyl jasmonate strongly improved the silymarin accumulation and enhanced Chalcone synthase activity. However temporal relationship between silymarin accumulation and increase in enzyme activity was not recorded [11].

Moreover, the presence of an arabinogalactan protein and its aminoacids was detected in cell suspension cultures in medium with the Yariv reagent [12]. The primary flavonolignans in the cultured roots of SM from *in vitro* germinated sterile plantlets with treatment of hormone-free Murashige and Skoog medium were detected to be silychristin, silydianin and flavonolignan precursor taxifolin (74.2, 8.1 and 40.8 µg/g of fresh weight), respectively. Likewise with report of Sampedro [11] methyl jasmonated also enhanced flavonolignans and taxifolin accumulation to approximately 300% compared to the control cultures and also increased 3,3',5,5',7-pentahydroxyflavanone content [13].

Developing of callus and shoot cultures from leaves and shoot tips explants with various concentrations of the precursor (coniferyl alcohol) was conducted by Rady *et al*. Authors found optimum and best medium for maintenance of friable callus and proliferation of high number of shoots (0.25 mg L^{-1} for each agent in formula: 2,4 –Dichlorophenoxy acetic + 0.25 mg L^{-1} Kinetin and Benzyl Adinine + Naphthalene acetic acid). Increasing silymarin accumulation in most callus cultures appeared when treatment with coniferyl alcohol in 30 µM concentration [14].

An insight research on influence of medium condition to silymarin accumulation in suspension cultures from seedlings of three typical chemotypes was performed. The results revealed that regioisomers silydianin and silychristin accumulation were strongly depended by the taxifolin: coniferyl alcohol concentration ratio [15].

The light quality influenced on biochemical indexes in *in vitro* grown leaf-derived callus cultures of *Silybum marianum*. Superoxide dismutase activities were strongly promoted in red light condition while highest peroxidase activities were recorded for the dark grown cultures. The highest flavonolignan accumulation was recorded under red and green spectrum conditions [16].

4. Reported Phytochemical constituents

The first indentified component of the flavonolignan complex of milk thistle, silybin (synonyms: silybinin, silibinin) including silybin A and silybin B was isolated and established by Pelter [17, 18]; Silydianin (synonym: silidianin) [19]; Silychristin (synonym: silicristin) and later renamed as silychristin A [20, 21]; Isosilybin A and Isosilybin B [22]; Isosilychristin [23]; 3-deoxyflavonolignans, silandrin and silymonin [24]; Silyhermin, neosilyhermine A and neosilyhermine B [25]; 2,3-dehydrosilybin [26]; 2,3-cis-silybin A, 2,3-cis-silybin B and neusilychristin [27]; Isosilandrin A and isosilandrin B [28] (Samu *et al*., 2004); Silychristin B, a diastereomer of silychristin A [29]; Silyamadin [30]; Isosilybin C and Isosilybin D [31]. Mariamides A and B, 4-hydroxy-N-{4-[(E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enamido]butyl}benzamide, 4-hydroxy-N-{4-[3-(4-hydroxy-phenyl)-E-acryloylamino]-butyl}-benzamide, N,N-1,4-butanediylbis (4-hydroxy-benzamide), 4,4′-diphenylmethanebis (methyl) carbamates, taxifolin, dihydrokaempferol, dihydroquercetin-4′-methylether, naringenin, naringenin 7-O-β-D-glucopyranosid, kaempferol, glucosyl methyl ferulate, coniferin, 3 -methylcarboxymethyl-indole-1-N-β-D-glucopyranoside, and dehydrodiconiferyl alcohol-4-β-D-glucoside [32]; donepezil, rutin, quercetin and morin [33]; Taxifolin [34]; (1R, 7R, 10R, 11R)-12-hydroxyl anhuienosol; 2-hydroxymethyl-5-(2-hydroxypropan-2-yl)phenol; 2-(hydroxymethyl)-5-(2-hydroxypropan-2-yl)phenyl β-D-glucopyranoside, (R)-2-(3-hydroxyl-4-methylphenyl) propan-1-ol [35]; Chlorogenic acid and caffeic acid [36]. Chemical structures of some silymarins are showed in Figure 1.

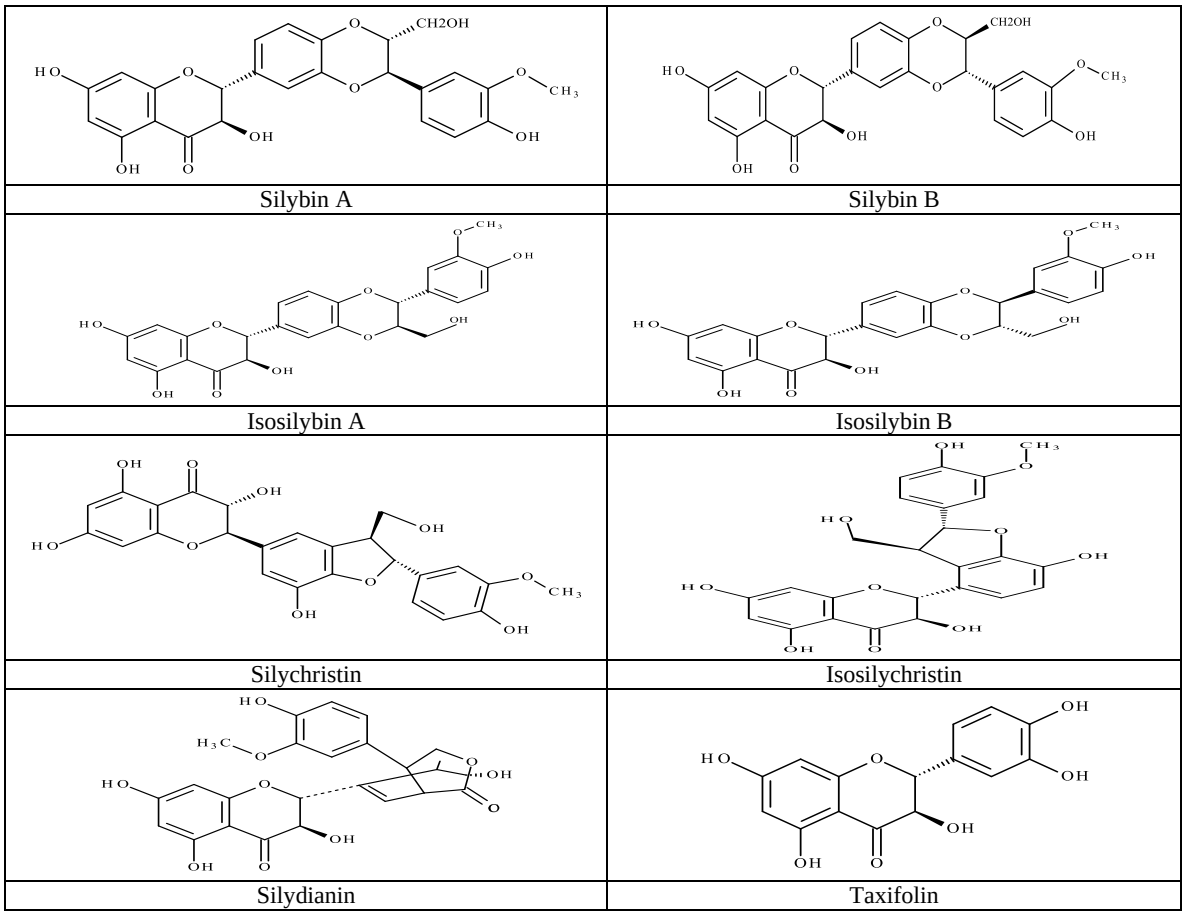

Fig 1: Chemical structures of several main silymarins in *Silybum marianum*

5. Extraction method reports

Some extraction methods use various solvents employed for isolation of selective bio-compounds. Each extract exhibits potent biological activities. Few studies on solvent extraction of the SM were reported.

6. Alcoholic extraction

The relationship between the silymarin content in tinctures and the alcohol strength was reported by Pendry [37]. Silymarin content could not be found or at low levels in tinctures extracted with 25 to 50% ethanol. Effective employed doses were detected only in tinctures with a concentration ratio herb to liquid 1:1 (kg/L) and an alcoholic content of 70%. To increase polarity, the alcoholic extract of the fruits was loaded onto a silica gel column and the column with benzene-ethyl acetate was established. Silymarin was isolated from the eluate by methanol precipitation, which gave 86% pure silybin (m.p. 167-180 ºC; reported values). However, which this method is uneconomical when used on an industrial scale due to the costly step of silica gel column chromatography [38].

Drying fruits by frozen method at -20 ºC for 24 hr allow to powdering easier. The major fatty oil portion was removed and defatted by extraction with hexane in a soxhlet extractor. Then, the defatted fruits were extracted with acetonitrile at 20-30 ºC. The crude silymarin fraction was stirred with cold dichloromethane at 5 ºC and filtered. The filtrate was dried with a slow purge of nitrogen gas. The silymarin was purified by suspension in acetonitrile and precipitation using water at 20 ºC. The pure silymarin was washed with distilled water and dried in a vacuum oven [39]. In another method, n-haxane or petroleum ether was used for defatting and defatted fruits were extracted by acetone. This way was regarded the most economic and least toxic solvent [40].

A substitute to the use of petroleum ether was recommended by Subramaniam *et al*., (2008) [41]. Pretreatment of the fruit meal with 1.5% H2SO4 (w/w) at 50 °C for 18 h and was extracted then with ethanol at 60 °C. All of the previous methods depend on a two-step phytochemical process for the preparation of silymarin from the crude fruit material.

7. Water extraction

Finally, hot water been advised as a green solvent used to extract silymarin from the SM [42]. Furthermore, water is a solvent with the benefits of low-purchase costs. The technical indexes can be controlled by adjustment of temperature and pressure. Interestingly, the defatting step was not required and the extraction time required decreases when increasing temperature. However, high temperatures could degrade silymarin content, which is a primary defect of the approach [43].

8. Advanced extraction method

An advanced method for the purification of silymarin from the fruits of the SM was reported. Determine of silymarin content in the pericarp extract was compared to that of the whole fruit extract using two orthogonal analytical methods. Silymarin content of the pericarp extract is higher (2.24-fold by HPLC and 2.12-fold by qHNMR) compared to acetone extract of whole fruits with hexane as a defatting solvent. Silumarin content of methanol pericarp extract is the highest and 2.72-fold higher than an acetone extract of whole fruits [44].

9. Pharmacological activity reports

9.1 Antioxidant activity

We read with great interest the recent article by Admah *et al*., (2013) [45]: "Evaluation of antioxidant activity and its association with plant development in *Silybum marianum* L." The authors only have evaluated DPPH-scavenging activity (DSA) of extracts from intact plants and leaves of the SM collected at different ages (10 to 100 days after germinating) and investigated effect of various radiation doses on DPPH-scavenging activity. The authors reported in articles abstract that maximum DSA was recorded in both leaves and intact plants in 80 days old plants (60 and 65.43 %), respectively, while the highest DSA value observed in table 1 of this text is 52.98 ± 03%. Additionally, authors did not conduct and performed any assays related to hepato-protection or anti-hepatotoxicity activity while these activities have complex mechanisms. Analyzing the antioxidant is only basic activity, which is not enough to evidence hepato-protective capacity but the authors reported in articles abstract: "this study suggested that not only the seeds but the whole plants of the SM can be used for the protection of live from toxins and infections", which is a mistakes and not relate to the study content.

The evidences of antioxidant capacity of compounds from the SM were developed by Lucini [36]. Authors reported that there was no correlation between the content of individual compounds, including silybin, and antioxidant potentials. Antioxidant capacity of constituents is higher compared to positive control in ABTS and FRAP assays. Taxifolin and dehydrodiconifery alcohol-4-β-D-glucoside exhibited the most DPPH inhibitory potent [32].

A recent paper by Nazir *et al*., (2018) [33], *in vitro* antioxidant effects of methanol extracts from SM were evaluated using DPPH and ABTS radical scavenging systems with IC50 values of 280 and 250 µg/mL, respectively. However, to assert the correct results, a detailed comment is as follows for return in method. The authors used "DPPH solution prepared by taking 24 mg in 100 mL of methanol and plant sample prepared in methanol in the concentration range of 1 mg/mL at various concentrations, then 0.1 mL of methanol extract were mixed with 3 mL of DPPH solution and incubated at 23 ºC for 30 min". We aimed to emphasize preparing of radical solution, which may be incorrect because the authors diluted concentration of DPPH solution being too high (24% w/v). We believe that this concentration is excess to make a suitable dilution and the chemical reaction may be not complete, hence it is difficult to interpret this results in the effect of methanol extracts on antioxidant capacity. Some typical studies as method references will help to clarify this opinion [46-50]. Moreover, in this assays authors used "plant sample" for determining DPPH radical inhibitory activity, while results displayed antioxidant effects of seeds. The technical terms are needed to use exactly to avoid reader confusions.

9.2 Inflammatory effects

Four compounds including (1R, 7R, 10R, 11R)-12-hydroxyl anhuienosol, 2-hydroxymethyl-5-(2-hydroxypropan-2-yl) phenol, 2-(hydroxymethyl)-5-(2-hydroxypropan-2-yl) phenyl and (R)- 2-(3-hydroxyl-4methylphenyl) propan-1-ol isolated from SM showed the potent NO inhibitory effects in murine microglial BV-2 cells model [35].

9.3 Antiviral activities

Silibinin from SM showed antiviral activiral activity against herpes simplex virus, type 2 (HSV-2) with IC50 value of 100 µg/mL and the therapeutic index of 3.8. This compound exhibited a more potent virucidal effect with an IC50 of 5 µg/ml and the therapeutic index of 76 [51].

9.4 Antidiabetic activities

In 2004, Maghrani *et al*., [52] reported the hypoglycaemic effect of the aqueous extract (AE) from the SM aerial parts on both normal and streptozotocin diabetic rat models. A significant decrease of blood glucose levels in both of models after administration of the AE with a single dose or 15 daily doses. Additionally, basal plasma insulin concentrations did not change after AE treatment in two *in vivo* models, which revealed that the SM extract exhibit effective hypoglycaemic and anti-hyperglycaemic activities in both of models, without affecting basal plasma insulin index.

In another study, also in streptozotocin (STZ)-induced type 1 diabete rats model, Silychristin A isolated from the SM significantly decreased the glucose level, increased insulin secretion, and improved the structure of b cells in tested rats. This compound significantly suppressed STZ or high concentration of glucose induced cell apoptosis and effectively inhibited α-glucosidase [53].

A clinical trial therapy investigation on type 1 diabetic mellitus (T1DM) patients by randomly taking placebo or baseline or *B. aristata/S. marianum* complex capsules (berberol) was conducted. Glycated hemoglobin index decreased with berberol compared to baseline, but not to placebo. Indexes of fasting plasma glucose and postprandial glucose also decreased with berberol compared with controls. Likewise, there was a decrease of total cholesterol, triglycerides, and LDL-cholesterol with berberol. This evidences that the supplementation of berberol to insulin therapy in patients with T1DM leads to a reduction of the insulin dose necessary to have an adequate glycemic control [54].

The silymarin administration did not induced side effects for type-2 diabetes mellitus (T2DM) patients. There were increases in superoxide dismutase, glutathione peroxidase activity and high-sensitivity C-reactive protein levels compared to patients taking the placebo. The silymarin supplementation significantly decreased malondialdehyde concentration compared to the baseline group [55]. Silymarin supplementation may improve the glycemic indices and lipid profiles of T2DM patients [56].

9.5 Anti-amnesia effects

The methanol extraction of the SM seeds exhibited a concentration dependent inhibition of acetylcholinesterase and buteryl cholinesterase with IC50 values of 110 and 130 μg/mL, respectively and also displayed anti-amnesia capacity in amnesia animal model induced by scopolamine. Additionally, querctin, rutin and morin were used to conduct induced fit docking and IFD score of all compounds were consistent with their experimental acetylcholinesterase inhibitory activities. However, these compounds are not main constituents of the SM seeds. We suggest that measuring and evaluating acetylcholinesterase inhibitory activity of flavonolignans are necessary in further research to give insight knowledge [33].

9.6 Cardio-protection

In a research with entitled "Silybum marianum provides cardioprotection and limits adverse remodeling post-myocardial infarction by miligating oxidative stress and reactive fibrosis" [57]. Authors have evaluated the effect of the SM administration on the acute phase of myocardial infarction, in remodeling period post-myocarial infarction, and in a non-infarcted heart by analyzing the antioxidant and anti-fibrotic properties in a pig trial model. The performing process and results in study had been discussed and explained clearly. However, choosing the most accurate and reliable oxidative stress markers to repress the correct results was accentuated in this argument. Fistly, measuring oxidant levels and evaluating antioxidant activities of molecules are impractical and their oxidant and antioxidant effects are additive. When only a few parameters are measured, their levels may be decreased or stable, even when the actual oxidant status in increased, or vice versa [58-60]. Secondly, the authors only used 8- hydroxyguanosine marker, dihydroethidium and xanthine oxidase to only analyze immunohistorchemical staining by RT-PCR, which cannot evidence the total oxidative status and it may be difficult to clarify relation between these results and effect of the SM on oxidative stress. Therefore, analyzing the total antioxidant oxidant status levels was recommended to record the best results helping demonstrated cardioprotection potential of the SM administration.

9.7 Hepatic protection

Ethyl acetate and ethanol seed extracts at concentration of 100 mg/kg BW were tested against liver demage induced by carbon tetrachloride (2 ml/kg bw) and compared with standard hepatic drug hepaticum at the same dose for 10 days. Ethanol extract exhibited the most significantly decrease in the liver enzymes and ethyl acetate showed the most increase for glutathione level and the risk factor HDL/LDL significantly. However, we suggest that a full compositional analysis to identify the active compounds in each extract is necessary to assert more reliable results [61].

Ethanol extract of the SM was evaluated on experimental nonalcoholic steatohepatitis (NASH) N-Mary rats induced by methionine and choline deficient (MCD) diet. Administration of the SM extract has abated the severity of nonalcoholic steatohepatitis among the MCD-fed rats. The alanine anmino transferase and aspartate amino transferase levels significantly reduced. Additionally, the elevated hepatic TNF-α and TGF-α mRNA and melondialdehyde levels dramatically decreased along with an increase in the glutathione. Lowing activation of procaspase-3 to active casepase-3 in the extract treatments also was observed [62]. Likewise, results in the recent study of Zhu [63] are agreement with this report. The results indicated that the SM oil could play a certain protective role against nonalcoholic fatty liver disease, and the protective effects might be associated with attenuating lipid accumulation, oxidative stress and inflammation, improving lipid metabolism.

The inhibitory capacity of individual compounds isolated from SM on CYP2C8 enzyme activity in human liver microsomes was evaluated. Isosilibinin exhibited the most potent inhibitor on CYP2C8 enzyme activity with IC50 1.64±0.66 mg/ml compared with another compounds. However, the observed IC50 values are unlikely to be achieved in humans orally administered with milk thistle extracts [64]. The effective hepatic-protective activities via enzyme kinetics of cytochrome-P450 isoenzymes in primary human hepatocytes and human liver microsomes also were reported by Doehmer [65].

9.8 Wilson's disease

The fact is that copper overload causes structural, biochemical and biophysical mitochondrial deficits in Wilson disease patients and related animal models. A recommendation which the application of milk thistle products with a high copper content should be limited for Wilson disease patients was

given [66]. However, we argue that determining a most suitable limited dose is necessary.

9.9 Obsessive-Compulsive Disorder

The efficacy of the SM in the treatment of obsessive – compulsive disorder was reported by [67]. A double-blind and randomized trial on thirty five adult outpatients randomly assigned to take either capsule of the extract or fluoxetine for 8 weeks (600 and 30 mg/day), respectively was conducted. There was no significant different between the herbal medication and fluoxetine in the treatment of obsessive – compulsive disorder. The efficacy of the SM is similar to fluoxetine on these symptoms, however, comparing to fluoxetine, the medicinal effects of extract capsules were recorded up one week late.

9.10. Sepsis and Burn prevents

The preventing sepsis induced by cecal ligation and perforation on acute lung and brain injury was evaluated. The TNF-α, IL-1β, IL-6; malondialdehyde levels and myeloperoxidase activity were increased while lactate dehydrogenase activity and tissue glutathione level were reduced in both the lung and brain tissues. Moreover, the presence of the oxidative damage was observed by increasing luminal and lucigenin chemiluminescence. Sepsisinduced remote organ injury was reduced by the treatment of silymarin, which may be due to its capacity of oxidant–antioxidant status balance, neutrophil infiltration inhibition and regulation of the release of inflammatory mediators [68].

The protective capacity against burn induced oxidative skin injury was reported by Toklu [69]. TNF-α and lactate dehydrogenase, malondialdehyde levels and myeloperoxide activity increased while glutathione levels and luminal-lucigenin chemiluminescense significantly decreased in the rats skin after burning for 48 hr. The silymarin merits consideration as a therapeutic agent in the treatment of burns.

10. Application on veterinary

Silybum marianum dietary supplementation reduced the mortality rate in growing rabbits under oxidative stress, thus being a promising natural feed additive in improving the sanitary status of a commercial rabbit farm. The application of the feed containing with the SM changed the sensory characteristics of rabbit loin [70].

In another study, results revealed that a mild effect on the growth performance of rabbits was recorded, but no effect on the majority of selected blood biochemical indexes and markers of oxidative stress after the dietary supplementation containing the SM constituents. However, when in one repetition a higher incidence of health problems connected with digestive disorders occurred, employing the diet with the highest content of the SM constituents was able to attenuate the morbidity and mortality of rabbits [71].

11. Conclusions

Silybum marianum (SM) has attracted substantial attention due to its outstanding benefits. Pharmacological studies on flavonolignans have been performed *in vitro* and also developed *in vivo* in animal models and human trials**.** Though several pharmacological mechanisms related to biological activity have already been explained, the comprehensive pharmacological mechanisms of the SM need to be elucidated. Based on phytochemical and pharmacological research, the silymarins responsible for the good anti-diabetic, anti-amnesia, hepatic-protective activities were selected as chemical markers to evaluate the quality of the SM and its products. However, pharmacokinetics studies on the main components, especially the bioactive components are still largely lacking, therefore firm evidence for further clinical application is necessary in order to assess the therapeutic potential of the SM and its pharmaceutical commodities such as cardio-protection activity.

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