

Silybum marianum: not just Silymarin and Flavonolignans

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Abstract: The use of *Silybum marianum* L. for therapeutic purposes has been known since ancient times. Its phytocomplex reduces transaminases and other biohumoral indices in the course of liver disease and also in hepato-renal syndrome. In particular, the flavonolignan component has shown properties that would partially explain the ability of the phytocomplex to induce a certain regeneration of liver cells, stimulate the cellular elimination of toxins and reduce the inflammatory component, present in fatty, alcoholic and hormonal therapies with steroids. *S. marianum* is also successfully used in the treatment of patients with symptomatic chronic hepatitis, with complete disappearance of clinical symptoms, such as asthenia, loss of appetite, severe meteorism, dyspepsia, and with normalization of transaminases. The same results can be obtained in patients undergoing heavy chemotherapy cycles. Modern herbal medicine uses it in decoction or infusion, however with some caution in patients suffering from hypertension, due to the presence of tyramine. In addition, the extracts of the roots have antioxidant, diuretic and febrifugal properties and those of the leaves have aperitif properties. It is therefore interesting to provide a picture of the different non-flavonolignanic components (terpenes, steroids and essential oils) of the plant and their properties, which have perhaps been wrongly neglected over the past few years.

Keywords: *Silybum marianum*; natural substances; silibinin; terpenes; steroids; biological activities. © 2021 ACG Publications. All rights reserved.

1. Introduction

Plants can provide bioactive compounds, generally secondary metabolites or even simple waste products of their metabolism [1]. In this regard, the study of phytotoxic activity of natural substances

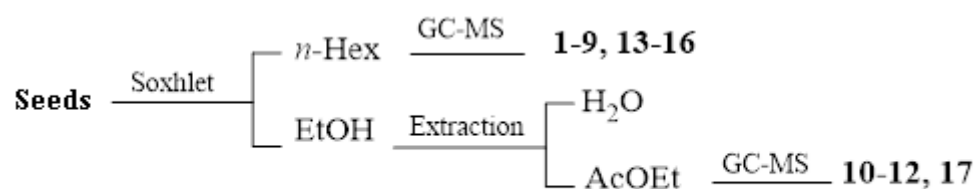
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plays an important role in the research of new eco-compatible active compounds to limit the proliferation of weeds, with undoubted advantages related to the identification of new chemical structures [2,3] that usually are quite difficult or impossible to obtain by synthesis [4–6]. Many reports highlighted as the study of substances from natural origin can facilitate the design for the synthesis of new molecules with phytotoxic activity [7–9], and increase the discovery of substances with sites and mechanisms of action different from those of synthetic herbicides aiming at a greater environmental safety [10–14]. But, during the last years, the use of natural products, pure or in mixture, has increased for food, voluptuous, aesthetic or therapeutic purposes, too [15]. In the continuous scientific research of new safe and effective drugs, there has been a rediscovery of natural substances as a potential reservoir of innovative therapeutic solutions for human health with the prospect of integrating and sometimes replacing conventional drugs [16,17]. Milk thistle is a wild herbaceous plant typical of the Mediterranean regions where it grows spontaneously in almost all climates and latitudes adapting to any type of soil. Usually, this plant is not the object of massive cultivation and can be a pest, only in few areas where the thistle is cultivated. The seeds are collected and subjected to a mechanical shaking action to encourage the distribution and growth of new plants. The plant is made up of a rose of thorny leaves. The flowers, which reach maturity in summer, are red-purple, while the fruits are oblong achenes of bright black color, stained with yellow. In practice, these are dried fruits that contain only one seed. The milk thistle belongs to the Plantae kingdom, of the *Asterales* order belonging to the *Asteraceae* family. The genus is the *Silybum* and the species is called *marianum*. It was first classified by Carl von Linné. In a second time, the milk thistle was reclassified by the German botanist Joseph Gaertner in his main work "*De Fructibus et Seminibus Plantarum*". In botany, the milk thistle has many synonyms: *Carduus marianus*, *Centaurea dalmatica*, *Silybum mariae*, *Silybum marianum albiflorum*, *Silybum pygmaeum*, *Silybum maculatum*, *Silybum marianum longispina*, *Silybum leucanthum* and *Mariana lactea*. The history of the milk thistle has very ancient origins, even the ancient Greeks knew its properties, in fact they used it in mixture with honey as a natural remedy for cough treatment. Its use has continued over the centuries widening also for the cure of other diseases related to the respiratory tract. In the Middle Ages, cultivation began mainly within monasteries and from this point on, many specific studies followed. Already in the mid-1700s the beneficial properties of the milk thistle against the liver were documented by the Swiss scientist Albrecht Von Haller, and subsequently were confirmed by medical scholars who extended the beneficial properties also to the spleen and the bile duct. The fruits produced an extract named silymarin that consist of a set of flavolignans concentrated in the outer protein layer of the fruit [18]. The main components of silymarin are silibinin, isosilybins, silicristin and silidianin [19]. Hepatoprotective action and efficacy in the treatment of degenerative liver diseases, such as cirrhosis, hepatitis C or liver disease caused by alcohol abuse or cardio drugs, has been confirmed by numerous scientific studies. The hepatoprotective activity is mainly due to the ability of silymarin as inhibitor of fat peroxidation, as regulator of cellular permeability and, more generally, as inhibitor of the formation of metabolites responsible for hepatic damage [20]. Of the flavonolignans present in silymarin to date the attention has been focused mostly on silibinin, the most abundant component that consists of a mixture of diastereoisomers silybin A and B, which are very difficult to be separated [21]. However, the use of silibinin is limited by its low solubility in water (0.4 mg/L) [22] that involves also in a low bioavailability [23,24]. Several research groups are involved in solving these problems using a pro-drug approach with the insertion of phosphate groups that considerably increases the water solubility of drug [25–27] or through the use of liposomes [28]. In addition to silymarin, the fruit contains other active substances with a protective action such as flavonoids, antioxidants, proteins, oleic, and linolenic acids, but above all sterols. Plant sterols, essential fatty acids and flavonoids play an effective protective action against cardiovascular diseases [29]. The protective and antioxidant effect is clearly enhanced by silibinin, which prevents fat oxidation and damage to cell membranes. Recent scientific research has confirmed that the silibinin could also be effective as a protector against some types of cancer, cardiovascular or renal diseases and Alzheimer disease [30,31]. Today it has been discovered that milk thistle also benefits many other organs, strengthens the capillaries, counteracts the action of free radicals, regulates blood pressure, and promotes intestinal regularity [32]. The plant is a vital remedy in case of mushroom poisoning, it is also recognized for its tonic and decongestant properties that can benefit people suffering from depression, food allergies and chronic fatigue problems, and is used in patients who are subjected to chemotherapy regarding to the purifying and detoxifying properties of its active principles. As a

natural remedy, milk thistle can be found in pharmacies, herbal medicine and organic stores in various formulations, including mother tincture, capsules, powder, liquid extracts, and decoction obtained from the extracts of the dry plant. The milk thistle is a well-tolerated herbal medicine and up to now it has no contra-indications. Adverse effects are rare, of mild intensity and of a gastrointestinal type. The majority of recently published reviews on the milk thistle mainly reported the chemistry and pharmacology studies on the silymarin extract. Conversely, this work aims to examine in depth the chemical composition of milk thistle keeping out the silymarin extract and reporting indeed the isolation and identification of the less abundant components of plant as terpenes and steroids with relative biological activities.

2. Extraction and Isolation of Mono and Sesquiterpenes

Recent papers have reported the abundance in terpenes of the seeds of milk thistle, and have highlighted their role as natural preservatives which can replace synthetic additives in a myriad of food products and not, thus preserving both health and environment [33,34]. In particular, the seeds were extracted with a Soxhlet extractor using *n*-hexane and then ethanol, for 6 hours (Scheme 1).



Scheme 1. Fractionation scheme of Soxhlet seed extracts

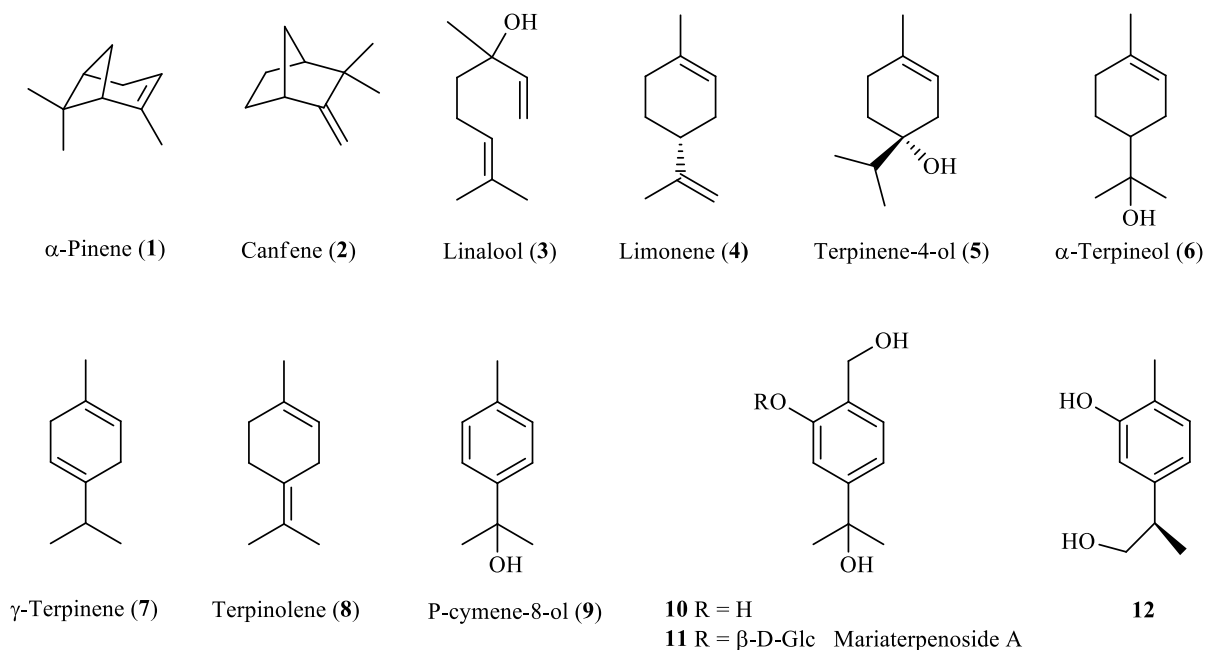


Figure 1. Monoterpenes isolated from Soxhlet seed extracts

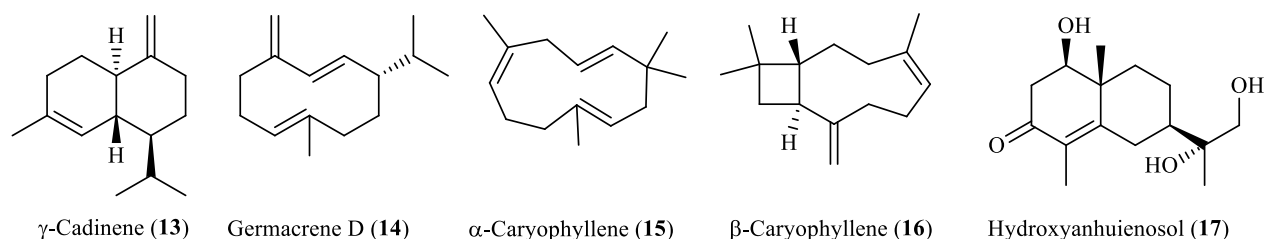


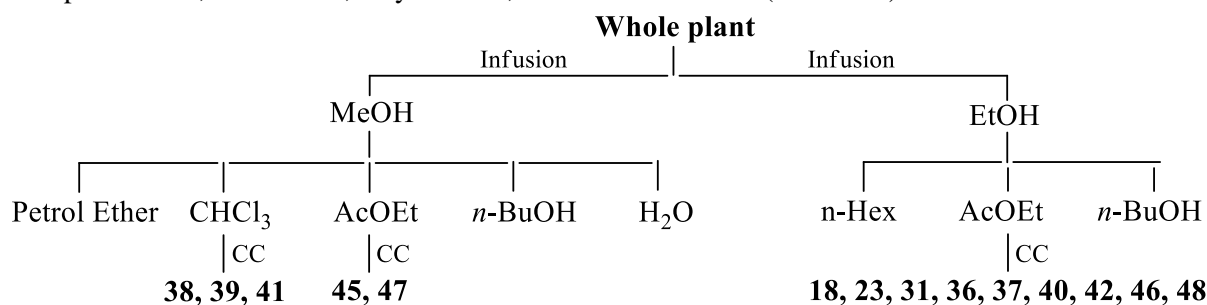
Figure 2. Sesquiterpenes isolated from Soxhlet seed extracts

All analyses were carried out by Gas Chromatography-Mass Spectrometry (GC-MS), the extract with *n*-hexane showed the presence of monoterpenes **1–9** (Figure 1) and sesquiterpenes **13–16** (Figure 2), together in amounts equal to 0.12% of the dry weight of the plant [33].

Sesquiterpenes **13–16** constitute 55% of the extract and compounds **13** and **15** are the most abundant, with percentages of about 50 and 4.5%, respectively. The monoterpenes are less abundant (39%) and between them hydrocarbons **1**, **2**, **4**, **7** and **8** are slightly more than 33%, in particular compound **1** with a percentage of 24.5%, while the oxygenated **3**, **5**, **6** and **9** are about 6%. Other components have been highlighted but not identified. The ethyl acetate fraction of ethanol extract contained the monoterpenes **10–12** (Figure 1) and sesquiterpene **17** (Figure 2), that were identified by comparison with reference compounds [34].

3. Steroids and Triterpenes

Regarding the determination of steroids and triterpenes, compounds **18**, **23**, **31**, **36–42** and **45–48** were isolated from the alcoholic extracts, obtained by infusion, of the whole plant (Figure 3a and 3b; Table 1). In particular, the methanolic extract was concentrated under reduced pressure to obtain a rubbery material which was then freeze-dried. The dried crude material was extracted in succession with petrol ether, chloroform, ethyl acetate, *n*-butanol and water (Scheme 2).



Scheme 2. Fractionation scheme of alcoholic extracts of whole plant

The chloroform extract was subjected to the Salkowski test which highlighted the presence of terpenes [3,35]. From this extract the compounds **38**, **39** and **41** were isolated and identified, interestingly these compounds represent the first lanostane-type triterpenes isolated from milk thistle [36]. The compounds silymin B (**45**) and silymin A (**47**) were isolated from the ethyl acetate extract, that gave positive Liebermann-Burchard test (acetic anhydride-conc. H₂SO₄) with the formation of a blue-green colour which highlighted the presence of triterpenes [37]. The ethanolic extract obtained by infusion was concentrated under reduced pressure to obtain a rubbery material which was then freeze-dried. The dried material was extracted in succession with *n*-hexane, ethyl acetate and *n*-butanol. From

the soluble fraction of ethyl acetate, the compounds **18**, **23**, **31**, **36**, **37**, **40**, **42**, **46**, and **48** [38] were isolated.

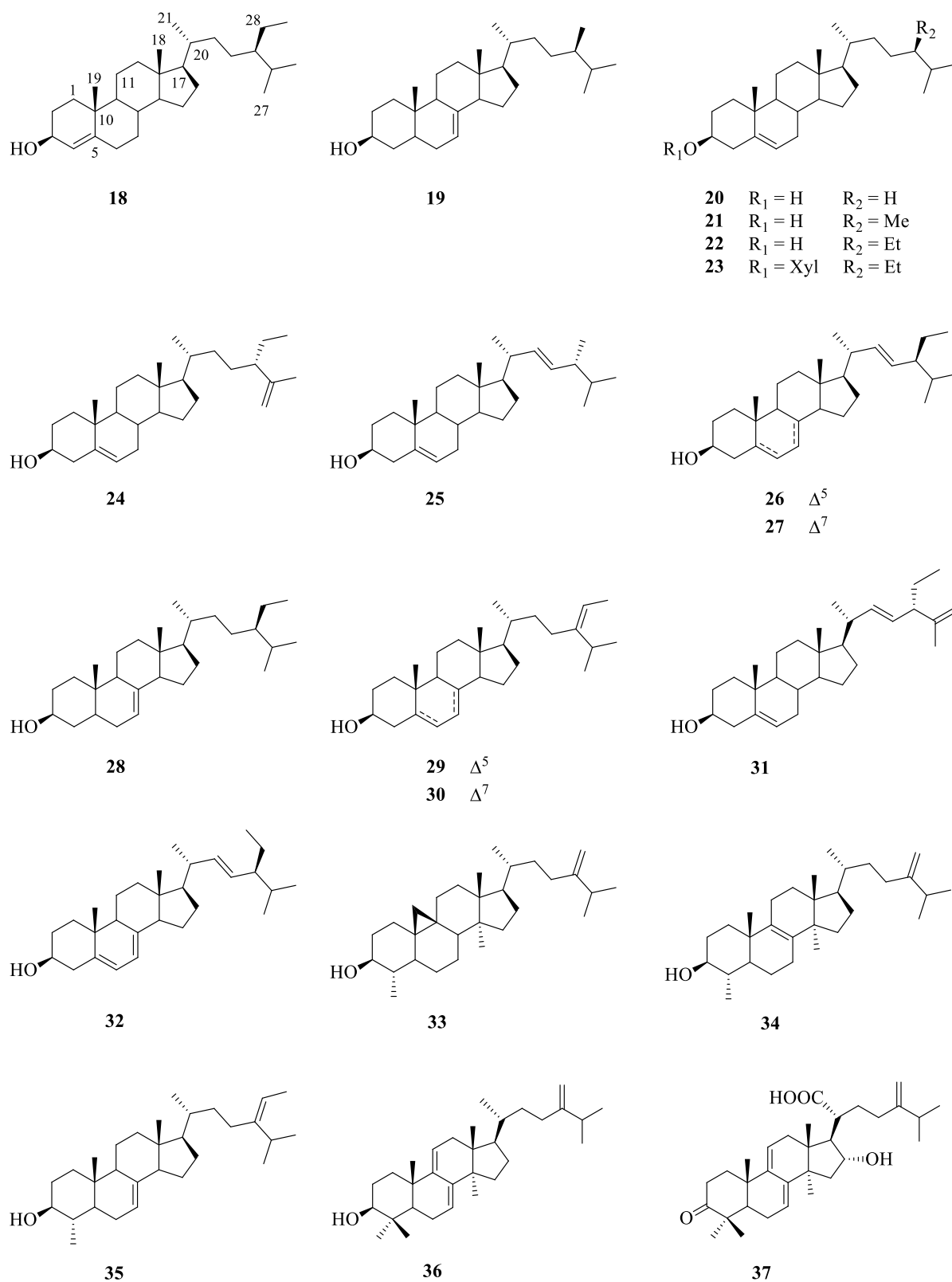


Figure 3a. Steroids **18-37** isolated by milk thistle

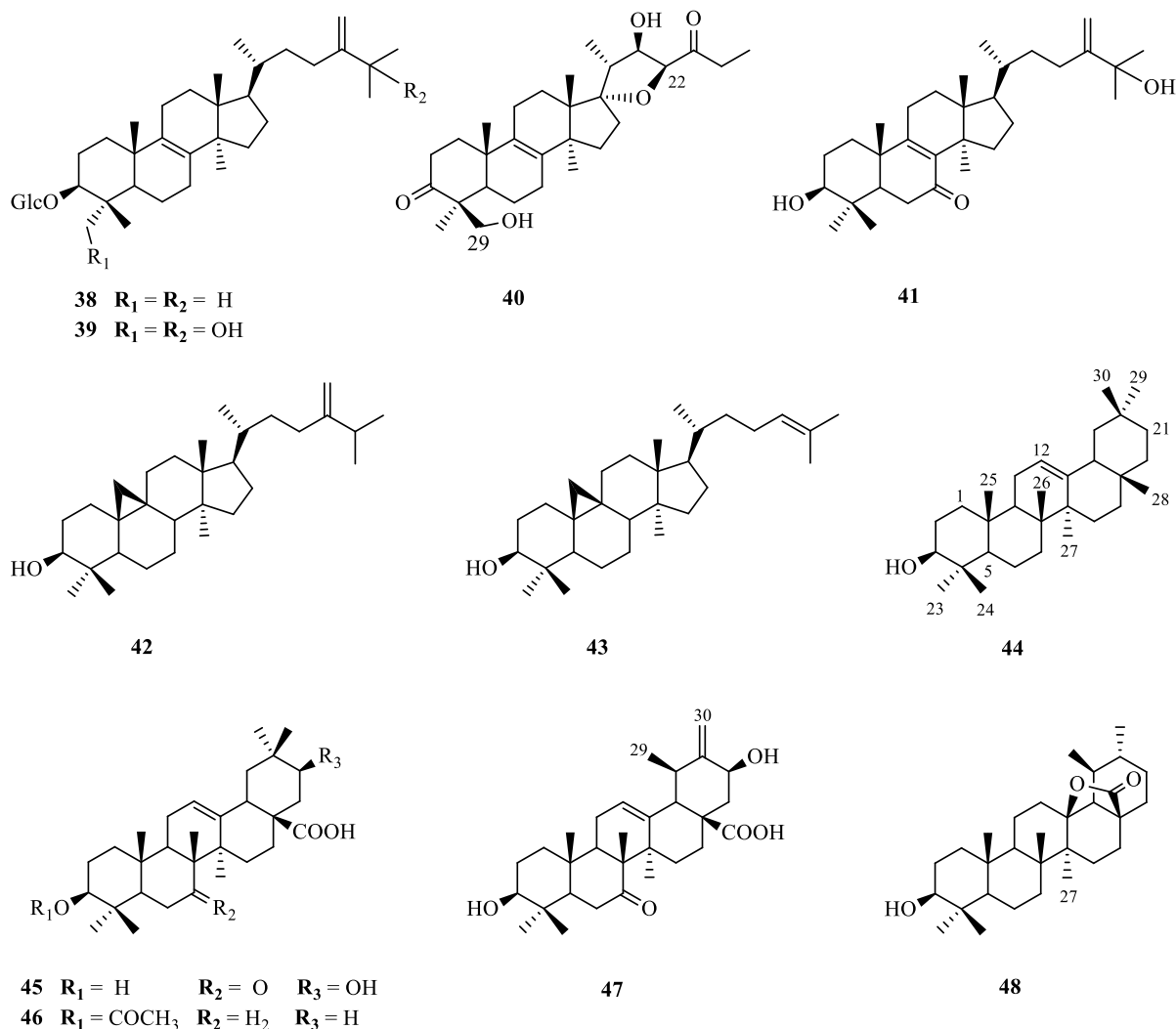
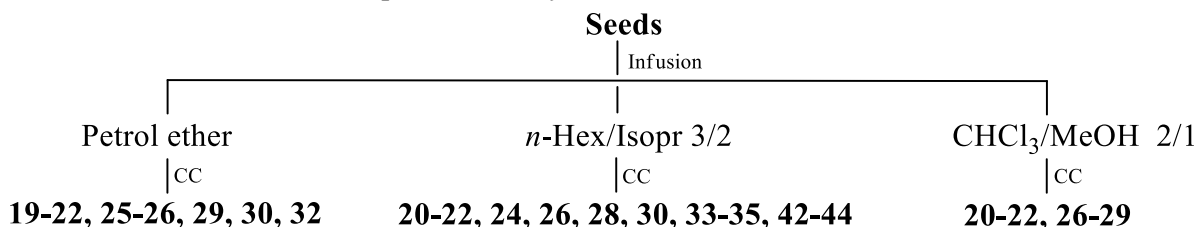


Figure 3b. Steroids **38–48** isolated milk thistle

Compounds **19–22**, **24–30**, **32–35**, and **43–44** were isolated from the seeds of *S. marianum* particularly rich in steroids ((Figures 3a and 3b; Table 1). From the Soxhlet extract with *n*-hexane, just over 6.2 g/kg, desmethylsterols **20–22**, **26** and, **28–30** [39,40] were isolated and identified by GC-MS analyses. The most abundant was β -sitosterol **22**, up to 27%, an interesting fact considering that this results in a decrease in the absorption of dietary cholesterol [41].



Scheme 3. Fractionation scheme of infusions by seeds.

Compounds **19–22**, **25–26**, **29–30**, and **32** were isolated from the infusion obtained with petrol ether (Scheme 3) [42]. From the infusion with *n*-hexane/isopropanol 3:2, slightly more than 2.3 g/kg has been obtained after saponification and its components have been identified by GC-MS analysis [43]. In particular, it has been isolated and identified compound **24**, some 4-monomethylsterols **33–35**, and among these the most abundant was the compound **35**, in addition to 4,4'-dimethylsterols **42–44**,

of which the **42** is the most abundant [44]. Finally, the compounds **20–22** and **26–29** were isolated from the infusion obtained with CHCl₃/MeOH 2:1 [45].

Table 1. Steroids isolated from *S. marianum*.

No.	Common name	Systematic name	CAS Number
18	β-Rosasterol	Stigmast-4-en-3-β-ol	134876-41-8
19	Δ ⁷ -Campesterol	3β,24R-Ergost-7-en-3-ol	70095-94-2
20	Cholesterol	Δ ⁵ Cholesten-3β-ol	57-88-5
21	Campesterol	24R-Methylcholest-5-en-3β-ol	474-62-4
22	β-Sitosterol	24R-Ethylcholest-5-en-3β-ol	83-46-5
23	β-Sitosterol 3-O-β-D-xylopyranoside	3β-Stigmast-5-en-3-yl β-D-xylopyranoside	93772-33-9
24	Cleroesterol	Stigmasta-5,25-dien-3β-ol	2364-23-0
25	Brassicasterol	Ergosta-5,22-dien-3β-ol	474-67-9
26	Stigmasterol	Stigmasta-5,22-dien-3β-ol	83-48-7
27	Spinasterol	5α-Stigmasta-7,22-dien-3β-ol	481-18-5
28	Δ ⁷ -Stigmastenol	Stigmast-7-en-3β-ol	6869-99-4
29	Δ ⁵ -Avenasterol/ Isofucosterol	3β-Stigmasta-5,24(28)-dien-3-ol/ 3β,24Z-Stigmasta-5,24(28)-dien-3-ol	18472-36-1/ 481-14-1
30	Δ ⁷ -Avenasterol	3β,5α,24Z-Stigmasta-7,24(28)-dien-3-ol	23290-26-8
31	22-Dehydrocleroesterol	24S-24 Ethyl cholesta-5,22E,25-triene-3β-ol	26315-07-1
32	Δ ⁷ -Stigmasterol	Stigmasta-5,7,22-trien-3β-ol	481-19-6
33	Cycloeucalenol	4β-Demethyl-24-methylenecycloartanol	469-39-6
34	Obtusifoliol	3β,4α,5α-4,14-Dimethylergosta-8,24(28)-dien-3-ol	16910-32-0
35	Citrostadienol	3β,4α,5α,24Z-4-Methylstigmasta-7,24(28)-dien-3-ol	474-40-8
36	24-Methyleneagnosterol	24-Methylenelanosta-7,9(11)-diene-3-β-ol	35624-74-9
37	Polyporenic acid C	Lanosta-7,9(11)-dien-21-oic acid, 16α-hydroxy-24-methylene-3-oxo	465-18-9
38	Marianoside B	(3β)-24-Methylidenelanost-8-en-3-yl β-D-glucopyranoside	886437-21-4
39	Marianoside A	24-Methylene-lanoesta-89-ene-25,28-diol 3-O-β-D-glucopyranoside	886437-20-3
40	-	22R,23S-17α,23-Epoxy-22,29-dihydroxy-27-norlanost-8-en-3,24-dione	713124-66-4
41	Marianine	(3β)-3,25-Dihydroxy-24-methylene-lanost-8-en-7-one	886437-19-0
42	24-Methylencycloartanol	3β-24-Methylene-9,19-cyclolanostan-3-ol	1449-09-8
43	Cycloartenol	Cycloart-24-en-3β-ol	469-38-5
44	β-Amyrin	Olean-12-en-3β-ol	559-70-6
46	Oleanolic acid 3-acetate	3β-3-Acetyloxyolean-12-en-28-oic acid	4339-72-4
45	Silymin B	3β,21β-3,21-Dihydroxy-7-oxo-olean-12-en-28-oic acid	926307-53-1
47	Silymin A	3β,21β-3,21-Dihydroxy-7-oxo-ursa-12,20(30)-dien-28-oic acid	926307-51-9
48	-	3β-Hydroursan-13β(28)-olide	29428-70-4

3.1. Biological Effects of Terpenes and Steroids Isolated by *S. marianum*

Only a few sesquiterpenes isolated from the milk thistle were tested to evaluate some biological properties. In particular, it has been assessed the ability to inhibit the production of LPS-induced nitric oxide NO in murine microglial cells BV-2 of compounds **10-12** and **17** [34]. The results showed that

the IC₅₀ (7, 15, 14, 18 μM respectively) were slightly higher than the value of the 2-methylthiopseudourea sulfate used as a reference; even more interesting, these compounds did not show significant cytotoxicity by MTT test at the concentrations used. Moreover, studies of molecular docking showed their strong interaction with the inducible nitric oxide synthase, responsible for the synthesis of NO during inflammatory processes that plays an important role in degenerative processes contributing to the neuronal death [34].

The terpenoids **18**, **23**, **31**, **36**, **40**, **42**, **46** and **48** were tested for the determination of antifungal activity on *Candida glabrata*, *Candida albicans*, *Microsporum canis*, *Fusarium solani*, *Trichophyton longifusus* and *Aspergillus flavus*, using Miconazole (**M**) and Amphotericin B (**AB**) as a reference standard [38] (Table 2).

Table 2. Antifungal activity of compounds **18**, **23**, **31**, **36**, **40**, **42**, **46** and **48** in agar tube dilution method

Fungal species	18	23	31	36	37	40	42	46	48	M	AB	Ref.
<i>T. longifusus</i>	+	-	+	++	++	++	-	-	++	70		
<i>C. glabrata</i>	++	-	-	++	++	++	-	-	++	110.8		
<i>F. solani</i>	++	-	+	+	++	+	-	-	-	73.25		38
<i>C. albicans</i>	-	-	+	++	+	+	-	-	+	110		
<i>M. canis</i>	-	-	+	+	+	-	-	-	-	98.4		
<i>A. flavus</i>	++	-	-	-	+	-	-	-	-		20	

- Values of inhibition of radial growth <50%; + values of inhibition of radial growth >50 and <74%; ++ values of inhibition of radial growth >75%. Miconazole (**M**) and Amphotericin B (**AB**)

Compounds **23**, **42** and **46** are very poorly active on fungal species, while the other ones are moderately active, especially compounds **48**, **40**, **18**, **36** and **37** on *T. longifusus* and *C. glabrata*. Hepatitis C virus HCV infection is one of the primary causes of chronic hepatitis, cirrhosis and hepatic carcinoma. In the NS protein group, the Chymotrypsin like serine protease known as NS3 appears to be the most promising target for new antiviral drugs. Compounds **38**, **39** and **41** were tested *in vitro* to evaluate their ability to inhibit Chymotrypsin, by spectrophotometric test using as substrate *N*-succinyl-phenylalanine-*p*-nitroanilide and monitoring the reaction at 410 nm with Chymostatin used as a reference. In general, proteases such as chymotrypsin have a fundamental role in the defense against pathogens and can cause damage to the digestive tract also fatal of certain insects. The compounds tested are able to inhibit the hydrolysis of the Chymotrypsin by 50% at concentrations ranging from 9.4 to 28.2 μM. Compound **41** was the most active of the three, with an IC₅₀ value almost equal to that of Chymostatin used as a reference. The results confirm the preliminary tests on the methanolic extract that showed a strong toxicity in the brine shrimp lethality and, in particular, the chloroform-soluble fraction from which the compounds **38**, **39** and **41** were isolated, showed a significant inhibitory activity against the enzyme chymotrypsin [37].

4. Conclusions

The beneficial properties of milk thistle in the treatment of liver disorders have long been known by folk medicine, which uses fruits and leaves of the plant. In fact, over the last 30 years a huge number of articles have been published focusing on silymarin, a complex mixture of flavonolignans, and on its individual components, highlighting their activities and limits. The milk thistle is also used as an antidote for poisoning with mushrooms of the genus *Amanita* and homeopathic medicine uses it in case of jaundice, biliary colic, hepatitis, stomach ache, peptic ulcers and venous circulatory insufficiency. However, there is not a general framework on the other classes of compounds isolated from this plant, primarily terpenoids and steroids, of which an insufficient and incomplete picture of biological activities has been defined. This work examines in depth the potentialities of milk thistle, reporting the identified terpenes and steroids extracted by the plant and their biological activities. Even if there are very few data performed determining the fungicidal and larvicidal efficacy of terpenoids, it is well known that essential oils exert an antimicrobial action and/or antifungal, at least to certain

species. Concerning terpenes isolated from milk thistle, it has been reported that the triterpenes, present in essential oils act as antifungals up to concentrations of 5-25 ppm. So, the mixture of terpenoids and steroids and their possible synergistic effects could delineate interesting applications in the food, nutraceutical, and even pharmacological fields.

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Author Contributions

All authors contributed to the draft of the article and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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