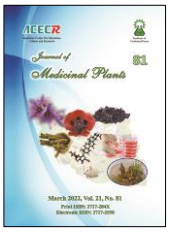
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Research Article

Purification, identification, and standardization of silybin A & B composition from *Silybum marianum* (L.) Gaertn.

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ABSTRACT

Background: *Silybum marianum* (L.) Gaertn. (Milk thistle) is a perennial herb with medicinal properties. The seeds of these plants contain silymarin compounds with flavonolignan structure and antioxidant, anti-inflammatory and hepatoprotective effects. The major bioactive constituent of *S. marianum* is silybin A and B. It is used in the treatment of various liver conditions and exhibits high anti-tumor promoting activity. **Objective:** The purpose of this study was to purify, identify, and standardize of silybin A and B from the seeds extract of *Silybum marianum*. **Methods:** At first, the milk thistle seeds were defatted with hexane and then extracted with methanol as solvent. Isolation and further purification of silybin A and B was carried out by column chromatography using Diaion HP-20 resin, silica gel and Sephadex LH-20 as stationary phase, respectively. ^{1}H-NMR and ^{13}C-NMR techniques were used to identify these compounds. Finally, the HPLC method has been used to standardize. **Results:** ^{1}H-NMR and ^{13}C-NMR techniques characterized the structure of silybin A and B extracted from *Silybum marianum* L. and standardization and determination of their purity was performed using HPLC. **Conclusion:** Our proposed system presented significant advantages in increasing efficiency and reducing cost, and the diastereoisomers of silybin A and silybin B in silymarin were successfully isolated with high purities.

1. Introduction

Silybum marianum (L.) Gaertn (milk thistle) is a medicinal plant that has been used for thousands of years as a remedy for various ailments [1]. The milk thistle is an annual or biannual plant from the Asteraceae family, flowering in July-August with reddish-purple flowers. Milk thistle grows in warm and dry climates [2]. Native habitats of milk thistle include southern Europe, southern Russia, Asia Minor and North Africa, and it is adapted to

Abbreviations: ^{1}H-NMR, Proton Nuclear Magnetic Resonance; ^{13}C-NMR, Carbon Nuclear Magnetic Resonance; HPLC, High-Performance Liquid Chromatography

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North and South America and South Australia [3]. The leaves have milky-white veins, which inspired its scientific name of *Silybum marianum.*

Silibinin (silybin) is composed of two diastereomeric compounds (silybin A and silybin B) in a 1:1 ratio. Silybin has been shown to have iron-chelating properties that produce hepatoprotective effects [4-6]. Silymarin, the extract of milk thistle, includes several flavonoglignans (silibinin, isosilibinin, silychristin, isosilychristin and silydianin). Among these, silybin is a major compound and has the most important biological effect. It also makes up about 70% of all silymarin compounds in the form of two diastereoisomeric compounds [7].

Silybin acts by turning off proinflammatory signals derived from NF-κB activation, which involves induction of cytokine syntheses such as TNF-α and interleukins (IL-1, IL-6) granulocyte-macrophage colony-stimulating factor (GM-CSF) [8]. In rheumatoid arthritis, silymarin extract is an anti-inflammatory agent that inhibits migration and neutrophil activation in articulations. Silymarin also acts as an estrogen modulator, insulin sensitizer, anticarcinogen, and antidiabetic agent [9]. The antioxidant activity has been from silibinin derivatives by scavenging free radicals and inhibiting lipid peroxidation both *in vitro* and *in vivo* [10]. Scientific evidence shows that silybin acts through interaction with various tissues. The action of silybin in modulating inflammation and apoptosis, along with its antioxidant power, has led to its use in various pathologies [7].

In addition, silybin induces apoptosis by modulating cytoplasmatic levels of BCL-2-like protein 4 (Bax) and B-cell lymphoma 2 (Bcl-2) proteins, cytochrome c release, and caspase-3 and nine activations. The protective effect derived from the silybin-phosphatidylcholine complex (SilPho) towards oxidative stress was demonstrated. *In vitro*, the use of SilPho can increase cellular vitality, which is assessed by MTT, under oxidative stress induced by the incubation of HepG2 and MKN28 cells, with xanthine oxidase and its substratum called xanthine. In addition, this study highlighted the effect of SilPho treatment on lipid peroxidation and cell necrosis [7].

Silybin could be an insulin sensitizer: it can reduce intrahepatic fat accumulation, lobular inflammation, ballooning, and serum fat can improve the homeostasis model assessment-IR Index (HOMA-IR) and insulin tolerance test (ITT) [11-13]. Moreover, silybin A has a vital role in reducing visceral fat accumulation, inducing lipolysis through the transcription of the adipose triglyceride lipase (ATGL) gene [14], and inhibiting gluconeogenesis silencing of some genes involved in the aforementioned metabolic pathway [15, 16]. However, in this work, the schedule of the administration of pure silybin without molecules that increase its oral bioavailability and high-fat diet (HFD) in the group of rats fed with HFD + silybin is not clear. Therefore, considering the low bioavailability of pure silybin, if administered daily, the result observed in this study cannot be ignored. The results of this study indicate that the reduction of fat absorption in HFD is due to the formation of non-absorbable complex with silybin, instead of relying on its essential role in disrupting the pathogenic mechanisms responsible for NAFLD [17].

Also, the antineoplastic effect of silybin can be associated with increased activity of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and decreased p-Akt production with modulation of signaling of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). In addition, it has an anti-angiogenetic effect [18].

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2. Materials and Methods

2.1. Plant Material

The seeds of milk thistle were purchased from the local market and authenticated by an expert botanist as *Silybum marianum*. A voucher specimen was deposited (No. PMP-1754) in the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

2.2. Extraction

500 g of plant seeds were ground entirely by the electric mill and passed through 18-45 mesh. For decolorization and defatting, powdered seeds were placed in Erlenmeyer, and 2 L hexane was added as solvent. Then placed the Erlenmeyer on a magnetic stirrer. Erlenmeyer contents were stirred at 500 rpm for three hours. Hexane solvent containing non-polar compounds was separated from plant seeds. Defatted seeds were dried in a shade until all of the hexane evaporated. The defatting seed powder is placed again into a five-liter Erlenmeyer flask and extracted with methanol solvent by a magnetic stirrer for 4 hours. The methanol extract is separated from the rest of the plant and dried by a rotary evaporator. Extraction efficiency with methanol was 2.08 %, and 10.4 g of extract was obtained. Finally, 200 ml water was added to the extract. Addition of water led to the appearance of a milky precipitate in the extract. The resulting precipitates were separated from the upper solvent by centrifugation at 3000 rpm and kept in the freezer until purification.

2.3. Isolation

The obtained extract was first dissolved in water and placed on Diaion HP-20 column (6×25 cm). The first moving phase was water. Water can remove high-polarity sugar impurities from the extract. The mobile phase was then converted to methanol to separate the remaining mixtures from the resin.

In the next step, column chromatography (5×60 cm) was used to separate silybin A and B. The decolorized fractions (2 g) were chromatographed on a silica gel column (230-400 mesh) eluted with chloroform: methanol (90: 10) as solvent.

The initial mobile phase contains chloroform: methanol (90: 10), the polarity of this solvent increased with each step by rising 5 % methanol. The solvent at the end of the process was chloroform: methanol (70: 30).

A total of 76 fractions with a volume of 25 ml were taken from the silica gel column. TLC was used to combine similar fractions with chloroform: methanol (80:20) as mobile phase. The standard silybin was used to combine the fractions (41 to 48) containing silybin A and B.

For further purification of silybin A and B, these fractions were applied on Sephadex LH-20 column (1×100 cm). 2.5 ml fractions were separated from this column. Each separated fraction was 2.5 ml, and silybin A and B were presented in fractions 8 to 11. This column can separate our desired composition into four completely pure fractions.

2.4. Structure elucidation

^{1}H-NMR and ^{13}C-NMR techniques were used to identify isolated compounds. Proton nuclear magnetic resonance (^{1}H-NMR) and carbon nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a Bruker 300 MHz AVANCE III HD NMR Spectrometer for proton and 75 MHz for carbon using tetramethylsilane (TMS) as the internal standard. The solvents were dimethyl sulfoxide-d6 (DMSO-d6).

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2.5. Standardization

After separating and purifying silybin A and B, the valid method (British Pharmacopoeia, 2017) was used to determine the amount of these substances in the instruction number PSA.AA.BB.CCC.312 [19]. Experiments to determine the levels of silybin A and B were repeated by three experts, three times on three different days. Finally, RSD, analysis accuracy and the silybin A and B percentage were determined. Purification was performed to standardize the samples using HPLC. The purified product was then standardized using the Sigma standard silybin. The mixture of silybin A and B was then weighed and was placed in dark glass vials.

2.6. High-performance liquid chromatography

Specifications and conditions of the device for quantitative analysis of silybin A and B have been shown in Table 1.

Table 1. HPLC Condition

HPLC device model	**Knauer**
Column type	Eurospher II-C_{18}
Column specifications	25 cm × 4.6 cm × 5 µm
pump	Knauer- P 6.1 L
Detector	Knauer- UV DAD 2.1 L: 288 nm
Flow rate	1 ml/min
Mobile phase	Phosphoric acid R, Methanol R, water R (6:30:64 V/V/V)
Temperature	20 ± 1 °C

3. Results

3.1. Extraction

Extraction efficiency with methanol was 2.08 %, and 10.4 g of extract was obtained.

3.2. Column chromatography

A total of 76 fractions with a volume of 25 ml were taken from the silica gel column. Silybin A and B were presented in fractions 8 to 11 from Sephadex LH-20 column (Fig. 1).

3.3. Structure elucidation

After recrystallization, white flat crystals were obtained from aqueous methanol. The ^{1}H-NMR spectrum (Table 2, Fig. 2) of silybin A showed the typical characteristics of 5,7-dihydroxy-substituted flavanonol by the signals at δ 5.91 (1H, d, J = 2.1 Hz, H-6), 5.86 (1H, d, J = 2.1 Hz, H-8), 5.07 (1H, d, J = 11.3 Hz, H-2), and 4.59 (1H, m, H-3). Six protons in the aromatic region can be attributed to two 1,3,4-trisubstituted aromatic rings, one belonging to the B-ring of a flavanonol unit and the other belonging to a cinnamic alcohol group. Four protons at δ 4.89 (1H, d, J = 7.9 Hz, H-7′), 4.15 (1H, m, H-8′), 3.54 (1H, dd, J = 12.3, 2.4 Hz, H-9′), and 3.32 (1H, dd, J = 12.3, 3.9 Hz, H-9′) can be assigned to a propanol moiety attached to a dioxane ring. The singlet peak in the 11.87 ppm is related to hydroxy-5, which is uncoated due to hydrogen bonding with the carbonyl group (Fig. 3).

The ^{13}C-NMR showed 25 carbon signals, characterized as one CH_3, one CH_2, twelve CH, and eleven C (Table 3, Fig. 4).

The ^{13}C-NMR spectra showed a carbonyl carbon signal at δ 197.77 (C-4), and signals at δ 82.53 (C-2), 71.46 (C-3), 163.29 (C-5), 96.06 (C-6), 166.80 (C-7), and 95.02 (C-8).

NMR signals at δ 116.58 (C-2'), 116.36 (C-5'), and 121.16 (C-6'), and two quaternary carbon NMR signals at δ 143.23 (C-3') and 143.62 (C-4') suggested a double-oxygenated aromatic C-ring.

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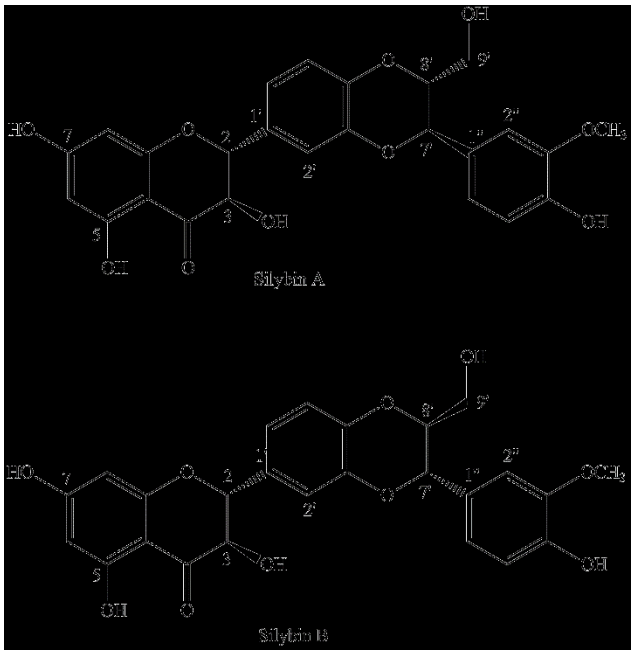


Fig. 1. Structure of Silybin A & B

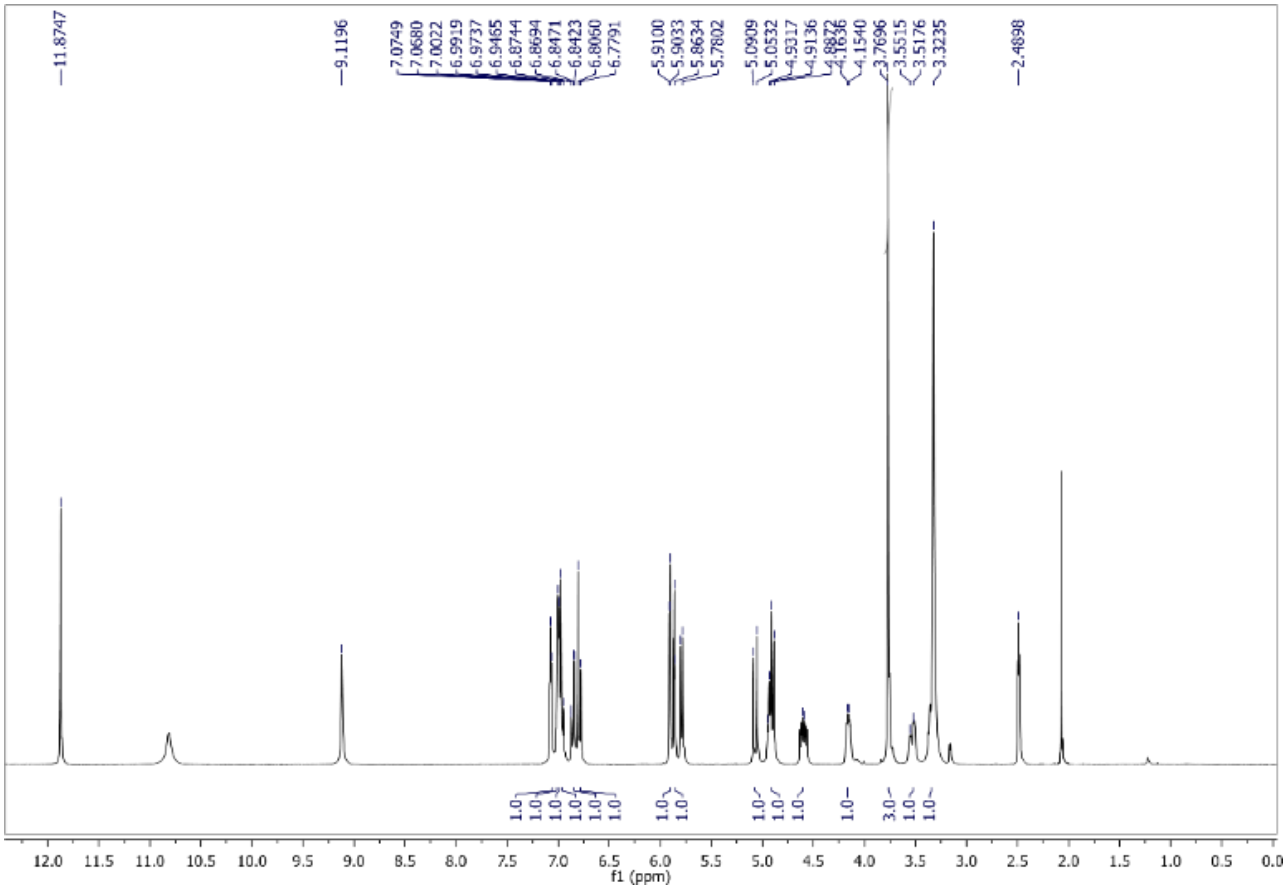


Fig. 2. [1]H-NMR Spectrum of the isolated compound-Silybin A & B

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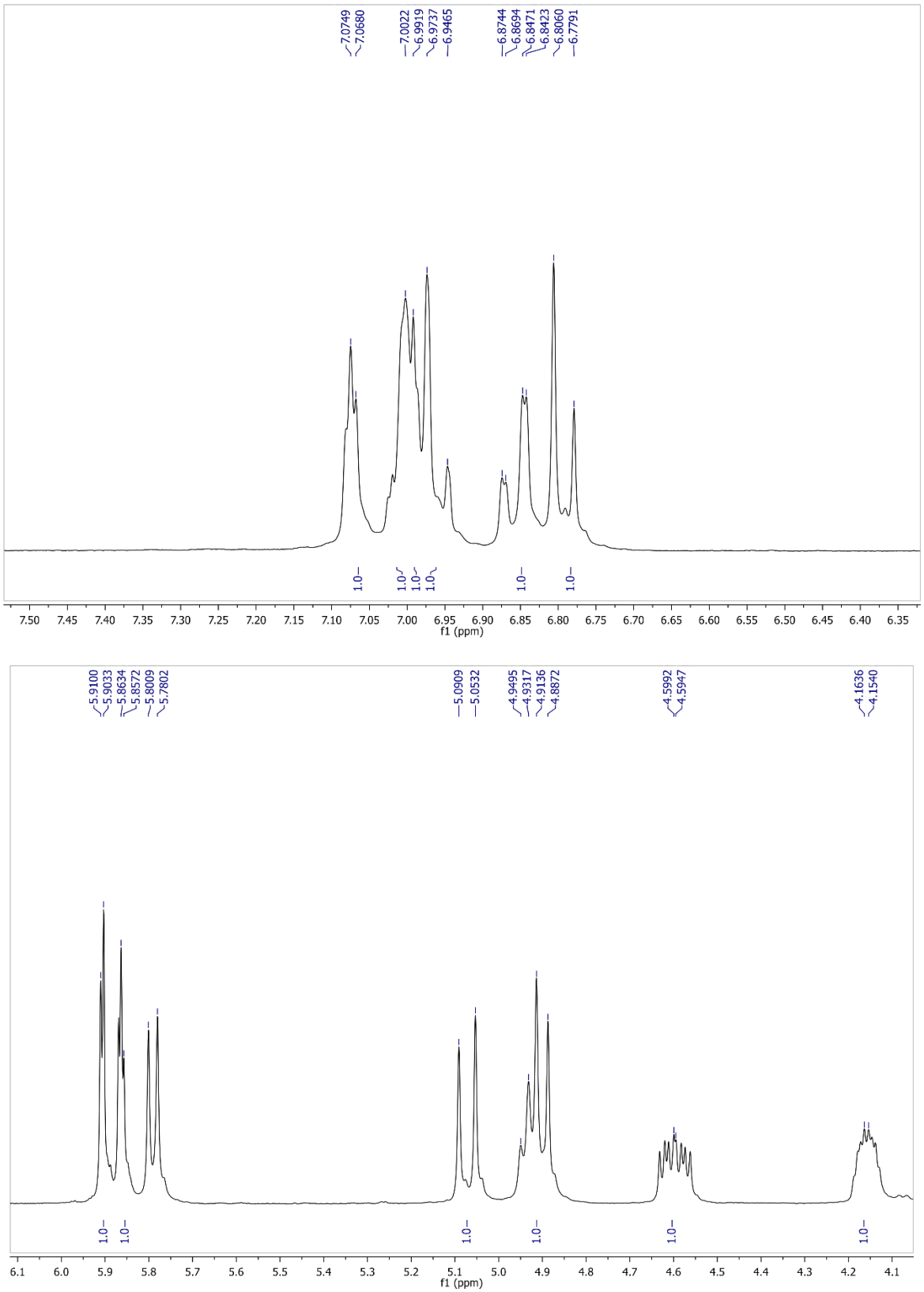


Fig. 3. Expanded ^{1}H-NMR spectra of the isolated compound-Silybin A & B

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Table 2. ^{1}H and ^{13}C-NMR data (ppm) and of Silybin A and Silybin B have been measured in DMSO-d6

Position	^{1}H-NMR data of Silybin A & Silybin B (multi, J in Hz)	^{13}C-NMR data of Silybin A & Silybin B
2	5.07 (d, 11.3 Hz)	82.53
3	4.59 (m)	71.46
4	-	197.77
5	-	163.29
6	5.91 (d, 2.1 Hz)	96.06
7	-	166.80
8	5.86 (d, 2.1 Hz)	95.02
9	-	162.47
10	-	100.46
1′	-	130.03
2′	7.07 (d, 2.0 Hz)	116.58
3′	-	143.23
4′	-	143.62
5′	6.95 (d, 8.1 Hz)	116.36
6′	6.99 (dd, 8.1, 2.0 Hz)	121.16
7′	4.89 (d, 7.9 Hz)	75.85
8′	4.15 (m)	78.11
9′	3.54 (dd, 12.2, 2.4 Hz)	60.18
9′	3.32 (dd, 12.2, 3.9 Hz)	-
1″	-	127.48
2″	7.00 (d, 2 Hz)	111.55
3″	-	147.62
4″	-	147.00
5″	6.80 (d, 8.1 Hz)	115.28
6″	6.86 (dd, 8.1, 2 Hz)	120.51
OMe	3.76 (s)	55.68

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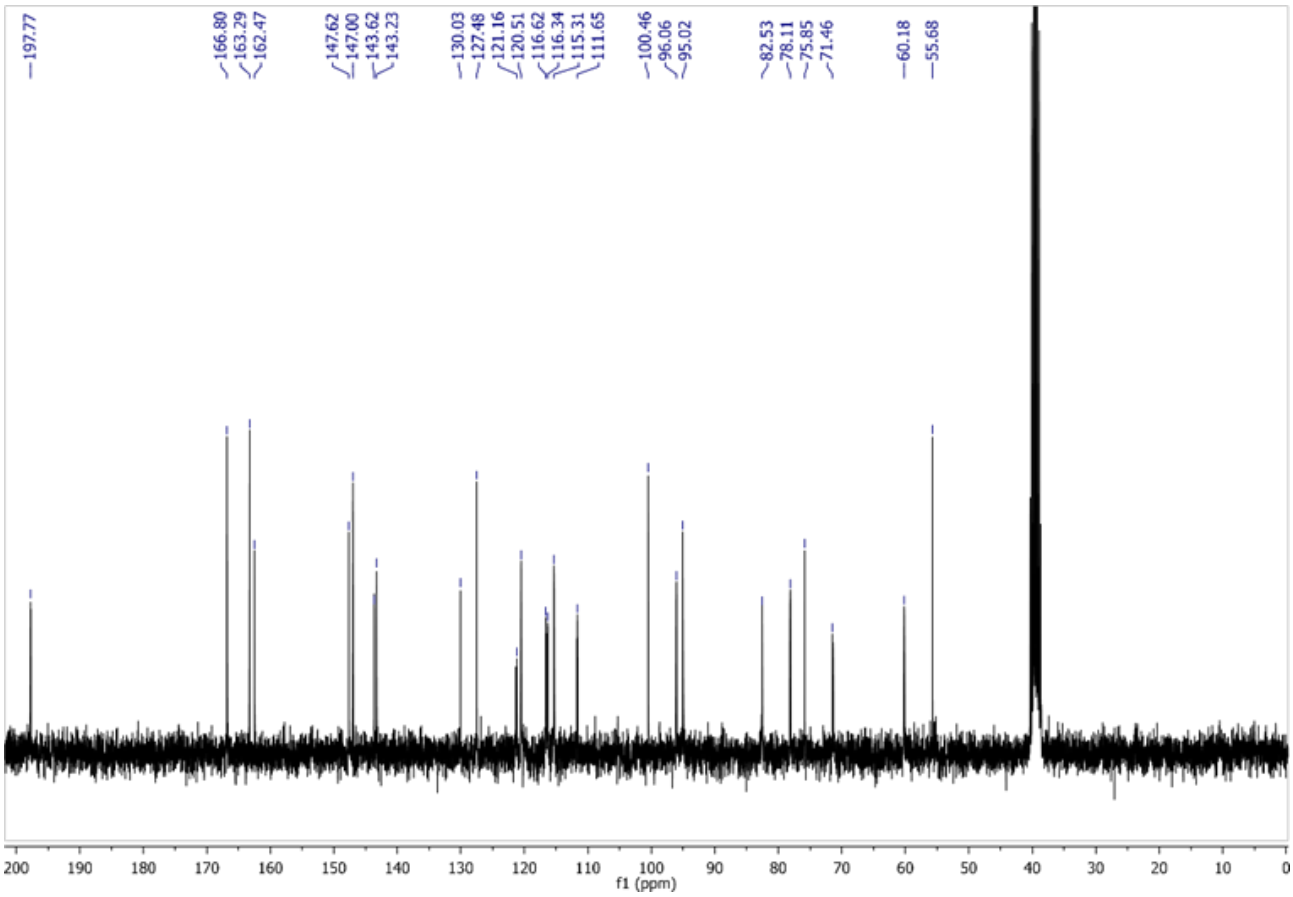


Fig. 3. ^{13}C-NMR Spectrum of the isolated compound-Silybin A, B

3.4. Standardization

After purification using HPLC and standardization using Sigma standard silybin, the standard silybin was placed in dark vials.

The label of standard vials includes compound name, Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR as manufacturer, approximate amount of the substance in container and batch number. Stabilization of standard vials should be done according to SOP number PSA.QA.IA.SOP-025.

4. Discussion

Many researchers have studied the isolation and structure of silybin over the past 60 years. Isolation of silybin diastereoisomers is a difficult problem that has not yet been completely resolved.

In summary, silybin A and B are fascinating chemical compounds. Their use as a dietary supplement is increasing worldwide, which explains the recent studies on increasing its oral bioavailability.

In this study, silybin A & B with high purity was obtained by a methanol extract from seeds of milk thistle and used the Diaion HP-20 resin, column chromatography, and thin-layer chromatography the purified compounds identified with ^{1}H NMR and ^{13}C NMR. Also, these compounds were quantified and standardized by the HPLC method [20]. The result of this study led to the completion of previous methods used to isolate these diastereoisomers in hundreds-of-milligram scale from milk thistle [21-26].

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As far as we know, few articles have been published on the separation of these two diastereoisomers from milk thistle extract. Our proposed system presented significant advantages in increasing efficiency and reducing cost, and the diastereoisomers of silybin A and silybin B in silymarin were successfully isolated with high purities. In all, this system is expected to be well adapted to isolate chemical constituents of interest from complex systems, including herbs.

5. Conclusion

In conclusion, silybin A and silybin B are one pair of diastereoisomers with different connectivities at C-7' and C-8'. These diastereoisomers have very similar ^{1}H and ^{13}C-NMR spectra and have no characteristic signals for the facile identification of individual isomers. In this study, initial separation was performed by column chromatography with stationary phase of Diaion HP-20 resin, silica gel and Sephadex LH-20, respectively. Reversed-phase HPLC can be applied effectively to identify and purify each of these diastereoisomers.

Author contributions

S. T: contributed to the study design and conducted the data analysis, R. H: contributed to the conducted the data analysis as well as the writing and reviewing of the manuscript, R. Gh : contributed to the study design and the writing and reviewing of the manuscript, M. Y: contributed to the NMR and HPLC data analysis as well as the writing of the manuscript, F. K.S: contributed to the conducted the data analysis as well as the writing and reviewing of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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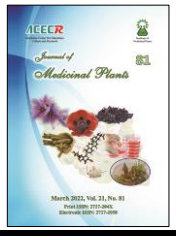
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مقاله تحقیقاتی

خالص‌سازی، شناسایی و استانداردسازی ترکیبات سیلی‌بین آ و ب از گیاه خارمریم

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اطلاعات مقاله

گل‌واژگان:
خار مریم
سیلی‌بین آ و ب
کروماتوگرافی ستونی
دیایون اچ پی-۲۰
سفادکس ال اچ-۲۰
کروماتوگرافی مایع با کارایی بالا
رزونانس مغناطیسی هسته

چکیده

مقدمه: خار مریم (*Silybum marianum* (L.) Gaertn) یک گیاه چند ساله با خواص دارویی است. دانه‌های این گیاه حاوی سیلی‌مارین، گروهی از ترکیبات با ساختار فلاونولینگانی و اثرات آنتی‌اکسیدانی، ضدالتهابی و محافظت از کبد می‌باشند. مهم‌ترین ماده فعال زیستی سیلی‌مارین، سیلی‌بین آ و ب است. این ترکیبات در درمان بیماری‌های مختلف کبدی مورد استفاده قرار می‌گیرند و فعالیت ضدتوموری بالایی را از خود نشان می‌دهند. **هدف:** هدف از این مطالعه خالص‌سازی، شناسایی و استانداردسازی سیلی‌بین آ و ب از عصاره دانه خارمریم بود. **روش بررسی:** ابتدا دانه گیاه خارمریم به کمک حلال هگزان چربی‌زدایی شده و سپس توسط حلال متانول عصاره گیری انجام شد. جداسازی و خالص‌سازی بیشتر سیلی‌بین آ و ب توسط کروماتوگرافی ستونی به ترتیب با استفاده از فازهای ساکن رزین دیایون اچ پی-۲۰، سیلیکاژل و سفادکس ال اچ-۲۰ انجام شد. برای شناسایی این ترکیبات از تکنیک های رزونانس مغناطیسی هسته‌ای پروتون و کربن استفاده شده است. سرانجام، از روش کروماتوگرافی مایع با کارایی بالا برای استانداردسازی استفاده شده است. **نتایج:** رزونانس مغناطیسی هسته‌ای پروتون و کربن ساختار سیلی‌بین آ و ب استخراج شده از خارمریم را تایید کردند و استانداردسازی و تعیین درصد خلوص آن‌ها به کمک تکنیک HPLC انجام شد. **نتیجه‌گیری:** سیستم پیشنهادی ما مزایای قابل‌توجهی در افزایش کارایی و کاهش هزینه ارائه کرد و دیاستریوایزومرهای سیلی‌بین آ و ب در سیلی‌مارین با موفقیت با خلوص بالا جدا شدند.

مخففها: ^{1}H-NMR، رزونانس مغناطیسی هسته‌ای پروتون؛ ^{13}C-NMR، رزونانس مغناطیسی هسته‌ای کربن؛ HPLC، کروماتوگرافی مایع با کارایی بالا

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