COMPARATIVE STUDY OF BIOACTIVE COMPOUNDS IN MILK THISTLE SEED VARIETIES IN SULAIMANI, IRAQ

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ABSTRACT

This study was conducted using two different varieties of milk thistle, var. *marianum*, and var. *albiflorum*. The plant samples were collected from the Sulaimani region to determine silymarin content, bioactive constituents, and antioxidant activity. HPLC analysis showed that var. *marianum* contains a high amount of silymarin (365.680±3.815 mg/g). Silybin B, Silychristin A, and Silybin A were the major flavonolignans in these varieties—the var. *marianum* contains silybins B, silychristin A, and silybin A (174.880±6.495, 86.720±2.113, and 54.960±2.483 mg/g) respectively. The hexane extract analysis of milk thistle seeds of the *marianum* and *albiflorum* varieties identified 26 (95.229±1.318) and 19 (94.547±6.541) phytochemical compounds, respectively. Both varieties contain a higher amount of oleic acid, which was found to be 14.800±0.481 and 31.940±0.726 in both varieties, respectively. This study evaluates the scavenging effect of milk thistle plants using a DPPH assay. The result indicated that the var. *marianum* had a maximum scavenging effect of 72.944% compared to the var. *albiflorum*, (61.846%). The stronger IC50 was obtained by var. *marianum* with 17.027±0.064 µg/mL.

Keywords: medicinal plants, *Silybum marianum*, var. *marianum*, var. *albiflorum*, antioxidant, HPLC, GC-MS.

دراسة مقارنة للمركبات الفعالة بيولوجياً لصنفين من الكلغان في السليمانية، العراق

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المستخلص

اجريت هذه الدراسة باستخدام صنفين مختلفين من نبات الكلغان *Silybum marianum*، هما الماريانم و البيفلورم والتي تم جمعهما في منطقة السليمانية لتحديد محتوى السيليمارين والمكونات النشطة بيولوجياً والنشاط المضاد للأكسدة. أظهر تحليل HPLC أن صنف الماريانم يحتوي على كمية عالية من السيليمارين (365.680±3.815 ميلغرام/غم). كان السيليبين B، السيليكريستين A، والسيليبين A من مركبات الفلافونوليجنين الرئيسية في هذين الصنفين. إحتوى صنف الماريانم على السيليبين B ، السيليكريستين A، والسيليبين A 6.495 ± 174.880، 2.113 ±86.720، و 2.483±54.960 ميلغرام/غم على التوالي. حددت تحليل مستخلص الهكسان لبذور الكلغان من صنفي الماريانوم والبيفلوروم 26 (1.318±95.229) و 19 (6.541±94.547) مركبًا كيميائيًا نباتيًا، على التوالي. يحتوي كلا الصنفين على كمية عالية من حامض الأوليك، بلغت 0.481±14.800و0.726±31.940 في كلا الصنفين، على التوالي. عند دراسة وتقييم تأثير نباتات الكلغان باستخدام اختبار DPPH تشير النتائج إلى أن صنف الماريانم كان له تأثير كسح أقصى قدره (72.94٪) مقارنة بصنف ألبيفلوروم (61.846٪). تم الحصول على IC50 الأقوى بواسطة صنف الماريانوم مع 0.064±17.027 ميكروغرام/مل.

الكلمات المفتاحية: النباتات الطبية، *Silybum marianum*، صنف ماريانم، صنف البيفلورم، مضادات الاكسدة، HPLC، GC–MS.

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INTRODUCTION

In flowering plants, the Asteraceae family is considered to be the largest family and contains about 1900 genera, including milk thistle *Silybum marianum* L. Gaertn, which is one of the family members (14,24). Milk thistle, a traditional medicinal herb, includes two varieties: the most widespread variety is *marianum*, which has a purple corolla, and the white corolla is the variety *albiflorum*. The fruits of the two varieties are achenes, characterized by an oblong shape and the presence of a lengthy white pappus fused at its base, which is subsequently shed. There exists diversity in seed coloration and size: seeds (achenes) of var. *marianum* are black, 8 mm and 4 mm in length and width, respectively, while those of var. *albiflorum* are brown with mottling, 6 mm and 3 mm in length and width, respectively(9). Milk thistle is an annual plant, sometimes known as a biennial plant (29), native to Asia and southern Europe (31). In 2016, the plant achieved respective sales figures of approximately $9.968 million and $17.077 million, positioning it as the 6th highest-selling herbal dietary supplement within the natural and health food sectors, and 16 within the mainstream multi-outlet channel market in the United States (34). All plant parts were consumed, like stalks, roots, and flowers. The leaves were used in salads, and the roasted seed was used as coffee (10). The fruits of both varieties accumulate flavonolignans (1). Silybin A and B, silychritin A, isosylibin A and B, isosilychristin, and silydaianin are the major active flavonolignans extracted from milk thistle fruit (5). Milk thistle leaves also contain silymarin, but the seeds contain a higher concentration (8). Milk thistle plants have been used as herbal medicine to treat liver disorders for centuries. Silymarin, the bioactive extract from milk thistle in preclinical studies, has been documented as having hepatoprotective and antioxidant properties (25). Silymarin can prevent and reduce the degeneration of liver cells, promote the purification of the liver, improve detoxification, and repair damaged liver cells (21). The plant exhibits attributes encompassing photoprotection, antidepressant properties, antihypertensive effects, and antimetastatic potential, and primarily acts as a hepatoprotective agent (7, 12, 35) Silymarin has been shown to boost glutathione synthesis in hepatocytes and superoxide dismutase activity in erythrocytes, in addition to its capacity to scavenge free radicals (18,30). Milk thistle is widely distributed in our region, and people have traditionally used it as a wild plant since ancient times without any knowledge about the useful content of plants, so this current study is important and aims to compare two milk thistle varieties grown naturally in the Sulaimani region in terms of phytochemical contents using HPLC and GC-MS techniques and its antioxidant capacity.

MATERIALS AND METHODS

Plant collection and identification

Silybum marianum var. *marianum* and *Silybum marianum* var. *albiflorum* plant samples were collected at the full bloom stage for identification in the 2022–2023 season. Sample specimens were identified and deposited in the University of Sulaimani, College of Agricultural Engineering Science Herbarium (SUFA, acronym according to Thiers, 2021) for references and further investigation. The fruits (achene) of the two varieties were collected from the Sulaimani-Bakrajo field in late May 2023 (4).

Sample preparation for HPLC analysis

Twenty grams of seed powder from the milk thistle were dissolved in 60 mL of acetonitrile and subjected to agitation in an ultrasonic bath (40 min). Subsequently, the extracted samples were filtered through Whatman filter paper (0.5 µm) to eliminate fibers and undissolved particulates. The extracts were then subjected to pre-concentration via a stream of N_2 until reaching a volume of approximately (0.5 mL), and the volume was then adjusted to (1 mL) using the mobile phase. Subsequently, 2 µL of the aqueous filtrate was injected into the HPLC column under optimized conditions for the effect of separation(4).

Sample preparation for GC-MS analysis

Two grams of each variety of milk thistle seeds were finely powdered using an electric blender and subsequently transferred into separate conical flasks. To each flask, 200 mL of hexane was added, and the mixture was then kept at room temperature for 72 hours to facilitate solvent extraction. Following the extraction period, the resulting mixture was

subjected to filtration using Wittman filter paper to separate the solid residues from the solvent extract. The solvent extract was stored at -2°C until used in chromatographic analysis (3).

HPLC analysis

The standard silymarin utilized in the study was procured from Sigma-Aldrich. The standards for various components of silymarin including silybin A, silybin B, silychristin A, silychristin B, silydaianin, Isosylibin A, and Isosylibin B were prepared according to the methods described in previous studies (11,26). Quantitative analysis was conducted using a monolithic Chromolith RP-C18 column (100×3 mm) with a guard cartridge Chromolith RP-18e (5×4.6 mm; both from Merck, Darmstadt, Germany) employing binary gradient elution (mobile phase: A = 5% acetonitrile, 0.1% formic acid; B = 80% methanol, 0.1% formic acid; gradient: 0 min 30% B, 12 min 60% B, 13 min 60% B, 14 min 30% B, 16.5 min stop), at a flow rate of 1.1 mL/min, with an increased flow rate of 1.5 mL/min during 14–16 min for faster re-equilibration of the column, at a temperature of (25 °C). Photodiode array (PDA) data in the (200–400 nm) range were collected with a rate of 40 Hz and, the time constant of 0.025 s, and signals at 285 nm were analyzed. The volume injection was maintained at (2 µL).

GC-MS analysis

The Agilent Technologies (7890A) gas chromatograph with a mass selective detector and Agilent Technologies (5975C) inert XL MSD mass spectrometer were used to analyze the seeds powder of Milk thistle plant samples. The Agilent 190915-433:325 °C (30 m × 250 µm × 0.25 µm) GC-MS column was organized to begin at (40 °C) and increase by (10 °C) per minute to a maximum temperature of (280 °C). The heater front inlet temperature was (250 °C), the injector port was also set to (290 °C), and the carrier gas was helium at a flow rate of (1 mL/min). The injection mode was split at a ratio of 5:1. At 70 eV, mass spectrometry was used in the electron impact mode (EI). Using the normalization of the GC peak areas, the percentages of the phytochemical components were calculated. The software automatically normalized the peaks by dividing each peak area by the total area and then multiplying by 100. Using the Wiley library, the relative proportions of the constituents were calculated (17).

Antioxidant properties

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay is a method used in chemistry and biochemistry to evaluate the antioxidant properties of various compounds. DPPH is a stable free radical compound with a deep violet color. The free radical scavenging activity of both milk thistle varieties was examined using previously reported procedures (30,32). Different concentrations (25, 50, 75, and 100 µg/mL) of both milk thistle varieties ethanolic extract were prepared. Ascorbic acid was used as a positive control with the same concentrations. The concentrations were mixed with 1mL of 0.1 mM DPPH. The solution was kept in a dark place for 30 minutes, and then the absorbance was measured at λ=517 nm. The following equation was used to measure the radical scavenging activity:

$$\textbf{Scavenging Effect}\ (\%) = \frac{[(\mathbf{A_{517nm}\ control} - \mathbf{A_{517nm}\ sample})}{\mathbf{A_{517nm}\ control}} \times \mathbf{100}$$

The IC50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using an inhibition curve. The lower absorbance of the reaction mixture indicated higher free radical activity. A linear graph of concentration versus percentage inhibition was prepared using various concentrations of ascorbic acid, and IC50 values were calculated (Figure 1)(22).

Statistical analysis

The tests were conducted three times, and SPSS software was utilized to analyze the data, calculating average values and standard deviation (averages±SD). A one-way ANOVA was employed to assess differences among the varieties, followed by LSD tests to compare means at significance levels of $p \leq 0.01$ and $p \leq 0.05$ (33).

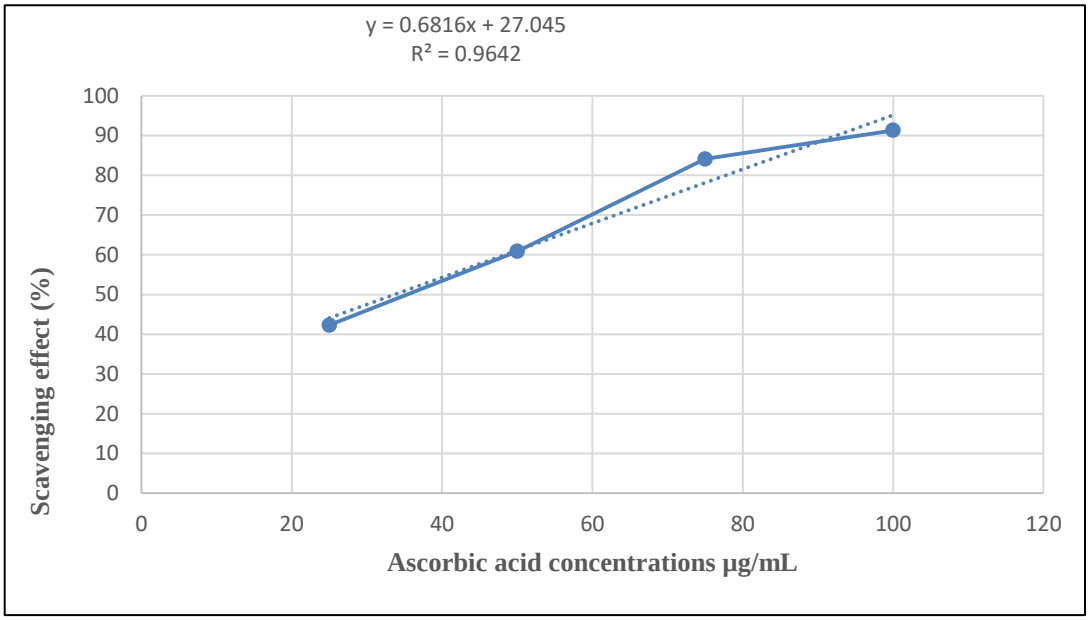

Figure 1. DPPH free radical scavenging affects the positive control (Ascorbic acid)

RESULTS AND DISCUSSION

HPLC analysis

A potential advantage of HPLC is the ability to separate and quantify the main silymarin components (2). For comparison, the two milk thistle fruits undergo the same extraction process. According to the HPLC analysis (Table 1 and Figure 2) var. *marianum* contains a higher total silymarin (365.680±3.815) compared to var. *albiflorum* (245.400±3.220). Silymarin content quantified through the HPLC ranged from 1669.5±1.05 to 1624.7±0.75 for var. *marianum* and 1653.7±1.71 to 1607.6±0.99 for var. *albiflorum* (8). In a study conducted by Friese *et. al* (20) who analyzed milk thistle plants from different locations in Egypt, the results illustrated that the var. *marianum* contained a higher amount of silymarin with 21.92±0.67 compared to var. *albiflorum* with 15.91±2.76. Silybin B, Silychristin A, and Silybin A were the major flavonolignans in these two varieties. Interestingly, var. *marianum* contains silybins B, silychristin A, and silybins A (174.880±6.495, 86.720±2.113, and 54.960±2.483 respectively) which were higher than var. *albiflorum* (131.040±8.142, 47.340±1.866, and 43.860±3.631 respectively). Silychristin B was the minor flavonolignan produced by both varieties, var. *marianum* and var. *albiflorum* (6.080±1.023 and 3.900±1.069, respectively). The analysis of different samples of milk thistle plants indicated that silybin B, silybin A, and isosylibin A were the most abundant among other detected silymarin, and var. *marianum* exhibited higher amount of var. *albiflorum* (20).

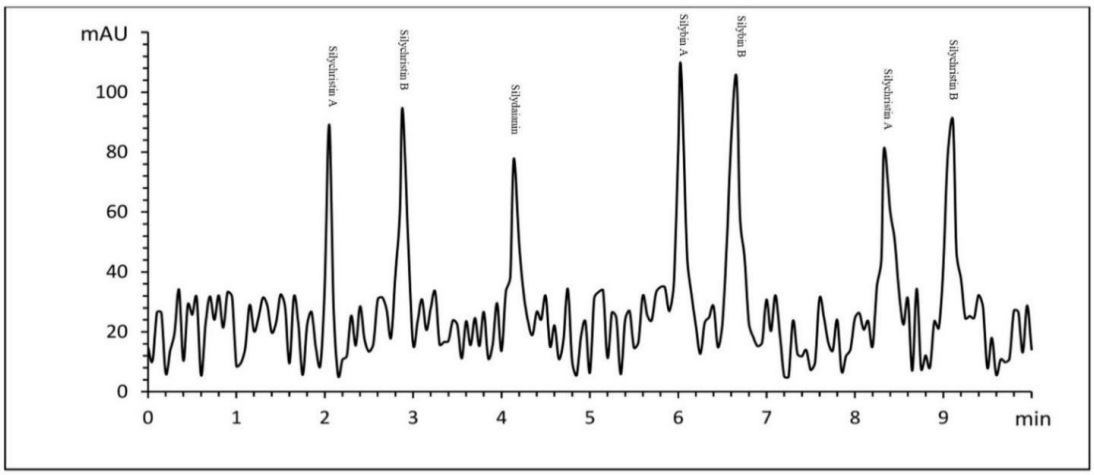

Figure 2. HPLC chromatogram standard for Silymarin content in seed extract of Milk thistle varieties

Table 1. Concentrations of Silymarin and its components in Milk thistle varieties

Phytochemical compounds	Retention Time (min.)	Concentrations (mg/g)	
		Var. *marianum*	Var. *albiflorum*
Silychristin A	2.05	86.720±2.113	47.340±1.866
Silychristin B	2.88	6.080±1.023	3.900±1.069
Silydaianin	4.14	9.600±0.629	6.300±1.080
Silybin A	6.03	54.960±2.483	43.860±3.631
Silybin B	6.66	174.880±6.495	131.040±8.142
Isosylibin A	8.33	20.480±1.596	12.000±1.818
Isosylibin B	9.11	12.960±1.480	9.960±0.903
Total Silymarin		365.680±3.815	245.400±3.220

GC-MS analysis

The most widely used method for phytoconstituent separation is gas chromatography-mass spectra. The analysis of *Silybum marianum* var. *marianum* and *Silybum marianum* var. *albiflorum* seed hexane extract identified 26 (95.229±1.318) and 19 (94.547±6.541) phytochemical compounds, respectively as shown in Tables (2), (3) and Figures (3), (4). The major compounds in var. *marianum* were 5-Hexenenitrile, and 2-methyl- (15.419±0.840), which were not detected in var. *albiflorum*. It is a byproduct of the production of trifluoromethyl compounds, more details about the trifunctionalization of 5 hexennitriles caused by cyano-group migration and photoredox-catalyzed trifluoromethyl radicals. The unsaturated fatty acid oleic acid was found to be (14.800±0.481) and (31.940±0.726) in milk thistle plants in both var. *marianum* and var. *albiflorum* respectively. A study by Eldalawy and Al-Ani (16) reported that the Iraqi milk thistle plant seeds contain oleic acid with a concentration of 33.7%. The GC-MS analysis of milk thistle fatty oil showed the presence of a high percentage of oleic acid with a concentration of 31.94%. Saturated fatty acid arachidonic acid was detected only in a *marianum* variety with a concentration of 11.246±0.362. Ciocarlan *et al*.(15), reported that the GC-MS analysis of milk thistle seed contains 2.63% arachidic acid. Heptanal aldehyde compound was identified in both varieties with (4.388±0.674) in *marianum* and a higher amount in var. *albiflorum* with (11.397±0.494). Heptanal is an aliphatic aldehyde, which has a wide range of odors and flavors (6,35). Nerolidol identified in var. *albiflorum* with a mount of (9.200±0.887) was not identified in var. *marianum.* Nerolidol is a sesquiterpene alcohol compound found in a wide range of plants that possess a floral odor (13). D-Mannose was identified in var. *albiflorum* with (8.700±0.156). A naturally occurring C-2 epimer of glucose, D-mannose is found in many plants and fruits (28) and previous research reported that these two varieties are different in morphological and chemical content. The chemical analysis of silymarin in two varieties, var. *marianum* and var. *albiflorum,* revealed concentrations of 4.3 µg/ml and 2.0 µg/ml, respectively. Both varieties exhibited high levels of total carbohydrates, phenolics, and crude protein in their seeds. Linoleic acid and oleic acid were identified as the major fatty acids, with var. *marianum* containing 46.7% and 28.6%, and var. *albiflorum* containing 42.5% and 27.1%, respectively. Amino acid analysis indicated elevated levels of phenylalanine, lysine, and valine in both varieties (9).

Table 2. Phytochemicals content of *Silybum marianum* var. *marianum* analyzing by GC/MS

Peaks	Compounds	Chemical Formula	RT (min.)	Abundance (%)	Similarity* (%)
1	4-Penten-1-ol, 3-methyl-	$C_6H_{12}O$	2.678	3.086±0.144	72
2	(+)-2-Bornanone	$C_{13}H_{30}O$	2.884	4.829±0.566	96
3	-Hexenenitrile, 2-methyl-5	$C_7H_{11}N$	3.338	15.419±0.840	69
4	Heptanal	$C_7H_{14}O$	4.139	4.388±0.674	96
5	Glucitol, 6-O-nonyl-	$C_{15}H_{32}O_6$	5.953	2.405±0.196	90
6	R-Limonene	$C_{10}H_{16}O_3$	6.088	2.335±0.281	96
7	Dodecane	$C_{12}H_{26}$	7.476	1.137±0.066	95
8	Oleic Acid	$C_{18}H_{34}O_2$	7.532	14.800±0.481	86
9	l-Gala-l-ido-octose	$C_8H_{16}O_8$	7.723	1.669±0.261	80
10	3-Hydroxy-α-ionene	$C_{13}H_{20}O_2$	8.144	1.283±0.040	96
11	Phytol	$C_{20}H_{40}O$	8.413	0.553±0.110	90
12	Desulphosinigrin	$C_{10}H_{17}NO_6S$	8.693	0.833±0.023	82
13	9-Tetradecen-1-ol, acetate, (E)-	$C_{16}H_{30}O_2$	9.027	3.200±0.353	83
14	d-Mannose	$C_6H_{12}O_6$	9.105	2.407±0.290	81
15	Maltose	$C_{12}H_{22}O_{11}$	9.245	1.833±0.110	90
16	Lactose	$C_{12}H_{22}O_{11}$	9.308	1.923±0.096	79
17	Arachidic acid	$C_{20}H_{40}O_2$	10.502	11.246±0.362	92
18	2-Hexadecanol	$C_{16}H_{34}O$	11.159	1.215±0.133	84
19	Guanosine	$C_{10}H_{13}N_5O$	11.211	1.058±0.053	93
20	Dodecanal	$C_{12}H_{24}O$	11.388	2.081±0.195	98
21	Nerolidol	$C_{15}H_{26}O$	11.579	2.846±0.099	92
22	Estradiol	$C_{18}H_{24}O_2$	12.24	0.63±0.121	99
23	Isopulegol	$C_{16}H_{34}O$	13.147	1.567±0.094	84
24	Trans-Isoeugenol	$C_{10}H_{12}O_2$	13.486	5.225±0.192	87
25	Erucic acid	$C_{22}H_{42}O_2$	14.072	4.544±0.189	90
26	Nonanal	$C_9H_{18}O$	15.716	2.717±0.205	83
	Total Components			95.229±1.318	

*** % of similarity relative to the reference library in the GC/MS**

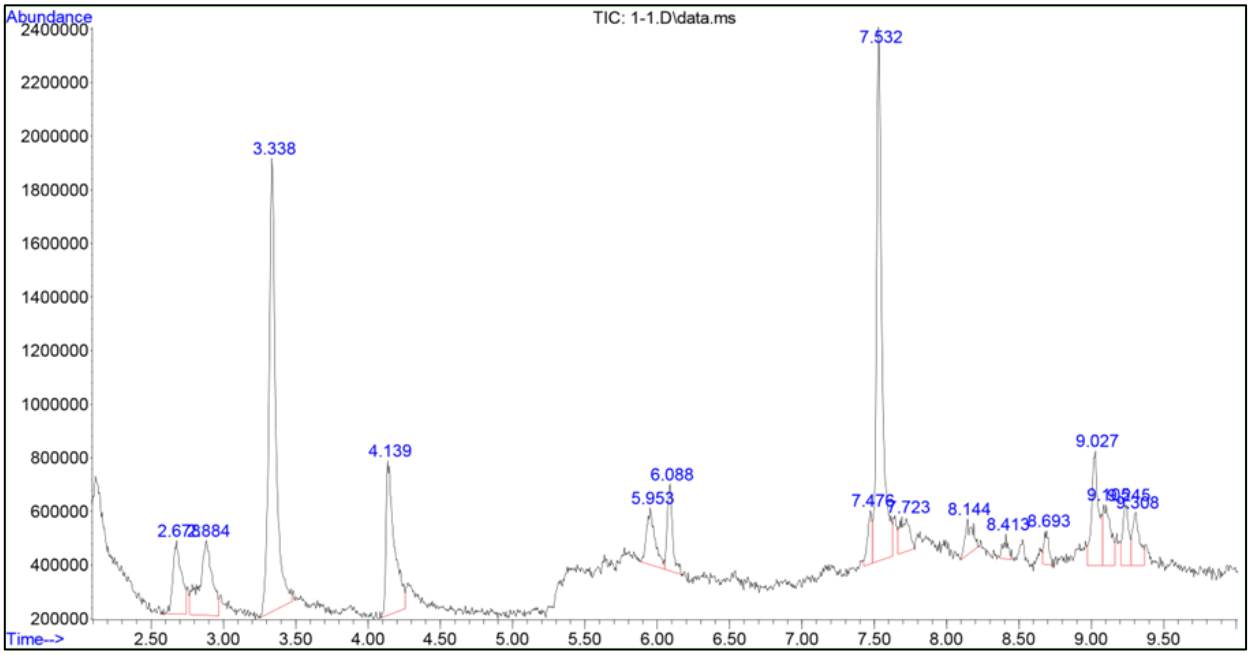

Figure 3. The GC-MS analysis of *Silybum marianum* (var. *marianum*)

Table 3. Phytochemicals content of *Silybum marianum* var. *albiflorum* analyzed by GC/MS

Peaks	Compounds	Chemical Formula	RT (min.)	Abundance (%)	Similarity* (%)
1	Rhodopin	$C_{40}H_{58}O$	2.227	3.703±0.479	83
2	Heptanal	$C_7H_{14}O$	2.643	11.397±0.494	79
3	(+)-2-bornanone	$C_{10}H_{16}O$	2.863	1.828±0.325	96
4	2-α-Isomethyl ionone	$C_{14}H_{22}O$	3.328	2.167±0.182	100
5	Bioallethrin	$C_{19}H_{26}O_3$	4.136	1.071±0.099	83
6	2-Decen-1-ol, (E)-	$C_{10}H_{20}O$	4.272	1.870±0.010	78
7	Santolinatriene	$C_{10}H_{16}$	5.134	2.484±0.307	69
8	Trans-11-Tetradecenyl acetate	$C_{16}H_{30}O_2$	5.316	0.334±0.042	92
9	d-Mannose	$C_6H_{12}O_6$	5.936	8.700±0.156	78
10	Isopulegol	$C_{10}H_{18}O$	6.079	0.670±0.062	85
11	Diglycolic acid	$C_4H_6O_5$	7.363	5.275±0.184	66
12	Phytol	$C_{20}H_{40}O$	7.468	3.407±0.369	99
13	Oleic acid	$C_{18}H_{34}O_2$	7.533	31.940±0.726	92
14	Erucic acid	$C_{22}H_{42}O_2$	8.4	1.320±0.075	88
15	dodecanal	$C_{12}H_{24}O$	9.025	1.970±0.240	90
16	Nerolidol	$C_{15}H_{26}O$	10.227	9.200±0.887	96
17	Cis-vaccenic acid	$C_{18}H_{34}O_2$	10.455	0.250±0.048	97
18	Stearic acid	$C_{18}H_{36}O_2$	11.596	1.681±0.178	93
19	Estradiol	$C_{18}H_{24}O_2$	15.939	5.280±0.221	85
	Total components			94.547±6.541	

*** % of similarity relative to the reference library in the GC/MS**

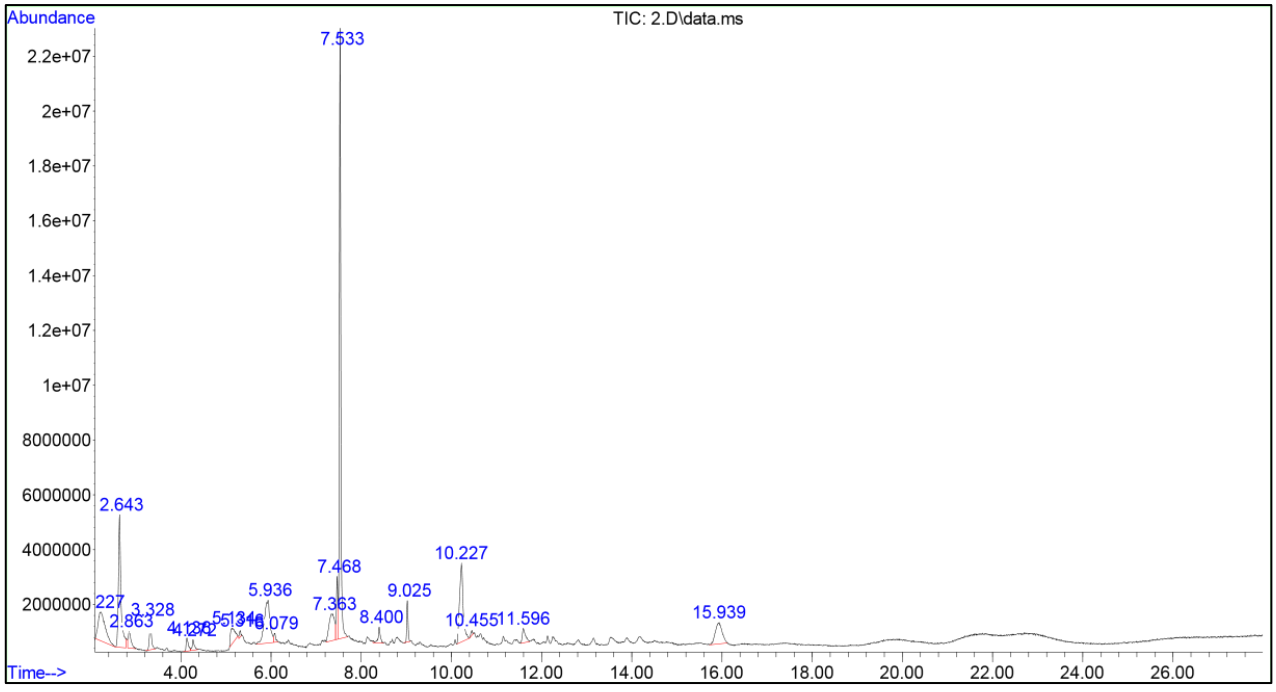

Figure 4. The GC-MS analysis of *Silybum marianum* (var. *albiflorum*)

Antioxidant activity of milk thistle varieties

DPPH is commonly utilized as a reagent to determine free radicals. To form a stable diamagnetic molecule, it must receive an electron or hydrogen radical. Antioxidant activity was defined as the antioxidant's ability to eliminate DPPH radical absorption at 517 nm. Figure. (5) indicates that the result of the scavenging effect of milk thistle varieties was statistically significant ($p \leq 0.01$). The var. *marianum* had a maximum scavenging effect of 72.944% in comparison with the var. *albiflorum*, (61.846%). The variations in silymarin and their content of bioactive compounds may be explained by the many antioxidant types found in the extracts, each of

which has a unique reaction with the applied radicals (19). According to a study by Serçe *et. al* (30), milk thistle seeds can stop lipid peroxidation and have good DPPH free radical scavenging ability. As a result, milk thistle has the potential to be a rich source of food preservatives and antioxidants. Hadaruga and Hadaruga (23) was reported that the milk thistle plant extract had a high antioxidant potential. The scavenging effect between the two milk thistle varieties concentration and DPPH radical, which linearly increased as the concentration increased from 25 to 100 µg/mL. The milk thistle seed extracts with 100 µg/mL exhibited 84.904 % activity, whereas the 25 µg/mL exhibited 49.816 % inhibition. The same result was obtained by Mishra *et. al* (27), who reported that by increasing concentration, the scavenging effect percentage increased.

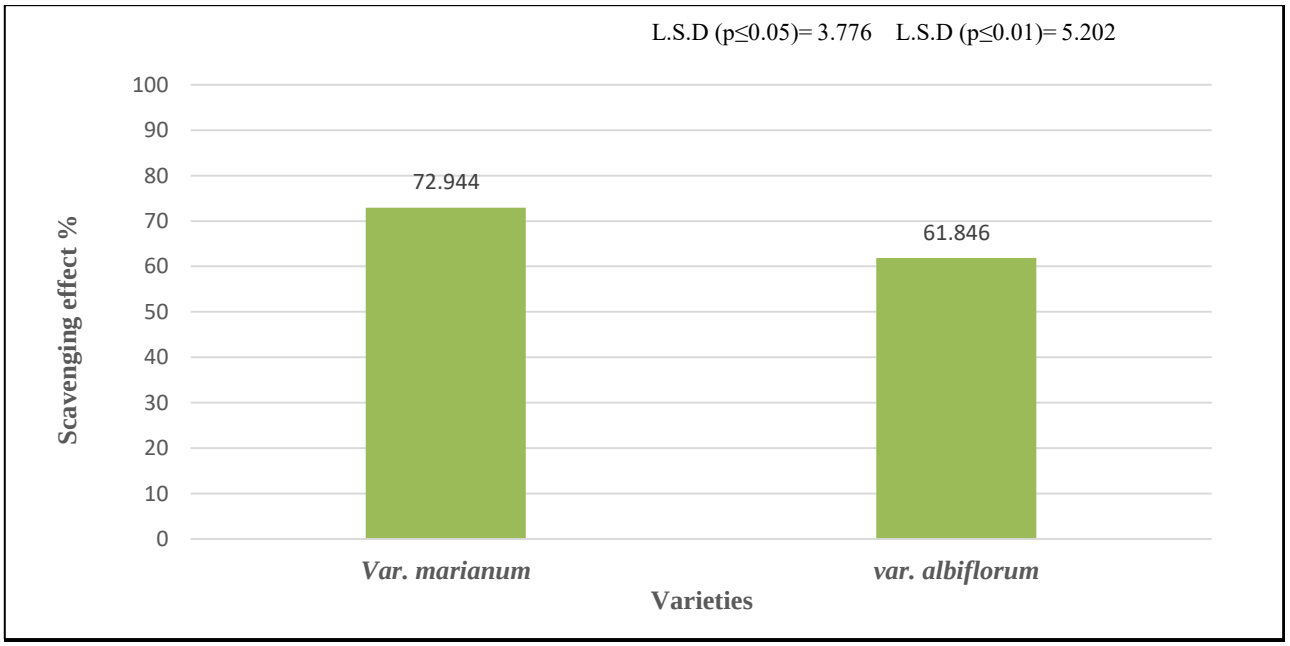

Figure 5. Milk thistle seed extracts scavenging effect (%)

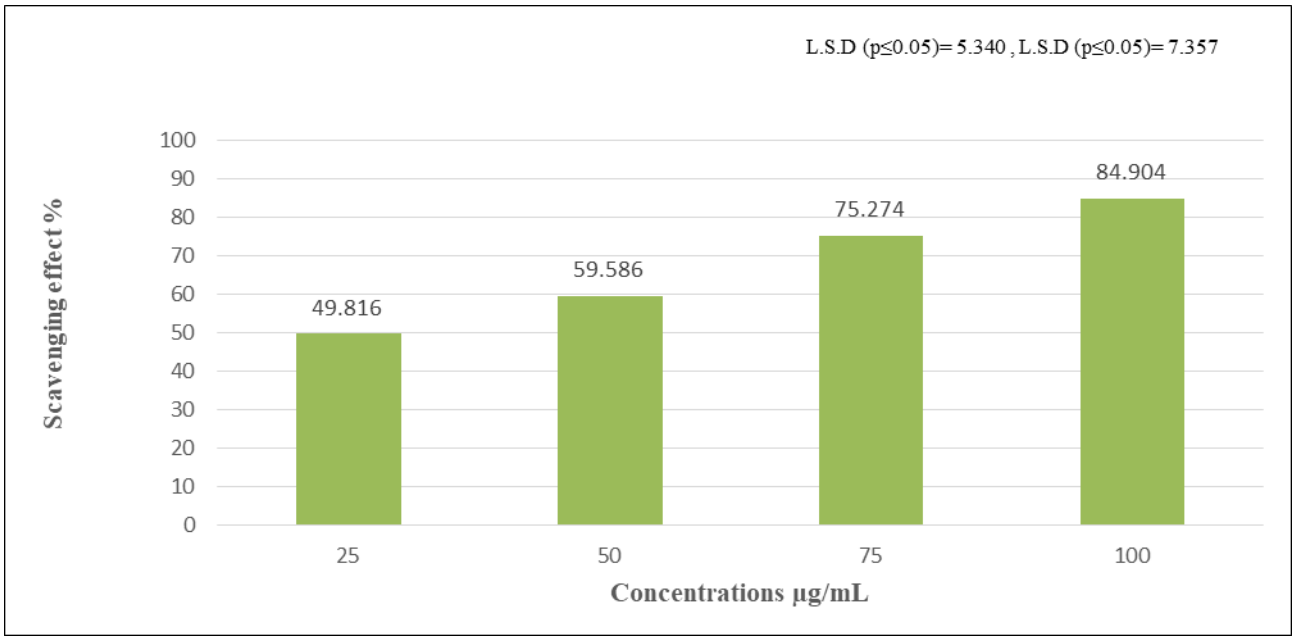

Figure 6. Milk thistle seed extracts scavenging effect (%) based on different concentrations

The interaction between varieties and seed extract concentrations on the scavenging effect is illustrated in Figure (6). The result indicated that the interaction effect was not statistically significant. At the same time, the highest scavenging effect was recorded by the

interaction between var. *marianum* and 100 µg/mL. The IC50 were 17.027±0.064 µg/mL, 33.678±0.618 µg/mL, and 36.922±0.208 µg/mL, var. *marianum*, ascorbic acid, and var. *albiflorum*, respectively. The var. *marianum* gained a stronger IC50 than the ascorbic acid and the var. *albiflorum*, and it was relatively less than the ascorbic acid. An investigation was conducted by Serçe *et al*. (30) who evaluated the antioxidant capacity of the milk thistle plant in comparison with butylated hydroxyanisole and butylated hydroxytoluene (both used as positive controls) at different concentrations. The antioxidant capacity of the interaction between the milk thistle plant and 150 µg/mL was higher than both positive controls.

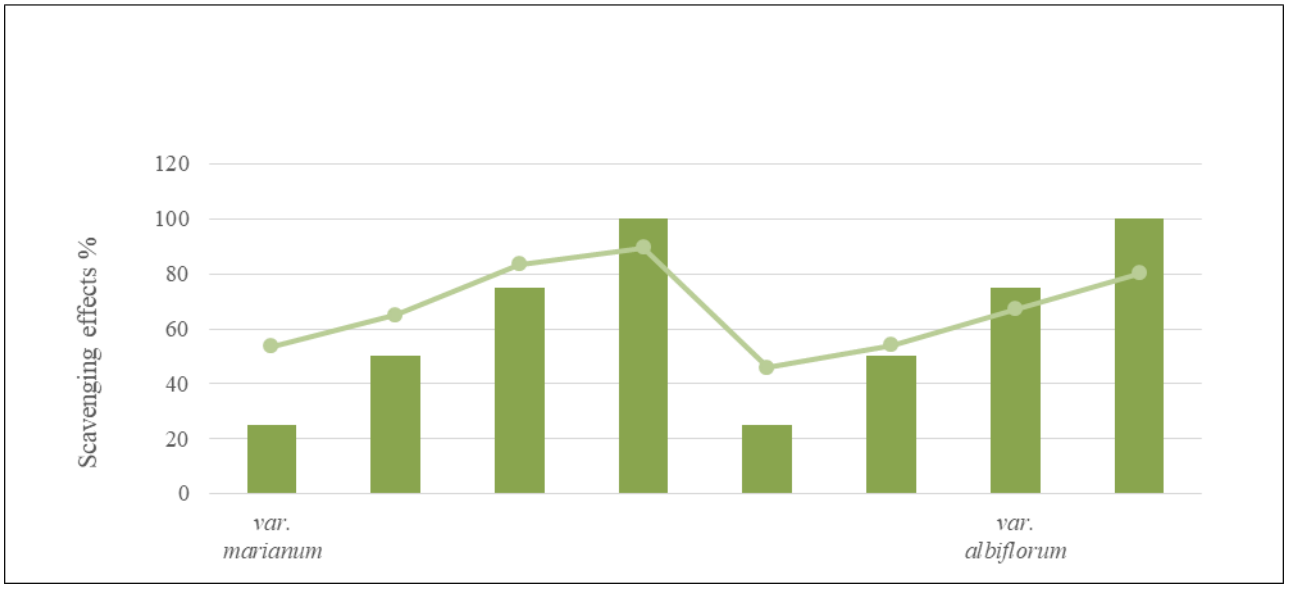

Figure 6. Milk thistle seed extracts scavenging effect (%) based on the interaction between varieties and concentrations var. *marianum* ($\hat{Y} = 0.5046X + 41.408, R^2 = 0.9698$), var. *albiflorum* ($\hat{Y} = 0.463X + 23.906, R^2 = 0.9889$)

CONCLUSIONS

In conclusion, these findings highlight morphological and chemical distinctions between the two milk thistle varieties and underscore their potential pharmacological and nutritional significance. HPLC analysis revealed var. *marianum* to contain a higher total silymarin content compared to var. *albiflorum*, with var. *marianum* also exhibiting higher levels of major flavonolignans. Gas chromatography-mass spectra analysis of seed hexane extracts identified varying phytochemical compounds between the two varieties, with notable differences in major compounds such as 5-hexenitrile and oleic acid. It was also indicated that antioxidant activity assays showed var. *marianum* to possess a higher scavenging effect on DPPH radicals compared to var. *albiflorum*, indicative of its potential as a rich source of antioxidants and food preservatives. Overall, the results could be useful as a good point to encourage researchers and farmers to cultivate different species of milk thistle by using different culture seasons under controlled conditions.

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