# The potential of cultivated milk thistle by-products as cancer chemopreventive and anti-inflammatory drugs

Sherin K. Ali[a], Nahla S. Abdel-Azim[a], Ali K. Khalil[b], Mohamed-Elamir F. Hegazy[a,c], Tarik A. Mohamed[a], Ahmed R. Hamed[a,d], Khaled A. Shams[a], Faiza M. Hammouda[a]

[a]Department of Chemistry of Medicinal Plants, [d]Biology Unit, Central Laboratory for Pharmaceutical and Drug Industries Research Division, National Research Centre, Giza,, [b]Department of Chemistry, Faculty of Science, Ain Shams University, Cairo, Egypt, [c]Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany

Correspondence to Sherin K Ali, MSC, Department of Chemistry of Medicinal Plants, National Research Centre, 33 El-Bohouth Street, Dokki, Giza 12622, Egypt.
Tel: +20 3739 2220; fax: +202 - 33370931;
e-mail: sheryali57@yahoo.com

**Received:** 18 July 2019
**Accepted:** 16 October 2019

**Egyptian Pharmaceutical Journal** 2019, 18:411–418

**Background and objectives**
Seeds of *Silybum marianum* (milk thistle), growing wild in Egypt, have been used since ancient times in traditional medicine. This study aims at identifying chemical and bioactivity properties of the oil of the seeds of cultivated *S. marianum*, obtained by cold pressing, as well as the extracts of the leaves. The prepared extracts were tested for their cancer chemoprevention and anti-inflammatory activities.
**Materials and methods**
The phytochemical constituents of cold-pressed seed oil and extracts of the leave were determined using gas chromatography–mass spectrometry and high-performance liquid chromatography (HPLC), respectively. The prepared extracts were tested for their cancer chemopreventive and anti-inflammatory activities.
**Results and conclusion**
HPLC profiling of leaves extract indicated that gallic acid and naringenin are the major phenolic acid and flavonoid, respectively. Additionally, HPLC analyses indicated the presence of seven main active components of silymarin in seeds. The total extract from leaves caused a moderate NAD(P)H-quinone oxidoreductase 1 protein induction and inducible nitric oxide synthase protein expression inhibition.
**Conclusion**
Cultivated *S. marianum* (milk thistle) by-products (oil and leaves) could have possible applications in food and pharmaceutical industry

**Keywords:**
Asteraceae, inducible nitric oxide synthase, NAD(P)H-quinone oxidoreductase 1, *Silybum marianum*, traditional medicine

Egypt Pharmaceut J 18:411–418

1687-4315

## Introduction

*Silybum marianum* (Asteraceae), commonly known as 'milk thistle,' is an annual or biennial plant, native to the Mediterranean area, North Africa, the Middle East, and in some parts of USA [1,2], growing wildly but can also be cultivated. The plant has been locally cultivated successfully in reclaimed lands of Egypt for medical uses of the seeds [3]. The active constituent of milk thistle is silymarin which is a complex of about seven flavonolignans, including silybin A and B, iso-silybin A and B, silydianin, silychristin, and the flavonoid taxifolin [1]. The seeds also contain other flavonolignans, betaine, apigenin, silybon, proteins, fixed oil, and free fatty acids [4] *S. marianum* has been known since ancient times and recommended in traditional medicine, mainly for treatment of liver disorders [5]. In recent years, there has been a growing interest in the properties of silymarin and other major metabolites in the medical, pharmaceutical, and veterinary sciences [1]. Silibinin is the most biologically active and exhibits anticancer and chemopreventive properties in various *in vitro* and *in vivo* models of various cancers, including lung [6], colorectal [7], breast, prostate [8], and brain cancers [9].

The oil of the seeds of the plant is useful for age-related diseases, including neurodegenerative diseases, and may be associated with diet as functional foods [10]. At the molecular level, the Kelch-like ECH-associated protein 1 (Keap1)/NF-E2 p45-related factor 2 (Nrf2) pathway orchestrates the protection against carcinogenesis [11]. In addition, inhibition of inflammatory pathways and targeting of cytokines have been strongly linked to cancer prevention [12,13] through nuclear factor-κB suppression.

The present study aims at identifying chemical and biological activities of the oil obtained from the cultivated plant seeds, as well as the leaves' extract.

The phenolics and flavonoids profiling in the extract of the leave was done using high-performance liquid chromatography (HPLC). Furthermore, experimental chemoprevention and inflammation models were tested to assess the potential of milk thistle extracts regarding the chemopreventive marker, NAD(P)H-quinone oxidoreductase 1 (NQO1), and the inflammatory marker, inducible nitric oxide synthase (iNOS).

## Experimental

### Plant material

Fresh leaves of *S. marianum* were collected in January 2015, and the seeds were collected at the end of February 2015 from Wadi Elsheh Farm, Assiut Governorate, Egypt, and identified by Prof. Dr. Ibrahim Ahmed ElGarf, Department of Botany, Cairo University. Herbal specimen is kept at NRC herbarium (voucher specimen #6411).

### Chemicals and instruments

Diethyl ether, chloroform, acetonitrile, and methanol were purchased from El-Nasr Company, Egypt. Sulfuric, acetic, and trichloroacetic acids and anhydrous sodium sulfate were purchased from Sigma-Aldrich (Taufkirchen, Germany). All of them were of analytical reagent grade. Plasticware for cell culture and assays were purchased from Greiner Bio-one (Frickenhausen, Germany).

Reagents for cell culture and *in vitro* models were purchased from Lonza (Verviers, Belgium) unless otherwise mentioned. Gas chromatography–mass spectrometry (GC-MS) analysis was carried out by using a TRACE GC ultra-GC (Thermo Scientific Corp., Miami, CA, USA) coupled with a thermo MS detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 mm×0.25 mm intradermal, 0.25 μm film thickness). HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using C18 column (4.6 mm×250 mm intradermal, 5 μm).

### Chemical analysis

Acid value, peroxide value, and iodine value of milk thistle seed oil were determined according to American Oil Chemists' Society (AOCS) [14].

### Preparation fixed oil

One kilogram of *S. marianum* seeds was cold-pressed at a commercial oil press in Cairo, Egypt, to obtain 200 ml of plant oil. The temperature was kept below 40°C, and no chemical or heating process was used.

### Fatty acid composition

Fatty acids were determined by the analytical methods described by Nazif [15]. In brief, the fatty acids were converted to fatty acid methyl esters before analysis. This was performed by shaking off a solution of 0.2 g of oil and 3 ml of hexane with 0.4-ml 2 N of methanolic potassium hydroxide. The fatty acid methyl esters were then analyzed by GC/MS. The injected volume was 0.2 μl. Helium was used as a carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1 : 10 using the following temperature program: 80°C for 1 min, rising at 4.0°C/min to 300°C, and held for 1 min. The injector and detector were held at 240°C. Mass spectra were obtained by electron ionization at 70 eV, using a spectral range of *m/z* 40–450.

### Silymarin extraction

The defatted seeds were extracted by soaking in ethyl acetate for 3 days. The collected extract was filtered through a Fisher brand QL100, 150-mm filter paper. Thereafter, the supernatant was evaporated till dryness under reduced pressure at 45°C, and then weighed and stored at −18°C for HPLC analysis.

### Preparation of standards

A methanolic solution of standard silymarin (0.7 mg/ml) was used to investigate the chromatographic behavior of the flavonolignan components of silymarin.

### Identification of silymarin by high-performance liquid chromatography

*S. marianum* defatted seeds' extract and standard silymarin were injected separately, to semi-prep HPLC for analysis using different proportions of $H_2O$ and methanol as mobile phase. The mobile phase used was 90 : 10 : 1 methanol : $H_2O$ : formic acid (solvent A) and $H_2O$ (containing 0.1% formic acid) (solvent B) at a flow rate of 0.7 ml/min. Injection volume was 5 μl. Detection was carried out by monitoring the absorbance signals at 288 nm.

### Leaves extraction

Fresh leaves of *S. marianum* were collected (1 kg) and dried in shadow to obtain 600 g. The dried leaves were ground and extracted with chloroform/methanol (1 : 1) in a percolator at room temperature for 3 days. After filtration, the combined extract was concentrated under reduced pressure at 40°C to dryness using a rotary evaporator to obtain 150 g.

### Identification and quantification of phenolics and flavonoids by high-performance liquid chromatography

HPLC chromatograms were detected using a photodiode array ultraviolet detector at wavelength

(280 nm) according to absorption maxima of the analyzed compounds. The mobile phase consisted of water (A) and 0.02% tri-flouro-acetic acid in acetonitrile (B) with a flow rate of 1 ml/min. The column temperature was maintained at 35°C. The injection volume was kept at 10 µl. A gradient elution was performed by varying the proportion of solvent B to solvent A. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A), 0–5 min (80% A), 5–8 min (40% A), 8–12 min (50% A), 12–14 min (80% A), and 14–16 min (80% A).

### Cell culture

The murine hepatoma cell line Hepa-1c1c7 was maintained as a monolayer culture in α-modified minimum essential medium Eagle supplemented with 10% (v/v) heat-inactivated and charcoal-inactivated fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate in humidified incubator (Sartorius CMAT, Germany, 5% $CO_2$/95% air). At about 80% confluence, cells were routinely subcultured with Trypsin EDTA solution.

Murine macrophage RAW 264.7 cells (ATCC) were maintained in complete Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), and 4 mM L-glutamine in a humidified 5% $CO_2$ atmosphere. For subculture and treatments, cells were scrapped off the flasks using sterile scrappers.

### Assessment of the induction of NAD(P)H-quinone oxidoreductase 1 in Hepa-1C1C7 cells

The induction of NQO1 in Hepa-1C1C7 cells was assessed. In brief, cells ($3\times10^5$ cells/ml) were seeded onto 6-well plates and left overnight to adhere and form semiconfluent monolayers. Monolayers were treated with either vehicle control (final concentration 0.1% v/v DMSO) or plant extracts (final concentrations of 50 and 100 µg/ml) for additional 24 h [16]. In parallel, sulforaphane was used as positive control for NQO1 induction. Monolayers were washed with ice-cold Dulbecco's PBS (2 ml/well). Cells were then scrapped in ice-cold homogenization buffer (25 mM Tris-Cl, pH 7.4, 250 mmol/l sucrose and 5 µmol/l FAD). Cell suspensions were then sonicated on ice for 5 s (20% amplitude). Sonicates were then centrifuged (15 000×g for 10 min) and the supernatants (cytosolic fractions) were aliquoted and stored at −80°C freezer until tested for protein expression.

### Western blot analysis

#### *NAD(P)H-quinone oxidoreductase 1*

Hepa-1C1C7 cells were cultured and treated as mentioned before. NQO1 protein expression was assessed in cell sonicates by Western blotting as previously described with some modifications [16]. Samples included vehicle control, positive controls (sulforaphane), and test samples (30 µg total proteins/lane). Samples were resolved under denaturing conditions by electrophoresis (SDS-PAGE) on 12.5% acrylamide/bisacrylamide gel (200 V for 1 h). Resolved proteins were then transferred to nitrocellulose membrane at 100 V for 60 min. Membranes were blocked in 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at 25°C and then probed overnight (4°C) with primary antibodies against NQO1 and β-actin (Abcam, Cambridge, UK). After three washes in TBST (10 min each), membranes were probed with 1 : 10 000 dilutions of appropriate secondary antibodies (Abcam) for 1 h at 25°C, washed three times in TBST, and then developed using enzyme chemiluminescence (ECL, Novex; Invitrogen, San Diego, CA, USA), and bands were detected using CCD camera (UVP, Cambridge, UK).

#### *Inhibition of inducible nitric oxide synthase induced by lipopolysaccharide*

Western blotting was also employed to analyze the relative protein expression of the pro-inflammatory marker iNOS based on Yang *et al.* [17] with some modifications. Overnight culture of RAW 264.7 (6-well plates, initially seeded as $1.5\times10^6$ cells/well) was treated with either 0.1% v/v DMSO [negative control lipopolysaccharide ($LPS^-$)] or milk thistle extracts (final concentrations of 50 or 100 µg/ml) in the presence of 100 ng/ml $LPS^+$ (Sigma-Aldrich). Indomethacin was used as iNOS positive control inhibitor (final concentration of 250 µmol/l). Following 24 h of exposure, cells were washed using ice-cold Dulbecco's PBS and scrapped in RIPA lysis buffer. After incubation for 20 min on ice, cell lysates were centrifuged at 15 000×g for 10 min at 4°C, and protein concentration was determined on a Thermo nanodrop spectrophotometer. Proteins in cell lysates were resolved on 10% PAGE gel (Bio-Rad Tetra Cell) and transferred onto nitrocellulose membrane. The membrane was blocked using 5% skim milk for 1 h at room temperature, followed by an overnight incubation at 4°C with 1 : 1000 dilution of iNOS primary antibody (Merck, Cambridge, Massachusetts, USA). Following four washes, the membranes were incubated with 1 : 10 000 dilution of horseradish peroxidase-conjugated secondary antibody (Abcam)

for 1 h at room temperature. Membrane proteins were detected using ECL (Novex; Invitrogen, USA), and bands were detected using CCD camera (UVP).

## Results and discussion

### Chemical analysis of seed oils

The physicochemical properties of seed oil of *S. marianum* cultivated in Egypt were determined in Table 1. The iodine value (107.1) was comparable to that found by Meddeb *et al.* [18]. Acid and peroxide values of the oil obtained from seeds of *S. marianum* were very low [19], indicating that the seeds oil of *S. marianum* are convenient for edible purposes [20,21].

### Analysis of fatty acid methyl esters by gas chromatography–mass spectrometry

Fatty acid composition of *S. marianum* seed oil is illustrated in Table 2, and GC/MS chromatograms are presented in Fig. 1. Ten fatty acids were detected, among which three were unsaturated. Linoleic and oleic acids were the most abundant and accounted for 30.27 and 28.93%, respectively.

The monounsaturated fatty acid content was higher than that of soybean oil (22%), corn oil (26.5%) [22], and sunflower (28.3%) [23]. The total saturated fatty acid of *S. marianum* oil was 36.57%, which rendered it strongly resistant to oxidative rancidity. Among the saturated fatty acids of the oil, palmitic and stearic acids were the highest, representing 12.90 and 8.76%, respectively. Behenic acid (6.73%) and arachidic acid (5.62%) were also detected. As the oil shows some similarity with some traditional edible oils, it could be used as a new potential source of edible oils, which could help to decrease the gap between local oil production and consumption. The obtained data suggested that cold-pressed seed oil could be considered as a rich valuable source for multipurpose products or by-products for cosmetic and pharmaceutical utilization.

**Table 1 Physicochemical characteristics of cold-pressed *Silybum marianum* seed oil**

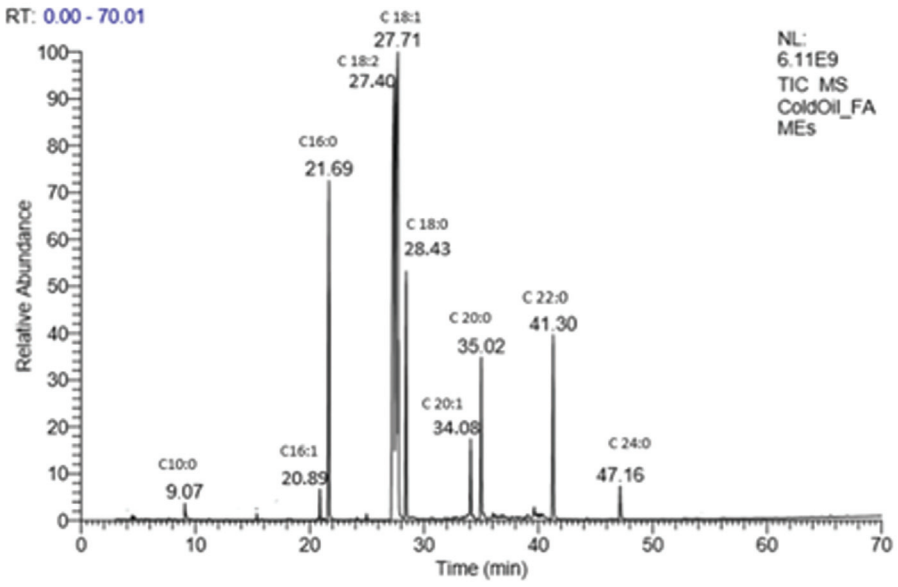
| Characterization | Oil extract by cold press |
|---|---|
| Acid value (mg KOH/g oil) | 4.49 |
| Peroxide value (meq $O_2$/kg oil) | 5.70 |
| Iodine value (g $I_2$/100 g oil) | 107.1 |

**Table 2 Fatty acid composition (%) of oil extracted from milk thistle seeds cultivated in Egypt**

| Compound name | Area (%) |
|---|---|
| C10:0 Nonanoic acid, 9-oxo-, methyl ester | 0.51 |
| C16:1 Methyl palmitelaidate | 0.91 |
| C16:0 Palmitic acid | 12.90 |
| C 18:2 Linoleic acid | 30.27 |
| C 18:1 Oleic acid | 28.93 |
| C 18:0 Stearic acid | 8.76 |
| C 20:1 Cis-11-Eicosenoic acid | 2.58 |
| C 20:0 Arachidic acid | 5.62 |
| C 22:0 Behenic acid | 6.73 |
| C 24:0 Lignoceric acid | 1.16 |
| SFA | 36.57 |
| MUFA | 31.51 |
| PUFA | 30.27 |

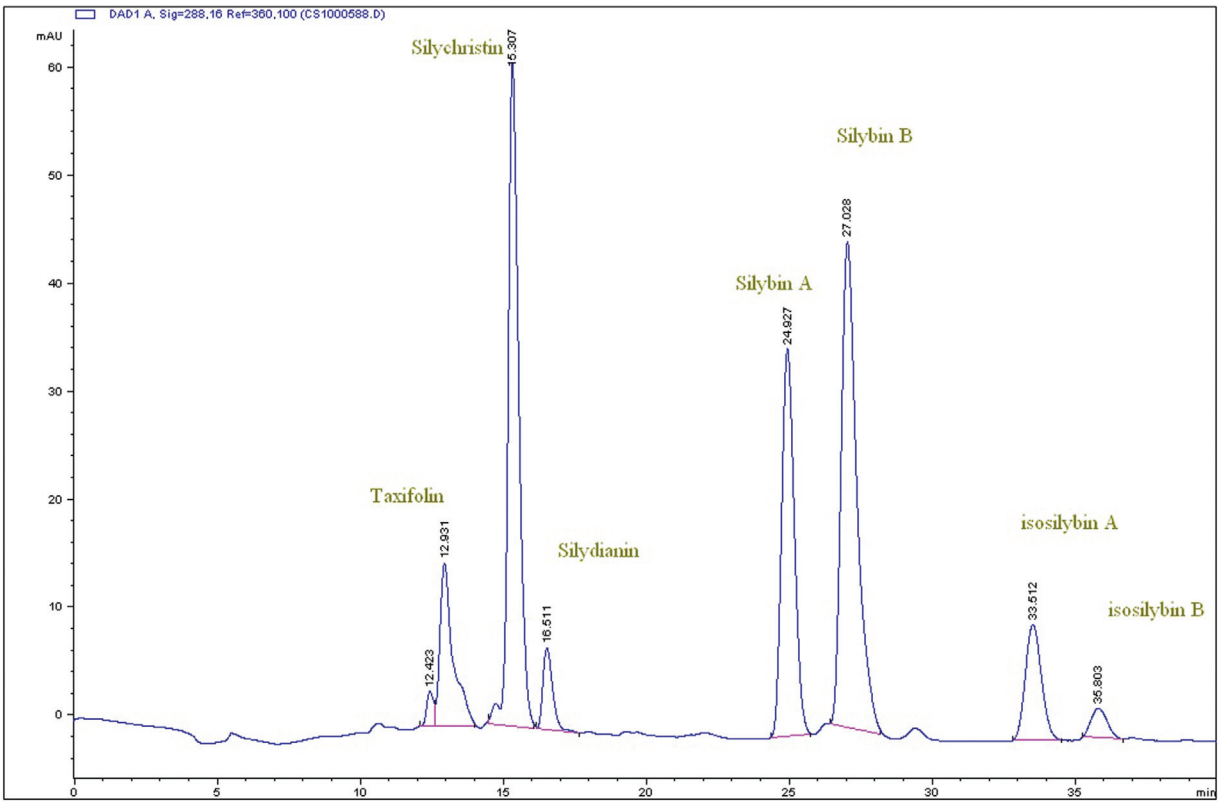
MUFA, monounsaturated; PUFA, polyunsaturated; SFA, saturated.

**Figure 1**



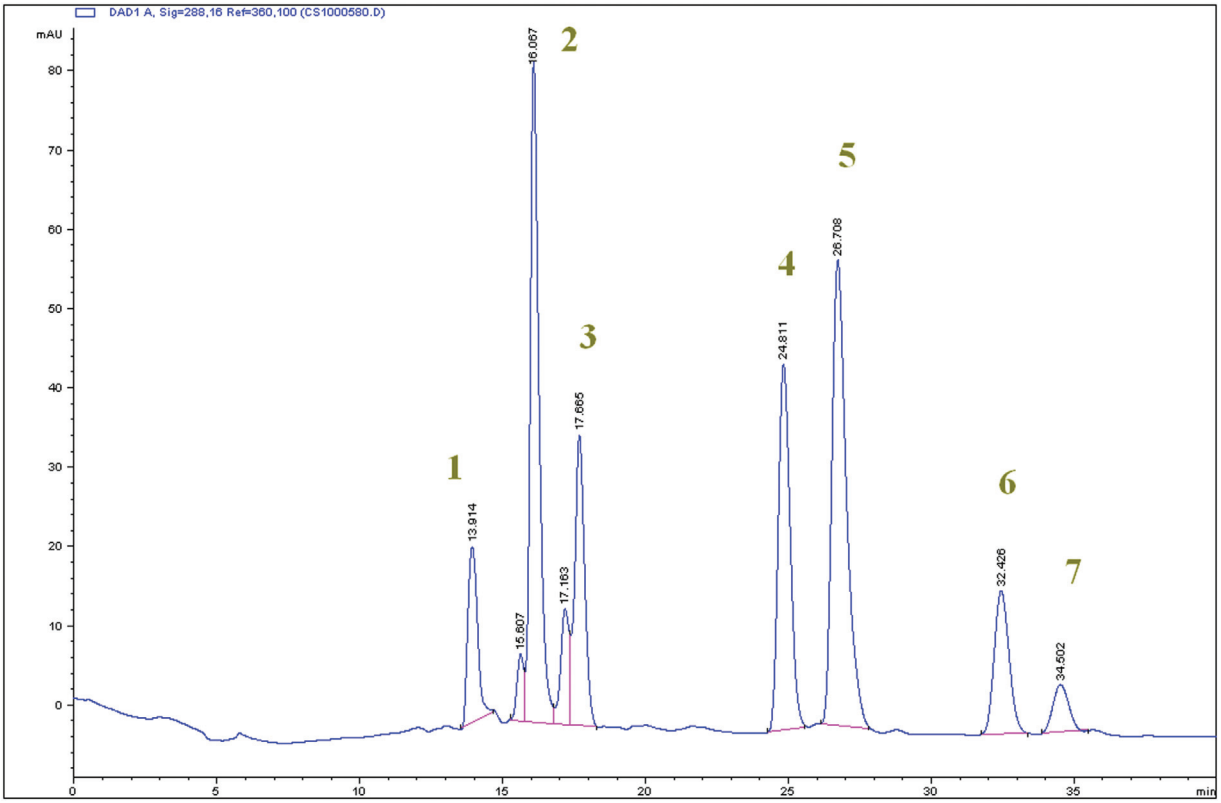

Gas chromatogram of *Silybum marianum* oil obtained by cold pressing.

**Figure 2**




HPLC chromatogram of standard silymarin. HPLC, high-performance liquid chromatography.

**Figure 3**




HPLC chromatogram of cultivated sample of *Silybum marianum* seed extract. HPLC, high-performance liquid chromatography.

### Identification of silymarin components in seeds

HPLC profiles of standard silymarin and defatted *S. marianum* seeds extract are presented in Figs 2 and 3, respectively. HPLC analysis showed the presence of seven main active constituents including taxifolin, silydianin, silychristin, diastereomers of silybin (silybin A and B), and diastereomers of iso-silybin (iso-silybin A and B) (Table 3).

Identification and quantification of phenolic and flavonoid components in plant leaves

The HPLC chromatogram is presented in Fig. 4, and the amounts of identified phenolic compounds are listed in Table 4. The most abundant phenolic acid was gallic acid (0.838 mg/g), which attracts the interest mainly for its wide range of pharmacological activities [24–29]. Additionally, naringenin as a major flavonoid (0.955 mg/g) was detected, along with coumaric acid (0.086 mg/g), ferulic acid (0.084 mg/g), caffeic acid (0.082 mg/g), and quercetin (0.038 mg/g).

### Induction of the chemopreventive marker NAD(P)H-quinone oxidoreductase 1

The induction of the cytoprotective protein NQO1 by phytochemicals has been a subject of interest to many investigators as a biomarker for cancer chemoprevention. The NQO1 protein is upregulated in response to phytochemical inducers through the Keap1/Nrf2 cellular pathway [11,30].

**Table 3 Composition of silymarin standard and cultivated seed extract**

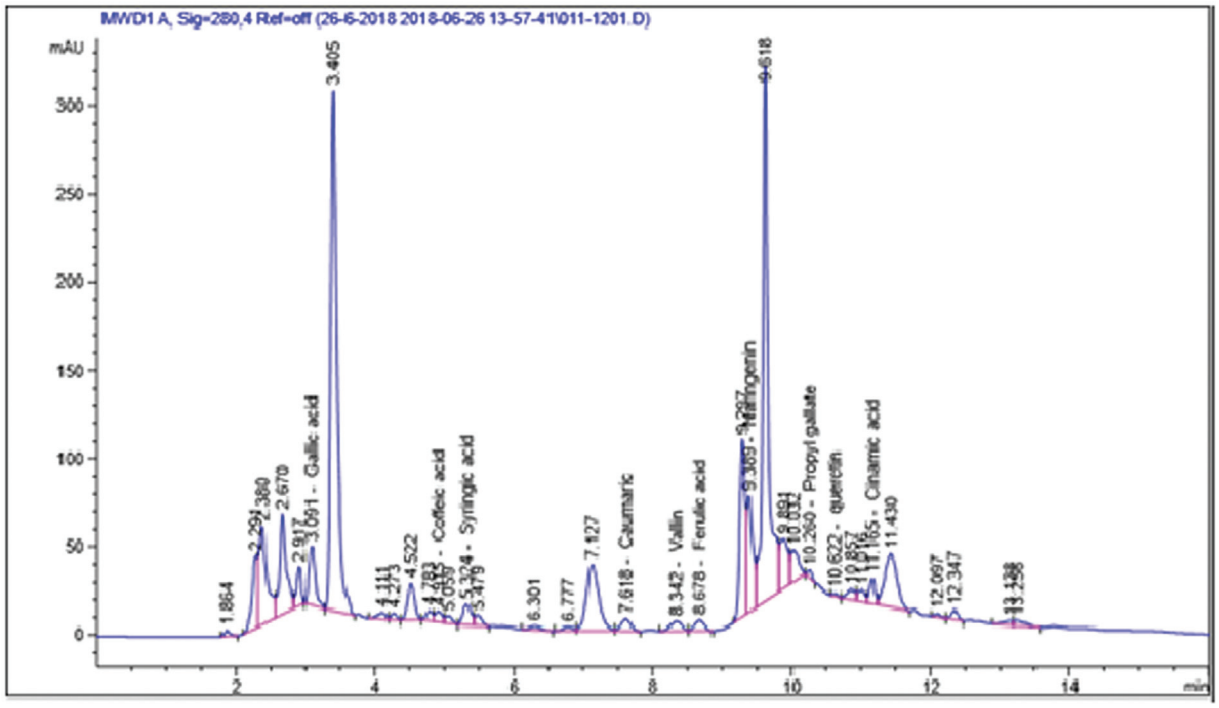
| Peak no | Silymarin compounds | Standard | Sample | |
|---|---|---|---|---|
| | | RT | RT | Area % |
| 1 | Taxifolin | 12.923 | 13.914 | 1.93 |
| 2 | Silychristin | 15.307 | 16.076 | 13.087 |
| 3 | Silydianin | 16.511 | 17.665 | 1.28 |
| 4 | Silybin A | 24.927 | 24.811 | 30.09 |
| 5 | Silybin B | 27.028 | 26.708 | 42.20 |
| 6 | Iso-Silybin A | 33.512 | 32.426 | 7.47 |
| 7 | Iso-Silybin B | 35.803 | 34.502 | 1.99 |

Retention time (min).

**Table 4 Determination of polyphenols in the chloroform : methanol (1 : 1) extract of leaves of *Silybum marianum* by using high-performance liquid chromatography method**

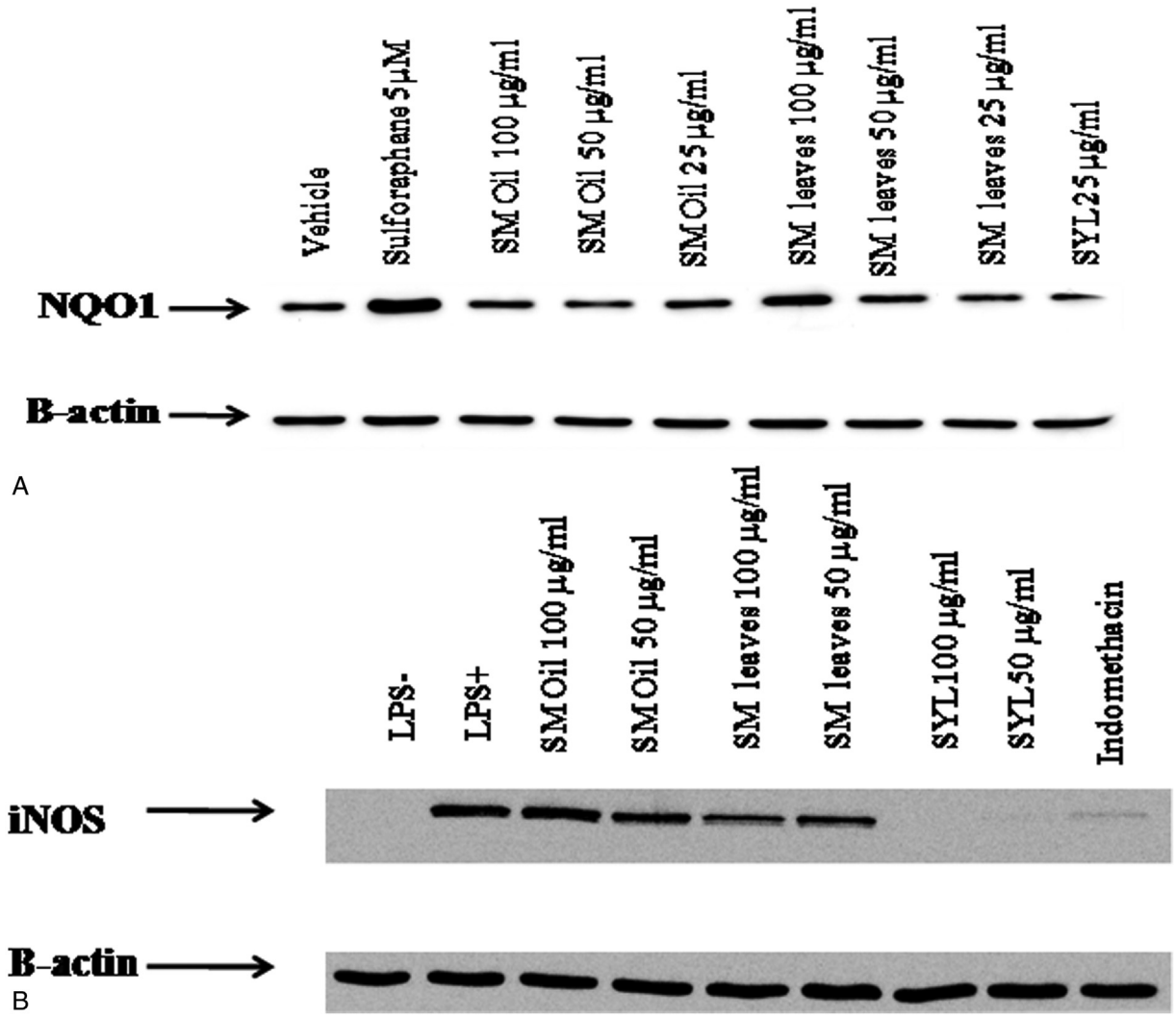
| Polyphenols | Area | Concentration (mg/g) | % |
|---|---|---|---|
| Gallic acid | 213.91 | 0.838 | 0.0838 |
| Caffeic acid | 42.93 | 0.082 | 0.0082 |
| Syringic acid | 91.58 | 0.224 | 0.0224 |
| Rutin | 0.00 | 0.00 | 0 |
| Coumaric acid | 67.28 | 0.086 | 0.0086 |
| Vanillin | 63.44 | 0.110 | 0.011 |
| Ferulic acid | 60.80 | 0.084 | 0.0084 |
| Naringenin | 364.65 | 0.955 | 0.0955 |
| Quercetin | 7.52 | 0.038 | 0.0038 |
| Cinnamic acid | 72.11 | 0.042 | 0.0042 |

**Figure 4**




HPLC chromatogram of the chloroform : methanol (1 : 1) extract of leaves of *Silybum marianum*. HPLC, high-performance liquid chromatography.

**Figure 5**




(a) Assessment of the cancer chemopreventive potential by Western blot analysis of the NQO1 protein expression. (b) Assessment of the anti-inflammatory potential by Western blot analysis of iNOS inhibition in RAW 264.7 macrophages. iNOS, inducible nitric oxide synthase; NQO1, NAD(P)H-quinone oxidoreductase 1.

In the present study, we employed western blotting to assess the chemopreventive potential of milk thistle leave extract, the oil fraction, and silymarin as inducers of the protein expression of NQO1 protein. As presented in Fig. 5, the total extract from leaves caused a moderate NQO1 protein induction of 60% compared with the vehicle control. However, the induction shown was at 100 µg/ml without apparent induction at 50 µg/ml. In a previous study [31] 2,3-dehydrosilydianin, an oxidized derivative from the flavonolignan silydianin isolated from milk thistle, induced the NQO1 mRNA level via Nrf2 activation in Hepa-1C1C7 cells. To the best of our knowledge, we report here NQO1 induction in milk thistle leaves for the first time.

### Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase protein expression in RAW 264.7 macrophages

Treatment of RAW 264.7 macrophages with LPS in the presence or absence of milk thistle samples and subsequent Western blotting analysis revealed the potency of silymarin to completely inhibit the LPS-induced iNOS protein expression at both tested concentrations (Fig. 5a). In addition, milk thistle leaves caused 41.5 and 48.5% inhibition of LPS-induced iNOS protein expression compared with the $LPS^+$ only-treated cells as revealed with densitometric analysis of bands in Fig. 5b. Our results are in agreements with previous studies that showed the potency of silymarin to inhibit iNOS expression [32,33]. However, this is the first report of the

potential of milk thistle leave constituents as anti-inflammatory agents.

### Conflicts of interest

There are no conflicts of interest.

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