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Quantitative identification of total silymarin in wild *Silybum marianum* L. by using HPLC

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Abstract
Silymarin, a mixture of flavonolignans exhibiting many pharmacological activities, is obtained from the fruits of milk thistle (*Silybum marianum* L. Gaertner). In this study, we measured the levels of total silymarin in the seeds of *Silybum marianum* from four different locations in Syria. Using high performance liquid chromatography. Results showed that, Silymarin concentrations varied according to the geographical location. Total silymarin concentrations in seeds ranged between 0.54 % and 2.91% dry weight. Significant differences in silymarin concentrations were found between various sampling locations as determined by LSD test at $P<0.05$. The highest concentrations of silymarin were in the seeds collected from Damascus (2.91%), and the lowest concentrations were found in seeds obtained from Homs (0.54%). seeds collected from Tartus and Lattakia contained (1.27%) and (1.39%) silymarin respectively. The *S. marianum* seeds is a cheap and wealthy source of silymarin in Syria.

Keywords: *Silybum marianum*, silymarin, high performance liquid chromatography

1. Introduction
Milk thistle (*Silybum marianum* L. Asteraceae family), is one of the most important medicinal plants in the pharmaceutical industry worldwide. This annual herb is native to Eastern Mediterranean and North African regions [1]. It grows as wild populations in open fields of the coastal, central and southern regions of Syria. This plant is well known in European and Asiatic traditional medicine for liver disorders treatment [2, 3]. Various parts of milk thistle, particularly the seeds, possess an antioxidant, antifungal and immune-modulator potential. It is used by alternative medical practitioners for treating a variety of disorders. It is a natural blend of many pharmaceutically important compounds which may contribute largely as a natural health healer for liver disorders. Two placebo-controlled clinical trials have also reported that administrating *Silybum marianum* seed extract to diabetic cirrhotic patients reduces insulin resistance and the need for exogenous administration of insulin [4-7]. The plant was also used to treat gallstones [8]. Intravenous preparations containing silibinin are licensed in a number of European countries to counteract Amanita mushroom poisoning in emergency rooms. Recent Seeds of this plant contains an isomeric mixture of flavonolignans collectively known as silymarin in a high concentration [9]. The principal components of silymarin are silybinin (~50 to 60%), isosilybinin (~5%), silychristin (~20%) and silydianin (~10%) [10]. Diastereoisomers of these compounds, (silybinin A, silybinin B, isosilybin A, isosilybin B) also exist; in addition to taxifolin [11, 12]. According to several earlier studies, silymarin is used as a strong antihepatotoxic therapeutic agent against almost every kind of human liver disease [13-15]. In addition to its antioxidant properties [16], it has been reported to have a high anti-tumor promoting activity [17], and antiproliferative effects [18], It also possess an anticancer actions on human prostate carcinoma *in vitro* and *in vivo* [19]. Recent research interest in this plant has been stimulated by studies showing its exceptional high antitumor activity. Extracts from this plant are now under intense studies in the experimental chemoprevention of cancer, and in the amelioration of chemotherapy side effects [20]. Many experimental tests have also reported that silymarin is effective in hepatitis C virus treatment [21, 22]. Silymarin shows anti-inflammatory and antifibrotic effects [23]. Previous in vitro studies reported a protective effect of silymarin on kidney cells against oxidative damage induced by paracetamol, cisplatin [24], aflatoxin B1 [25], fumonisin B1 [26], and ischemia/reperfusion injury [27]. Milk thistle is growing wild in Syria and covers large areas. Some local pharmaceutical companies plan to invest this plant commercially. However, no reports are available about silymarin concentrations in this local plant. In this study we investigated the concentrations of silymarin Milk thistle seeds found in Syria using high performance liquid chromatography (HPLC).

2. Materials and Methods

2.1 Sampling

Samples were harvested in May 2016 during maturation (dark brown color) from four wild plant populations located: at Lattakia province (coastal region), Tartus province (coastal region), Homs (inland region), and Yafoor site located in Rif-Damascus province (semi-arid region). The plants were identified by Prof. M. Oudat (taxonomist, AECS). Voucher specimens have been deposited in the laboratory of the plant biotechnology department at the Atomic Energy Commission of Syria (AECS). General important annual average meteorological parameters data for collection sites were also included (table1). For every location, three separate individual plants were collected; from which fruits (seeds) were taken. The raw materials were cleaned and oven dried at 40 °C, till constant weight was achieved.

Table 1: Milk thistle collection sites and their general meteorological parameters

Location	Longitude	Latitude	Altitude [m]	Average rainfall [mm/year]
Damascus	36° 7'25.77"	33°30'34.65"	945	212
Homs	36° 36'59.74"	34°43'6.13"	500	435
Tartus	35° 01'50.11"	35°54’26.96"	7	848
Lattakia	35° 54'20.98"	35° 1'49.57"	4	799

2.2 Samples extraction

Dried samples were extracted according to Çağdaş *et al.* [28] method. Briefly, 10g of nonfat milk thistle powder was weighed accurately and dissolved in 50mL of methanol using a homogenizer for 15 minutes at 3000 rpm/min. Intensified by nitrogen to the volume of 10mL. The mixture was then filtered through Whatman no.1 filter paper and the extract was stored at -8 ˚C until analysis. All samples were extracted in triplicates.

2.3 Chemicals, Standards solution

Silymarin Standard purchased from Sigma Aldrich and contains (Silybin A, Silybin B, Isosilybin A, Isosilybin B, Silychristin, Silydianin, Apigenin-7-glucoside, and Taxifolin). Solvents and other reagents were obtained from Merck. Silymarin stock solution was prepared at 1 mg/ml concentration in methanol. The standard working solutions used to build calibration curve were prepared by serial dilutions: 100, 250, 500μg/ml of stock solutions with methanol.

2.4 High performance liquid chromatography (HPLC) analysis

Chromatographic separation was achieved with an LC system from Agilent (Infinity 1260) equipped with a diode array detector (DAD), using a reversed phase Eclipse C18, (150×4.6mm, 3.5 μm) column from Agilent Co. The separation was conducted at 35 °C and the mobile phase consisted of [A] water with 0.1% formic acid; [B] methanol (1:1). The flow rate was 0.6 ml/min, and the injected volume was 20μl, with UV detection at 254 nm.

2.5 Statistical Analysis

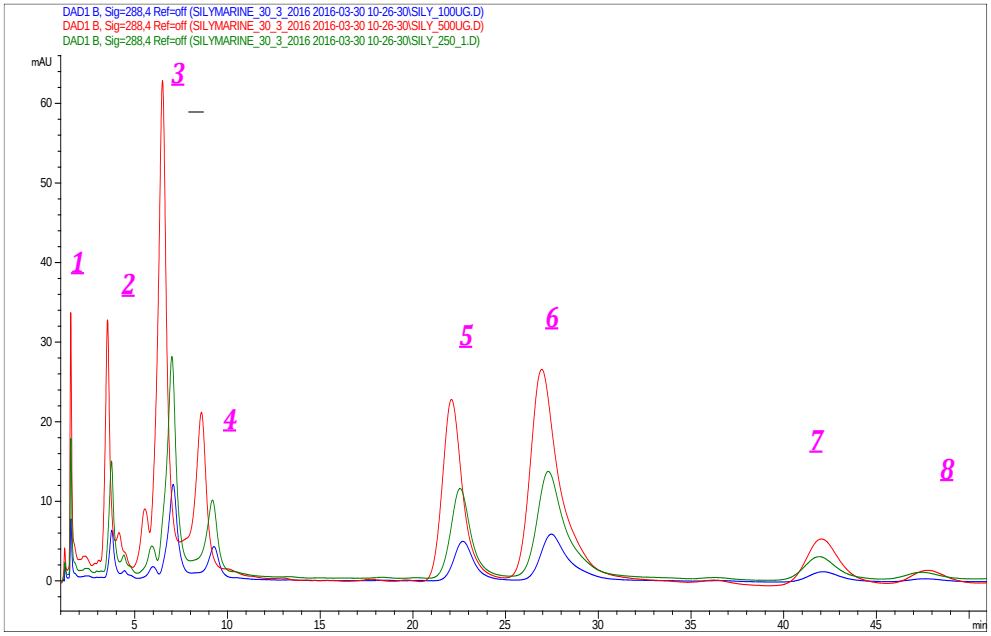
Measured concentrations were expressed as means. One way analysis of variance (ANOVA) was used to assess the significance of differences among variables. SPSS software (v, 17) was used to perform multiple comparison test by applying the least significant difference LSD at *p* values less than 0.05. Microsoft Excel program was also used to generate statistical histograms.

3. Results

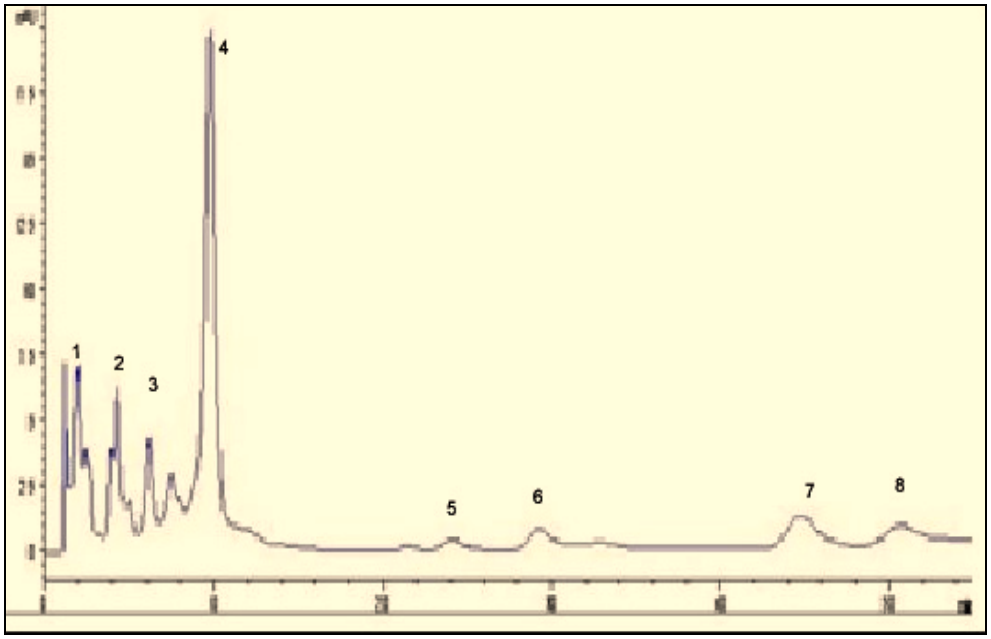
Figure 1a, shows the HPLC profile of polyphenol standards for silymarin. Whereas, Figure 1b illustrates the chromatograms of methanolic extracts of dried nonfat milk thistle samples in comparison to silymarin standard**.** As seen in chromatograms, eight principal peaks were observed, each peak was identified as one of the flavolignan constituents of the silymarin samples. The peaks were identified as: (1) Taxifolin, (2) Silychristin, (3) Apigenin-7-glucoside, (4) Silydianin, (5) Silybin A, (6) Silybin B, (7) Isosilybin A, and (8) Isosilybin B, respectively. A good correlation of linearity has been achieved (n=3; R^2 = 0.99904 - 0.99999) in the range of 100-500 μg/ml for silymarin. Limits of detection (LOD) and quantification (LOQ) values are summarized in table 2.

The concentrations measured in samples analyzed by HPLC are reported in table 3. Results showed that silymarin constituent’s concentrations in seeds extracts varied according to the geographical location and metrological conditions. The highest concentration of silydianin in silymarin samples were found in seeds collected from Rif-Damascus location then from Lattakia, and Tartus. The least concentration was in the seed sample collected from Homs location. The highest amounts of other components such as isosilybin B, silychristin, isosilybin A were 4.22, 3.87 and 2.88 mg/g, respectively, in silymarin samples from Rif-Damascus location.

Quantitative analyses showed that the amount of total silymarin in seeds ranged from 0.54% to 2.91% of dry weight, for tested location ns. Total silymarin concentration showed the highest value in Rif-Damascus location (2.91%), and the lowest concentration of silymarin was measured in Homs location (0.54%) compared with (1.27%) and (1.39%) in Tartus and Lattakia, respectively (table 3). The total silymarin concentration in samples from Rif-Damascus site was 2 times higher than that present in seeds samples from Lattakia site. 5.3 times higher than that recorded in seeds samples from Homs site, and 2.3 times higher than that recorded in samples from Tartus site (table 4).



A



B

Fig 1: Retention time (min) Typical HPLC chromatograms of chemical standards and tested samples.
A: chemical standards for silymarin b: Chromatogram of silymarin in the Seeds of *S. marianum*. (1= Taxifolin, 2= Silychristin, 3= Apigenin 7-glucoside, 4= Silydianin, 5= Silybin A, 6= Silybin B, 7= Isosilybin A, 8= Isosilybin B).

Table 2: LOD and LOQ results for silymarin

Sample	LOD µg/ml	LOQ (µg/ml)
Seeds	12	18

Table 3: Concentrations of silymarin constituents in seeds samples of *S. marianum* (mg/g) dry weight

Silymarin constituents	Location			
	Damascus	Homs	Tartus	Lattakia
Taxifolin	1.29±0.03	0.72±0.02	2±0.02	2.07±0.02
Silychristin	3.87±0.03	0.49±0.01	1.44±0.03	1.54±0.01
Apigenin 7-glucoside	0.70±0.01	0.10±0.01	0.17±0.01	0.22±0.01
Silydianin	15.33±0.01	3.07±0.01	5.95±0.03	6.32±0.02
Silybin A	0.39±0.02	0.10±0.01	0.15±0.01	0.19±0.01
Silybin B	0.50±0.02	0.05±0.01	1.10±0.02	0.10±0.02
Isosilybin A	2.88±0.01	0.60±0.02	1.2±0.01	1.50±0.01
Isosilybin B	4.22±0.01	0.71±0.01	1.79±0.01	1.95±0.03

Data are expressed as mean±SD (n=3)

Table 4: Total silymarin content (mg/g dry weight, percentage) in seeds samples of *S. marianum*.

Location	Total silymarin
Damascus	29.1 (2.91%)
Homs	5.4 (0.54%)
Tartus	12.7 (1.27%)
Lattakia	13.9 (1.39%)

Silymarin concentration expressed as mg/g derived from the average of three independently extraction replicates.

4. Discussion

Milk thistle is among the top selling herbs in the world [29]. Demand on this plant increased significantly due to the increased uncontrolled spread of hepatitis worldwide [30]. It is well-known that silymarin content varies between 1.5 and 3.5% in the fruits, with 3 to 6% considered of a high quality [31]. Results of chemical analyses showed that all silymarin samples were the same in view of their components, but they were different in their flavonolignans content and geographical location. Analysis of variance using LSD test showed statistically significant differences (P=0.000<α=0.05) in silymarin concentrations between various sampling locations.

Previous studies reported that total silymarin content in seeds ranges from 0.77 to 1.37g/100g [28]. Wallace *et al*. [32], found that the yield of total silymarin was 1.6g/100g seeds. Radjabian *et al*. [33], reported that the levels of silymarin in seeds harvested from Iranian locations ranged from 23.98% to 45.46%. A concentration of 4.1% DW for total silymarin also reported in seeds samples originated from Croatia [34].

Since milk thistle is very adaptable to many different habitats, this hints that certain ecotypes with more favorable amounts of flavonolignans are suitable for domestication and intended purposes [33]. Despite the economical and pharmaceutical values of milk thistle, efforts on domestication and breeding of this plant have been low [35]. There is evidence of genetic differences between ecotypes of milk thistle with regard to their content of silymarin and its constituents**.** Silymarin content and composition in milk thistle is affected by many factors such as sowing depth, harvesting and post harvesting treatment, fruits during maturity, plant parts. In addition, Different environmental conditions such as rainfall, the altitude above sea level, soil texture, phenological stage and etc,), plays an important role in silymarin content [36-39].

5. Conclusion

Silymarin concentrations in *S. marianum* L. seeds collected from four different regions in Syria were in Rif-Damsacus > Lattakia> Tartus> Homs. This imposes the economic importance of *S. marianum* seeds and plant as a cheap and wealthy source of silymarin in Syria.

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