Silymarin content in *Silybum marianum* populations growing in Egypt

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Abstract

Chemical variation of *Silybum marianum* growing in the north, middle, and south of Egypt was investigated. Variation was assessed according to the content of the individual silymarin components in the fruits of the plant. The fruits were distinguished according to location, plant variety, and fruit color (maturity). Accelerated solvent extraction was used to standardize the silymarin extraction. Quantitative analysis of the content of silymarin components was carried out using HPLC with qNMR-controlled reference standards of taxifolin and seven major flavonolignans including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin. The quantification method was validated in accordance with ICH guidelines. Principal component analysis and hierarchical clustering were carried out to create homogeneous clusters of samples based on the content of the silymarin components. Taxifolin had the lowest correlation relative to other silymarin components, whereas silybin A was positively correlated with silybin B. The samples clustered into three classes: silydianin-rich samples, samples with an average silymarin content of <18.8 mg/g, and one class enriched in silymarin (>18.8 mg/g). *S. marianum* growing in the Nile delta showed the highest silymarin content. No correlation was found between fruit color and silymarin content, indicating that the fruit maturity stage has no significance.

Keywords

1. Introduction

Milk thistle [*Silybum marianum* (L.) Gaertn., Asteraceae] is an annual or biennial herb, native to the Mediterranean and North African regions (Morazzoni and Bombardelli, 1995). The plant has an erect ridged stem that reaches to heights 150 cm and deeply lobed leaves

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characterized by white patches along its veins. The fruits of *S. marianum* are of achene type having shiny dark brown–black color. Two varieties of this species occur in Egypt; variety *purple* with purple corollas and variety *albiflorum* with white corollas (Boulos, 2002). The two varieties are growing wild along canal banks, roadsides, and waste ground. The plant is classified according to its ecological amplitude as a frequent species in the canal bank habitats of the Nile in Egypt (Mashaly et al., 2010).

Silymarin, in the form of a standardized extract prepared from milk thistle fruits, is widely used as hepatoprotective agent. Silymarin exerts its hepatoprotective activity through antiviral, anti-inflammatory, antioxidant, and immuno-modulatory actions in the liver and immune cells (Polyak et al., 2007; Ahmed-Belkacem et al., 2010; Morishima et al., 2010; Wagoner et al., 2010). Silymarin has also shown in vitro efficacy as a cancer chemopreventive agent by arresting human prostate carcinoma proliferation in cancer cell cultures (Tyagi et al., 2002) and in human cancer models (Singh et al., 2002). The name, silymarin, was originally introduced by Wagner et al. (1965) when describing the flavonolignans mixture as containing silybin, isosilybin, silydianin, and silychristin. Subsequently, this mixture has been further resolved into so far seven flavonolignans and one flavonoid (Fig. 1) that altogether comprise 65–80% of the milk thistle extract. Both silybin and isosilybin were subsequently resolved into pairs of diastereoisomers, silybin A & B and isosilybin A & B, respectively. The isosilybins A and B are regioi-somers of silybin A and B, respectively. The absolute configurations of these four isomers studied using 2D NMR and CD spectroscopy (Kim et al., 2003).

In 2014, the plant was placed at the sixth position among the twenty top-selling herbal dietary supplements in the natural and health food market and position among the forty top-selling herbal supplements in the mainstream multi-outlet channel market in U.S., at about $9.2 million and $16,4 million, respectively (Smith et al., 2015). According to the European Pharmacopoeia and the United States National Formulary, mature fruits of *S. marianum* yield not less than 1.5–2% of silymarin.

It is well known that medicinal plants collected from different locations may considerably differ in their active constituents, resulting in potentially different therapeutic efficacy. These differences are caused by various environmental conditions and habitats in which the medicinal plants are grown. The plants develop locally adopted subpopulations, known as ecotypes (Shokrpour et al., 2007). Variation in the ecotypes can be characterized by chemical or genetic means (Mahmood et al., 2010; AbouZid, 2014). Analysis of chemical variation by chromatographic or spectroscopic provides a means of measuring and standardizing the metabolites responsible for the therapeutic effects of milk thistle.

The present study was aimed at differentiating wild populations of *S. marianum* growing in Egypt based on their silymarin content as a potential source for silymarin products. The study was also designed to determine whether links exist between the chemical variation and the location, plant variety, or fruit color as an indicator of maturity of the fruits.

2. Materials and methods

2.1. Chemicals

Silymarin (S0292-10G, Sigma, China), silybin primary reference standard 92.53% (HWI ANALYTIK GMBH pharma solutions, Rülzheim, Germany), and taxifolin analytical standard 85% (Sigma–Aldrich, St. Louis, MO, USA) were used as reference compounds. Isosilychristin, silychristin, and silydianin were prepared by preparative column chromatography and identified by ^{1}H NMR (Abraham et al., 1970; Diep et al., 2007; Napolitano et al., 2013). The solvents used in this study, *n*-hexane and acetone (Pharmaco-AAper, Brookfield, CT, USA) were of reagent grade and re-distilled. For analytical HPLC, methanol (Fisher Scientific, Fair Lawn New Jersey, USA), water and formic acid (Sigma–Aldrich, St. Louis, MO, USA) were of HPLC grade. Silica gel 60A was used for column chromatography (Fisher Scientific, New Jersey, USA, 200–425 mesh). Sephadex LH-20 (Sigma, Sweden) was used for column chromatography. Pre-coated silica gel plates ALUGRAM® SIL G/UV$_{254}$ for TLC (E-Merck, 10×20 cm) were used for analysis of the extracts, fractions, and isolated compounds. A BRUKER Avance digital NMR spectrophotometer AV400 (9.4 T/400 MHz, Oxford magnet) equipped with 5 mm Z-gradient probe BBO (broadband observe) networked with MS Window-based computer with XWIN-NMR for acquisition and processing of the spectra, was used for acquiring the ^{1}H NMR spectra.

2.2. Plant material

S. marianum fruits heads were collected from populations growing in nine different locations in Egypt during the period 26/3-15/4/2014. The altitude and latitude of the locations are shown in Table 1. Botanical authentication was carried out by Dr. Abdel Halim Mohamed, Flora and Phytotaxonomy Department, Agricultural Research Center, Cairo, Egypt. The fruits were manually separated from the heads and freed of the pappus. Color and size of the fruits were examined (Table 1). Length, width, and thickness of the fruits were measured (mean of six measurements) using a Manostat 15-100-500 (Switzerland) caliper.

2.3. Silymarin extraction

Accelerated Solvent extraction was carried out with a Dionex ASE350 instrument (Dionex Corporation, Sunnyvale, CA, USA) according to a reported method (Wianowska and Wi niewski, 2015) with modifications. The whole ground fruits (1 g) was placed into a 34-ml stainless steel extraction cell containing filter paper at the bottom. The cell was tightly closed and placed in the extractor. The content of the cell was extracted with acetone at a temperature of 70 °C, at a pressure of 1700 psi, for 15 min. Two extraction cycles were used. For defatting, *n*-hexane was used at 50 °C, 1600 psi for 30 min. The extraction volumes were 61–64 ml. The extraction solvent was evaporated to dryness under vacuum, dried in a vacuum desiccator, weighed, and re-dissolved in methanol for further chromatography. Three replicates were extracted per each sample.

2.4. Flavonolignan analysis

High performance liquid chromatography analysis was performed on Waters 2695 system using Agilent ZORBAX SB-C18 column (5 μm, 4.6 × 250 mm, PN 880975-902, SNUSCLO10981, USA). A Waters 996 PDA detector was used to monitor the absorbance of the eluent at 288 nm. The mobile phase consisted of MeOH:H$_2$O/0.1% formic acid with a gradient run initiated with 30:70 and increased linearly to 60:40 over 32 min. Flow rate was 1 ml/min. Ambient temperature was used. Empower™ software (Waters Corp., Milford, MA) was used to acquire, process, and analyze chromatographic data.

2.5. Validation of chromatographic method

For determination of linearity, different concentrations of silybin reference standard working solution were transferred to a series of 5-ml volumetric flasks and diluted to volume with methanol, to furnish eight solutions containing 3–630 μg/ml. Each of these solutions was injected in triplicate, and peak areas of silybin were recorded. A calibration curve was constructed relating peak area to the corresponding concentration, and the regression equation was calculated. Repeatability, as intra-day variation, was determined by triplicate analysis of sample 3P64B. Reproducibility, as inter-day variation, was determined by analysis of the same sample on three consecutive days. The limits of detection and quantification were determined in accordance with ICH recommendations. The accuracy of the method was assessed by analysis of medium and high concentrations of pure silybin primary reference standard. The results were compared with the expected results from the regression equation, and accuracy was calculated.

2.6. Isolation of silydianin, silychristin, and isosilychristin

S. marianum crude extract was monitored by thin layer chromatography (TLC) using chloroform (0.1% formic acid):acetone—9.5:0.5, 5:5, 8:2. The TLC plates were examined under ultraviolet light short (254 nm) wavelength, and sprayed with 10% sulfuric acid reagent. The crude extract (2.3 g) was loaded on silica gel (5 g) and the dried loaded silica was applied onto a silica gel column (120 g) using methylene chloride (0.1% formic acid) as a mobile phase and increasing the polarity by acetone (5–50%). Forty fractions (50 ml) were collected. The fractions obtained from column chromatography were concentrated under reduced pressure and monitored by TLC using chloroform:acetone:formic acid (75:16.5:8.5) as a solvent system and 10% sulfuric acid as a spray reagent. The fractions were monitored against authentic silymarin and silybin. Fractions 22 and 23, tested positive for the presence of silychristin, were pooled and further purified on Sephadex LH-20 column chromatography using methanol as a mobile phase. Thirty sub-fractions were collected, concentrated under reduced pressure, and monitored by TLC using chloroform (0.1% formicacid):acetone—9.5: 0.5, 5:5, 8:2. The TLC plates were examined under ultraviolet light short (254 nm) wavelength, and sprayed with 10% sulfuric acid spray reagent. Sephadex CC fractions 7 and 8 showed one spot at R_f value 0.32. These fractions were further analyzed by HPLC and NMR. Fractions 17 and 18, showed a distinct spot under the silybins at R_f value 0.44, were pooled and further purified on Sephadex LH-20 column chromatography using methanol as a mobile phase. Twenty two sub-fractions were collected, concentrated under reduced pressure, and monitored by TLC using chloroform

(0.1% formic acid):acetone—9.5:0.5, 5:5, 8:2. The TLC plates were examined under ultraviolet light short (254 nm) wavelength, and sprayed with 10% sulfuric acid. Sephadex CC fractions 5 and 6 showed one spot at R_f value 0.44. These fractions were further analyzed by HPLC and NMR. Fractions 13–16 from the first silica column, tested positive for the presence of silybins, were pooled and further purified on Sephadex LH-20 column chromatography using methanol as a mobile phase. Thirty sub-fractions were collected, concentrated under reduced pressure, and monitored by TLC using chloroform (0.1% formic acid):acetone–9.5:0.5, 5:5, 8:2. The TLC plates were examined under ultraviolet light short (254 nm) wavelength, and sprayed with 10% sulfuric acid spray reagent. The isolated compounds were identified by comparing their spectroscopic data with available literature (Abraham et al., 1970; Diep et al., 2007; Napolitano et al., 2013).

2.7. Purity determination of isolated flavonolignans

An absolute qHNMR method was used for the determination of the purity of silydianin, silychristin, and isosilychristin, using an externally calibrated residual protonated solvent signal as internal calibrant (Pauli et al., 2014). DMSO-d_6 was used as NMR solvent. The content of residual protonated DMSO-d_5 in the DMSO-d_6 lot was established using the internal calibration qHNMR method with silybin primary standard sample as calibrant. The purity of the calibrant was assayed by qHNMR method according to the manufacturer certificate of analysis to be 92.53%. Samples were weighed (0.01 mg accuracy), dissolved in 600 μl of DMSO-d_6 (Lot no. 10E-645, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) using a Pressure-Lok gas syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA), and transferred into 5-mm standard NMR tubes (NORELL Inc., Landisville, NJ, USA). The qHNMR spectrum was measured for the calibration sample using quantitative acquisition and processing parameters as follows: single 90° ^{1}H excitation pulse with GARP carbon decoupling for measuring silydianin and isosilychristin spectra; and standard 30° ^{1}H excitation pulse for measuring silychristin spectrum; relaxation delay 60 s, acquisition time 4 s, number of scans 64, non-spinning mode, sample temperature 25 °C, data points 64 K, dummy scans 4, and pre-acquisition delay 59.57 usec. Probe tuning and matching were performed on each sample. The ^{1}H NMR data were analyzed using ACD NMR Manager, version 12.01. Processing involved zero filling to 262,144 data points, Fourier Transform, manual phase correction, baseline correction, calibration, integration, and peak assignment.

2.8. Statistical analysis

Results are expressed as mean ± SEM. Principal component analysis was used to visualize the correlation between *S. marianum* samples growing in Egypt using Microsoft Office XLSTAT 2015. Hierarchical clustering was used to create homogeneous clusters of the samples.

3. Results and discussion

3.1. Collection of S. marianum *fruit samples*

Fruits of *S. marianum* were collected from nine different locations including one from Upper Egypt, two from Middle Egypt, three from Nile delta, and three from Mediterranean flora (Table 1). Coordinates, altitude and latitude for these locations are shown in Table 1. The

fruits were distinguished according to the plant variety; var. *purple* and var. *albiflorum*. They were further separated according to their color as creamy white, brown, or black.

3.2. Extraction of S. marianum *fruits by accelerated solvent extraction*

Silymarin is traditionally extracted from *S. marianum* fruits using a two-step process according to European Pharmacopoeia. The fruits are first defatted using *n*-hexane in a Soxhlet extractor for 8 h, and then silymarin is extracted using methanol for 5 h. This method is time consuming, uses large amounts of solvents and generates waste solvent, and importantly is associated with major losses of silymarin during the defatting process. The use of accelerated solvent extraction in the present study reduced time and solvent requirements, used a controlled temperature, and allowed static and dynamic extraction. Its use of solvents at elevated pressure, i.e., temperatures above the boiling points, increased the dynamic of the extraction; enhanced analyte diffusion through cell walls; and decreased solvent viscosity and surface tension. Acetone was used as an extraction solvent as it has been reported to produce the highest silymarin yield (Wianowska and Wi niewski, 2015).

3.3. Development of a chromatographic method for silymarin determination

An HPLC method was developed to quantify individual silymarin components in the extracts. HPLC has been reported previously as a suitable method for quantitative analysis of silymarin components (Quaglia et al., 1999), comparing RP-18 and RP-8 sorbents for their ability to achieve baseline separation of the flavonolignans. While RP-18 showed good resolution for silybin and isosilybin, silydianin and silychristin resolution was insufficient. Increasing the water concentration in the mobile phase allowed the separation of the two diastereomeric silybins. On the other hand, while RP-8 stationary phase improved the resolution of peaks related to all flavonolignans, it did not resolve the two silybin diastereomers. In the current study, taxifolin and seven flavonolignans representing the major silymarin components were baseline separated (Fig. 2). Available reference standards allowed us to assign retention times for the following silymarin components: taxifolin (R_t = 13.168), silybin A (R_t = 27.138), silybin B (R_t = 28.139), isosilybin A (R_t = 30.174), and isosilybin B (R_t = 30.815). Silydianin, silychristin and isosilychristin were isolated using a two-step column chromatography scheme and identified based on their fingerprint ^{1}H NMR data (Napolitano et al., 2013). The ^{1}H NMR spectra of silydianin, isosilychristin, and silychristin, isolated in the current study as well as silybin (A + B) as primary reference standard are shown in Fig. 3. An absolute qHNMR method was used for the purity determination of these reference materials using the externally calibrated residual protonated solvent signal of DMSO-d_6 as internal calibrant. The qHNMR purities and the HPLC retention times were as follows: isosilychristin 73.96% (R_t = 17.805), silychristin 80.80% (R_t = 19.687), and silydianin 84.83% (R_t = 21.455).

3.4. Validation of the chromatographic method

Next, the HPLC quantification method was validated in accordance with the ICH guidelines. Stock solution of silybin primary reference standard was diluted to a series of concentrations with methanol to test the limits of quantification (LOQ) and detection (LOD). The intra- and inter-day precision was determined for one sample (3P64B) by analyzing three replicates on the same day and on three consecutive days, respectively. The relative standard deviation

(RSD%) was taken as a measure of precision. Accuracy was determined using a recovery test upon addition of medium and high amounts of authentic standard samples of silybin (A + B). Each sample was analyzed in triplicate. The validation parameters for the developed method are shown in Table 2.

3.5. Analysis of samples

Using the developed and validated HPLC quantification method, the extracts prepared from the samples of *S. marianum* fruits collected throughout Egypt were analyzed. The silymarin content in the fruits from different populations growing in Egypt varied between 0.06–2.19% (Table 1). Table 3 shows the content of individual silymarin components in different samples. The data revealed that the two samples of variety *albiflorum*, 1W + 293 and 3W64, collected from northern Egypt at 29° and 30° N, from Cairo–Alexandria desert road and El-Beheira governorate (north of Egypt), respectively, exhibited the same qualitative flavonolignan patterns typically found in purple varieties. On the other hand, W154 sample (variety *albiflorum*) collected from Assiut city 27° N (south of Egypt) showed a different flavonolignan profile.

The accessions of fruits represented nine different locations, the two varieties *purple* and *albiflorum*, and three different colors of fruits: creamy white, brown, and black (Fig. 4). The color of *S. marianum* fruits (achenes) changes during maturation from creamy white to a light brown or even black color, depending on conditions of the maturation and/or storage. *S. marianum* deposits pigments in the sub-epidermal layer of the pericarp during its development (Upton et al., 2010; Martinelli et al., 2014), and this process depends on the influence of exterior factors (Stoiljkovi et al., 2007). It was previously reported that the silymarin content increases concomitantly with the ripening stage of the fruits (Elwekeel et al., 2013). There are two metabolic processes associated with the ripening of the fruits: pigment deposition, responsible for changes in the fruit pericarp, and silymarin formation and/or deposition. Therefore, it was a reasonable hypothesis that these two processes are related; i.e., that black fruits contain more silymarin than the lighter-colored ones. This would have potential implication for the selection of the fruits used for silymarin production in an industrial process or by consumers of the whole fruits. Usually the fruits available commercially exhibit a range of pericarp colors. In the case of *S. marianum* fruit samples collected from Egypt, samples from two populations (3P64 and 1P + 293) showed all three different colors of the fruits (Table 1), which were separated and analyzed for silymarin content. Notably, based on the present findings, the different-colored fruits are ripe, but immature with regard to pigment deposition. This finding contrasts a previous report that showed silymarin content changes with development of the fruits, i.e. fruits changing their color from creamy white (unripe & immature) to brown to black (ripe & mature).

However, Table 1 shows that there is no significant difference in the silymarin content in fruits of different colors collected from population 3P64. This result is confirmed in the overlaid chromatograms of the three ripening stages of the fruits: white, brown, black. The white fruits showed more silymarin content in case of samples collected from population 1P + 293 (Fig. 5).

3.6. Principal component analysis

Principal component analysis (PCA) was carried out to study the relationship between *S. marianum* growing in Egypt, using Pearson's correlation coefficient to show their degree of similarity. Cumulative variability (in%) was used to determine the number of factors to be used for data interpretation. Due to the high correlations between the entire variable as shown from the correlation matrix, there were 8 factors for the initial 8 silymarin components. F1 (76.60%) and F2 (16.48%) represented 93.08% of the total variability. Therefore, the variables were represented in the space of these two factors (Fig. 6). The correlation matrix showed that taxifolin has the lowest correlation with other silymarin components. In addition, silybin A and silybin B are positively correlated ($r = 1$). The outliers are the silydianin-rich samples (1P64, 1W + 293, P263, and 2P293), and *S. marianum* var. *albiflorum* collected from Assiut city that showed very low concentrations for the analyzed silymarin components.

3.7. Agglomerative hierarchical clustering

Agglomerative hierarchical clustering (AHC) was carried out to create homogeneous clusters of samples based on the content of the individual silymarin components. ACH clustered the samples into three classes; the dotted line in the dendrogram (Fig. 7) represents the automatic truncation leading to three classes. The first class has four samples; the second and the third class each have six samples. The first class, displayed in green color, represents the silydianin-rich samples, as shown by PCA in the current study. The second class includes samples containing an average silymarin content (<18.8 mg/g). The third class is comprised of the populations that are most rich in silymarin content. All of the samples belonging to the high-content class were collected from the Nile delta, except for P154.

4. Conclusions

This study focuses on a wild plant of medicinal importance, *S. marianum*, growing in Egypt. Due to anthropogenic pressures and demand from economic development projects, the areas of wild plants are decreasing with a great loss of the natural biodiversity. Our data show that significant differences in the yield of therapeutically active phytoconstituents exist in Egypt's wild milk thistle populations, and that these parameters as well as optimized extractions protocols can be used to drive the sustainable use this natural resource.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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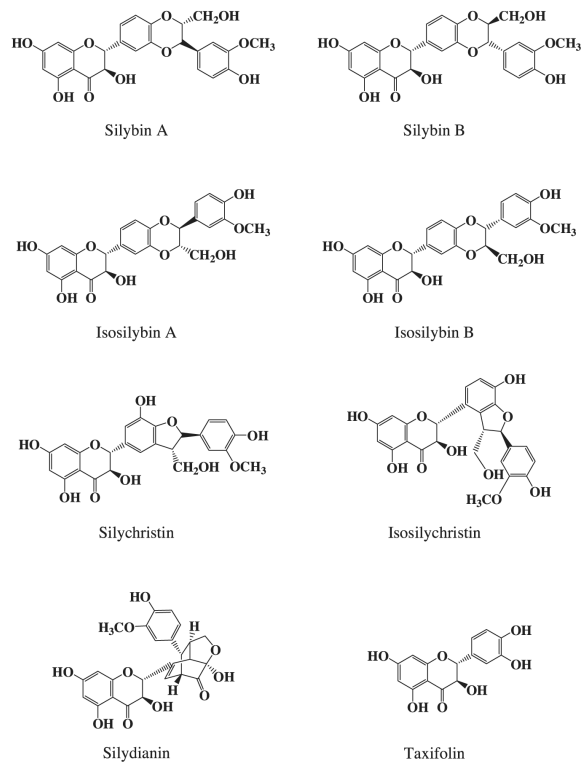
Fig. 1.
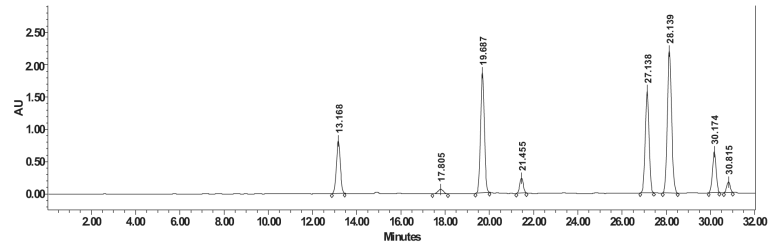Chemical structures of silymarin components.

Fig. 2.
HPLC chromatogram showing the analysis of silymarin components. The key to peaks: taxifolin (R_t = 13.168), isosilychristin (R_t = 17.805), silychristin (R_t = 19.687), silydianin (R_t = 21.455), silybin A (R_t = 27.138), silybin B (R_t = 28.139), isosilybin A (R_t = 30.174), and isosilybin B (R_t = 30.815).
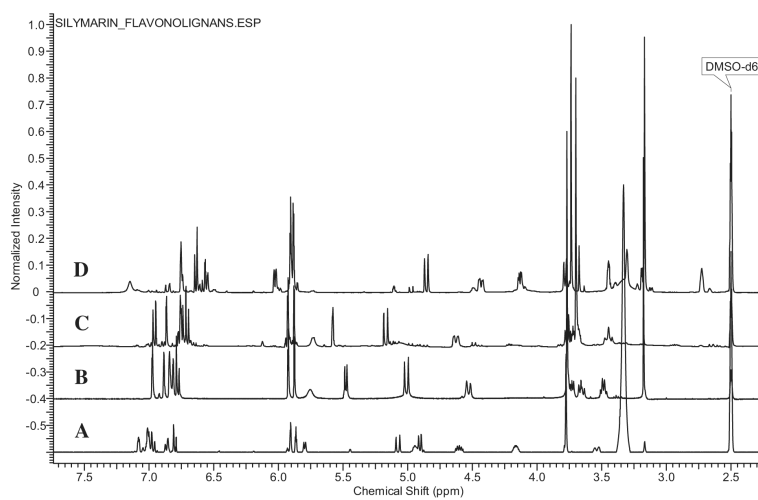
Fig. 3.
Collected ^{1}H NMR spectra of isolated flavonolignans: primary reference standard sample of silybins (A), silychristin (B), isosilychristin (C), and silydianin (D).

Fig. 4.
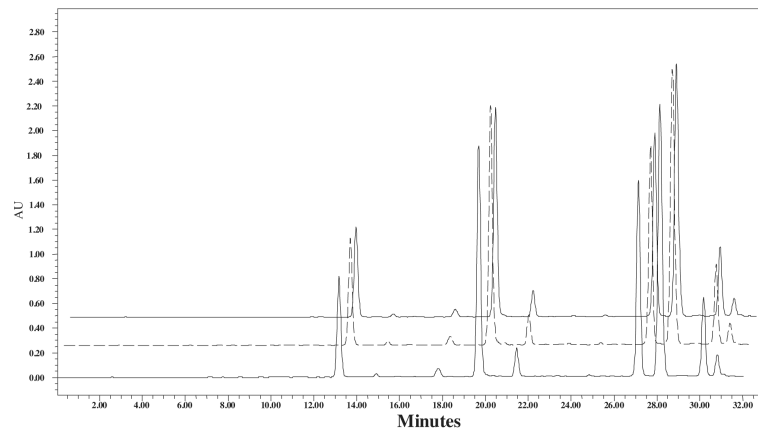The three different colors of *Silybum marianum* fruits.

Fig. 5.
Chromatograms of *Silybum marianum* fruits (population 3P64) of white (front, solid line), brown (middle, dashed line), and black (back, dotted line) colors.

Fig. 6.
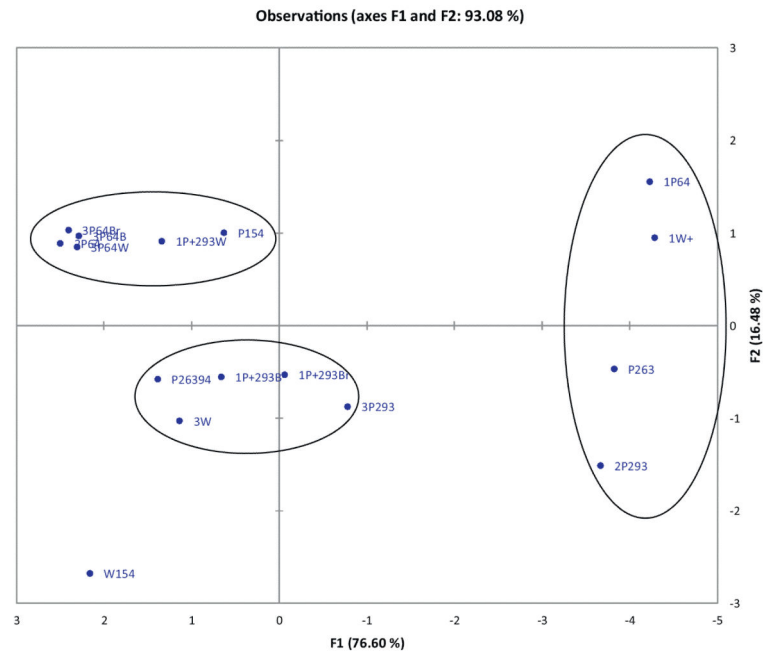Principal component analysis of the content of silymarin components in *Silybum marianum* samples growing in Egypt. Plot of the observations in the space of F1 and F2 representing 93.08% of variability. Fruit sample codes correspond to those in Table 1.
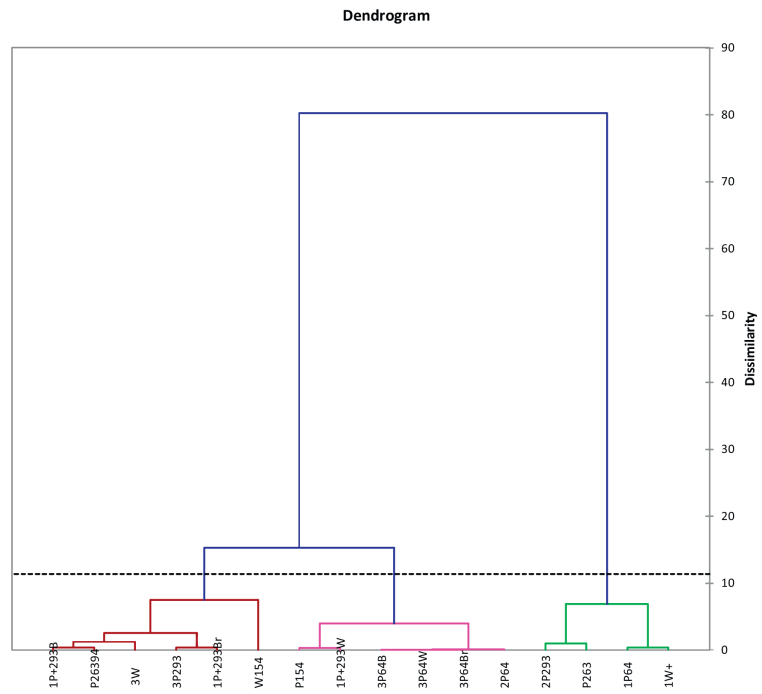
Fig. 7.
Agglomerative hierarchical clustering for *Silybum marianum* samples growing in Egypt.
Fruit sample codes correspond to those in Table 1.

Table 1

Silybum marianum fruit samples collected from Egypt, three replicates were analyzed for each sample.

Location governorate/city	Coordinates	Population code	Fruit sample code	Variety	Fruit color	No. fruits/head	Fruit dimension (L, W, T in mm)	Silymarin content (mg/g)
Assiut/Assiut	27°11′58.56″N	P154	P154	*purple*	brown	81±28.4	6.8, 2.9, 1.8	18.89±0.51
	31°10'22.22"E	W154	W154	*albiflorum*	brown	66±23.2	6.7, 2.9, 1.8	0.58±0.03
Beni-suef/Beni-suef	29°5'4.69"N	P263	P263	*purple*	brown	137±13.4	6.8, 2.8, 1.9	8.60±0.59
	31°6'19.91"E							
Beni-suef/Alwasta	29°25'47.17"N	P26394	P26394	*purple*	brown	135±20.5	7.1, 2.7, 1.9	18.63±1.54
	31°11'33.77"E							
Qaliubiya/Dandna	30°19'53.69"N	1P64	1P64	*purple*	brown	56±23.0	6.6, 2.9, 1.9	10.14±0.69
	31°12'5.58"E							
Menofia/Kewisna	30°33'31.51"N	2P64	2P64	*purple*	brown	58±9.7	7.3, 2.8, 2.0	21.92±0.67
	31°8'5.11"E							
El-beheira/Itai Elbaroud	30°56'34.57"N	3P64	3P64W	*purple*	white	77±28.0	7.6, 3.0, 2.1	21.40±2.48
	30°35'13.98"E							
			3P64Br	*purple*	brown	77±28.0	7.2, 2.9, 1.9	21.49±1.37
			3P64B	*purple*	black	77±28.0	6.8, 2.8, 1.9	21.20±1.57
		3W64	3W64	*albiflorum*	black	88±27.4	7.0, 2.8, 1.9	15.91±2.76
Cairo–Alexandria Road (1)	30°57'45.27"N	1P + 293	1P + 293W	*purple*	*white*	116±40.7	7.6, 2.8, 1.8	19.69±1.00
	29°49'58.05"E							
			1P + 293Br	*purple*	brown	116±40.7	7.6, 2.8, 1.8	14.20±0.81
			1P + 293B	*purple*	black	116±40.7	7.0, 2.8, 1.9	15.54±0.48
		1W + 293	1W + 293	*albiflorum*	*brown*	119±43.1	7.4, 2.8, 1.8	9.16±0.16
Alexandria/Borg Al-arab	30°58'33.40"N	2P293	2P293	*purple*	brown	142±49.8	7.0, 2.9, 2.0	5.91±0.64
	29°39'41.40"E							
Cairo–Alexandria Road (2)	31°0'8.45"N	3P293	3P293	*purple*	black	91±7.7	6.8, 3.3, 1.9	11.02±1.02
	29°48'20.91"E							

Table 2

Validation parameters of the HPLC method for analysis of silymarin components.

Validation parameter	Value
Linear range (mg/ml)	0.003–0.630
Regression equation	$Y = [2.10^{\wedge}(-07)]X - 0.066$
Regression coefficient	0.999
LOQ (mg/ml)	0.003
LOD (µg/ml)	0.005
Precision (repeatability RSD%, $n = 3$)	
Intra-day	0.216
Inter-day	2.785
Accuracy (recovery%)	
Medium concentration	94.797
High concentration	93.490

Table 3

Content of silymarin components in *Silybum marianum* samples collected from Egypt (mg/g fruits). Values are averages of three replica±standard error. For fruit sample codes, refer to Table 1.

Fruit sample	Taxifolin	Isosilychristin	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
P154	1.64±0.03	0.48±0.03	4.12±0.11	1.29±0.10	3.59±0.10	5.69±0.14	1.59±0.05	0.48±0.01
W154	n. d.	n. d.	0.27±0.01	n. d.	0.07±0.03	0.22±0.01	0.01±0.01	n. d.
P263	0.95±0.04	0.54±0.18	0.56±0.08	4.40±0.53	0.20±0.05	0.53±0.08	0.89±0.03	0.53±0.03
P26394	1.00±0.11	0.34±0.19	4.66±0.36	0.44±0.05	4.08±0.34	6.36±0.45	1.47±0.13	0.29±0.05
1P64	1.18±0.07	1.01±0.08	0.55±0.02	4.36±0.33	0.27±0.01	0.70±0.03	1.30±0.12	0.77±0.07
2P64	2.18±0.08	0.12±0.03	5.40±0.18	0.52±0.04	4.62±0.16	7.08±0.19	1.65±0.03	0.34±0.01
3P64W	2.25±0.27	0.16±0.04	5.11±0.03	0.58±0.25	4.46±0.49	6.81±0.72	1.61±0.20	0.32±0.05
3P64Br	2.40±0.17	0.14±0.01	5.19±0.30	0.54±0.05	4.41±0.32	6.85±0.40	1.63±0.09	0.32±0.04
3P64B	2.31±0.18	0.16±0.01	5.08±0.36	0.54±0.05	4.41±0.35	6.75±0.46	1.62±0.12	0.33±0.04
3W64	1.35±0.25	0.08±0.03	3.86±0.67	0.39±0.08	3.42±0.58	5.35±0.88	1.23±0.22	0.24±0.05
1P + 293W	1.61±0.08	0.37±0.03	4.55±0.21	0.76±0.10	4.02±0.19	6.24±0.11	1.69±0.09	1.69±0.03
1P + 293Br	1.08±0.09	0.35±0.02	3.17±0.17	0.75±0.09	2.79±0.15	4.41±0.23	1.27±0.08	0.39±0.03
1P + 293B	1.26±0.03	0.58±0.02	2.92±0.11	1.94±0.01	2.43±0.10	3.97±0.14	1.51±0.05	0.60±0.02
1W + 293	1.13±0.04	1.01±0.02	0.46±0.02	4.14±0.10	0.17±0.01	0.52±0.01	1.13±0.02	0.67±0.02
2P293	0.48±0.03	0.50±0.03	0.33±0.03	2.87±0.50	0.14±0.03	0.38±0.03	0.74±0.03	0.48±0.04
3P293	0.57±0.07	0.04±0.01	2.77±0.24	0.24±0.03	2.47±0.23	3.91±0.35	0.86±0.08	0.14±0.02