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Short communication

Tracking and quantifying the major silymarin flavonolignans in the fruit extracts of milk thistle (*Silybum marianum* L.) using a validated HPTLC-MS method

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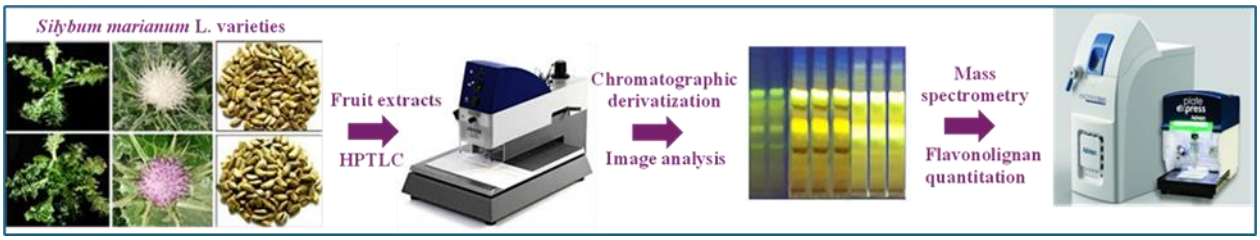

Abstract:

High performance thin layer chromatography hyphenated with mass spectrometry (HPTLC-MS) was implemented for the first time for the simultaneous tracking and quantitation of the major flavonolignans in fruit extracts of milk thistle (*Silybum marianum* L.*)* varieties; marianum and albiflora. A mobile phase of chloroform: acetone: formic acid (110: 16.5: 8.5 v/v/v) was utilized for the development of the tracked flavonolignans followed by their visualization under UV 366 nm after NP/PEG derivatization. The tracked flavonolignans were identified, using mass analysis of the developed flavonolignans directly from the developed plate, and quantified. The developed method's linearity, accuracy, and precision were all confirmed in accordance with ICH requirements. Linear calibrations for the quantified flavonolignans were obtained with a correlation coefficient > 0.98. Precision was confirmed with a relative standard deviation ranging between 1.34 and 1.83%. In addition, acceptable accuracy was obtained with recovery exceeding 97%, as well as appropriate limit of detection (LOD) and limit of quantitation (LOQ).

Keywords: HPTLC-MS, *Silybum Marianum* L. Varieties, Flavonolignans.

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1. Introduction

Silybum marianum (L.) Gaertn., frequently referred to as 'Milk thistle', is a valuable Asteraceae member with a distinct spiny appearance. It is represented by two varieties; var. marianum and var. albifora, distinguished by the color of their floral corolla [1,2]. Milk thistle content of flavonolignans is called silymarin which is responsible for its reputative hepato-protective potential [3], in addition to cardiovascular-, neuro- and skin-protective capabilities exerted via different mechanisms including antioxidant and anti-inflammatory ones [4,5]. Silymarin is utilized to maintain liver function against triggers for its impairment through hepatic cell regeneration and reconstruction and its cellular membrane stabilization opposing different stresses and free radicals [6]. Silymarin flavonolignans are natural compounds comprising 3', 4' dihyroxy flavone 'taxifolin' and hemilignan 'coniferyl alcohol' moieties conjugated via dioxane or furan rings or even resulted from B-ring fission [7,8]. There are various chemotypes of *Silybum marianum* with different proportions of silymarin flavonolignans mainly silybin, isosilybin, silychristin, silydianin [9].

The fluctuation in the proportions of the various silymarin constituents in *Silybum marianum* varieties is a crucial subject to be examined as different biological potentials have been attributed to the individual flavonolignans [10]. Therefore, a feasible analytical method should be utilized for tracking the different flavonolignans in *Silybum* fruits.

Accordingly, this study aims at hyphenating high performance thin layer chromatography (HPTLC) - image analysis to mass spectrometry for profiling and quantitation of the major flavonolignans in *Silybum* fruit extracts in a reproducible validated manner.

2. Experimental:

2.1. Samples collection and preparation:

The two varieties of *Silybum marianum* (L.) Gaertn.; variety marianum and variety albiflora were collected from different geographical locations in Alexandria. Three individual fruit samples were prepared for each variety. Freshly collected samples (500 g) were exposed to alcoholic extraction and ultra-sonicated at 45°C for 30 minutes, filtered and dried completely under vacuum. Samples were prepared for HPTLC application via the precise dissolving of 1 g of each fruit extract with 10 ml methanol in volumetric flasks followed by filtration using syringe filter (0.2 µm).

2.2. Standard solution preparation:

Silybin, isosilybin, and silychristin reference standards were purchased from Sigma-Aldrich, USA. Taxifolin, and silydianin standards were ordered from LGC standards, UK. Silyamandin was obtained from Biorbyt bioreagents, US.

The purchased flavonolignans were prepared as stock solutions of concentrations 50 µg/µl silybin, 30 µg/µl isosilybin, 15 µg/µl silydianin , 10 µg/µl taxifolin 20 µg/µl silychristin and 5 µg/µl silyamandin. Calibration curves were constructed using serial dilutions of the mentioned flavonolignans for attaining silymarin mixture, to be utilized for their HPTLC quantitative analysis in the examined fruit samples.

2.3. High performance thin layer chromatography HPTLC

8 µL of the different representative samples along with 6 µL of the standard silymarin mixture solution were applied on sample plates using a 100 µL syringe of Linomat V Camag spray applicator (Muttenz, Swizerland) programmed with WinCats manager software (Camag, 2008) using the following settings: 10 mm were left from the margins, the plate bottom was adjusted at 15

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mm, with bandwidth 8 mm and bands were spaced by 4 mm. The plate was then developed in a double twin trough CAMAG glass chamber filled with 50 ml mobile phase composed of chloroform: acetone: formic acid (110: 16.5: 8.5) [11,12].

2.4.Post-chromatographic derivatization:

The developed plate was submerged in 0.5% methanolic 2- aminoethyl diphenyl borane (Natural Product NP) reagent, followed by immersing in 5% methanolic polyethylene glycol (PEG) 400. The plate was then dried and photographed under UV light at λ 366 nm using digital camera (18 megapixel, Samsung, Korea).

2.5. Image processing:

The obtained image of the derivatized plate was analyzed using ImageJ 1.51h software (Wayne Rasband, NIH, USA) yielding two-dimensional plots of the examined samples for pixels' intensities against their distances along a fixed line.

2.6. Mass spectrometry analysis:

Prominent bands corresponding to individual flavonolignans in the resultant fruit profiles were identified using mass spectrometry hyphenated to HPTLC of the already developed HPTLC plate using automated Advion plate reader Express ™ of TLC-MS interface where compounds were eluted directly from the plate using solvent mixture of methanol: chloroform (4:1) with 0.2 ml/min flowrate. Both positive and negative modes switched during analysis and were utilized for data acquisition using ESI as an ion source for a mass range of 100-600 Da with the aid of Advion compact mass spectrometer (CMS) NY/ USA.

2.7. HPTLC quantitative analysis:

Identified flavonolignans' peak areas were determined using ImageJ software from the derivatized HPTLC plates for quantitation of these compounds. The developed quantitation method was then evaluated and validated in accordance with ICH guidelines regarding linearity, specificity, accuracy and precision, in addition to limit of detection (LOD) and limit of quantitation (LOQ) [13,14]. LOD and LOQ were calculated according to the equations LOD = 3SD/σ and LOQ = 10SD/σ, where SD is the standard deviation of the response (y-intercept) while σ is the slope of the calibration curve. Quantitation data were employed for the investigation and differentiation of *Silybum marianum* L. fruit extracts. Accuracy was evaluated using the standard addition analysis method where known amounts of each standard were added to prequantified samples in the analytical range then accuracy was calculated as recovery and relative standard deviation (RSD%) of the standards (tracked flavonolignans) in the samples. Intermediate precision was estimated as RSD% of six repeated measurements of the standards at 100 % of test concentration over 6 days.

3. Results and discussion

3.1. Investigating the major silymarin constituents in the fruit extracts of S*ilybum marianum;* var. marianum and var. albiflora

HPTLC has been routinely approached for the quantitative estimation of the individual flavonolignans. It is considered one of the easiest and quickest analytical tools, where the chromatogram scan can be detected directly under UV at 254 and 366 nm, or after using different visualization/spraying reagents [15,16]. Based on the characteristic ultraviolet absorption of flavonolignans, natural products-polyethylene glycol (NP/PEG) reagent is commonly used where relevant bands are detected at 366 nm [17].

Based on the developed and derivatized chromatogram (Figure 1), bands at R_f values of 0.71, 0.62, 0.51, 0.45, 0.43 and 0.34 were found to be the most prominent in fruit profiles. Thus, they were defined as the most contributing variables to samples'

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variability. Bands at the specified R_f values were accurately scrapped and analyzed using mass spectrometry for their identification. These bands were identified as silybin, isosilybin, silydianin, taxifolin, silychristin and silyamandin respectively; their identities were confirmed via mass spectral data comparison **Fig.1**. Identification of these compounds was based on comparison of the fragmentation pattern of each compound with those mentioned in literature as well as authentic reference standards [18–20].

The six identified flavonolignans were then targeted for quantification via a validated HPTLC method using ImageJ® software. Band color, R_f value and mass spectra of reference substances were employed to confirm the identity of the mentioned flavonolignans. Quantitative assessment of the developed chromatographic plate, based on the spot area of the specified flavonolignans, was validated in accordance with ICH guidelines' parameters.

Linearity was represented by linear responses covering the specified calibration concentration range with correlation coefficient (r^2) exceeding 0.98. Selectivity was assured based on R_f values of the identified flavonolignans and bands targeted on the developed plate. LOD and LOQ were estimated relying on the constructed calibration curves for the individual flavonolignans in addition to total silymarin. LOD was estimated for both total silymarin (17.37 µg/band) and its individual components; silybin, isosilybin, silydianin, taxifolin, silychristin and silyamandin (5.91, 3.55, 2.47, 1.03, 3.94 and 1.22 µg/band, respectively) while LOQ was found to be 52.63 µg/band for total silymarin and 17.93, 10.77, 7.49, 3.12, 11.95 and 3.72 µg/band for silybin, isosilybin, silydianin, taxifolin, silychristin and silyamandin, respectively. **Table 1** Precision was attainable with a standard deviation ranging from 1.34% to 1.83%. In addition, accuracy was achievable with recovery exceeding 97%.

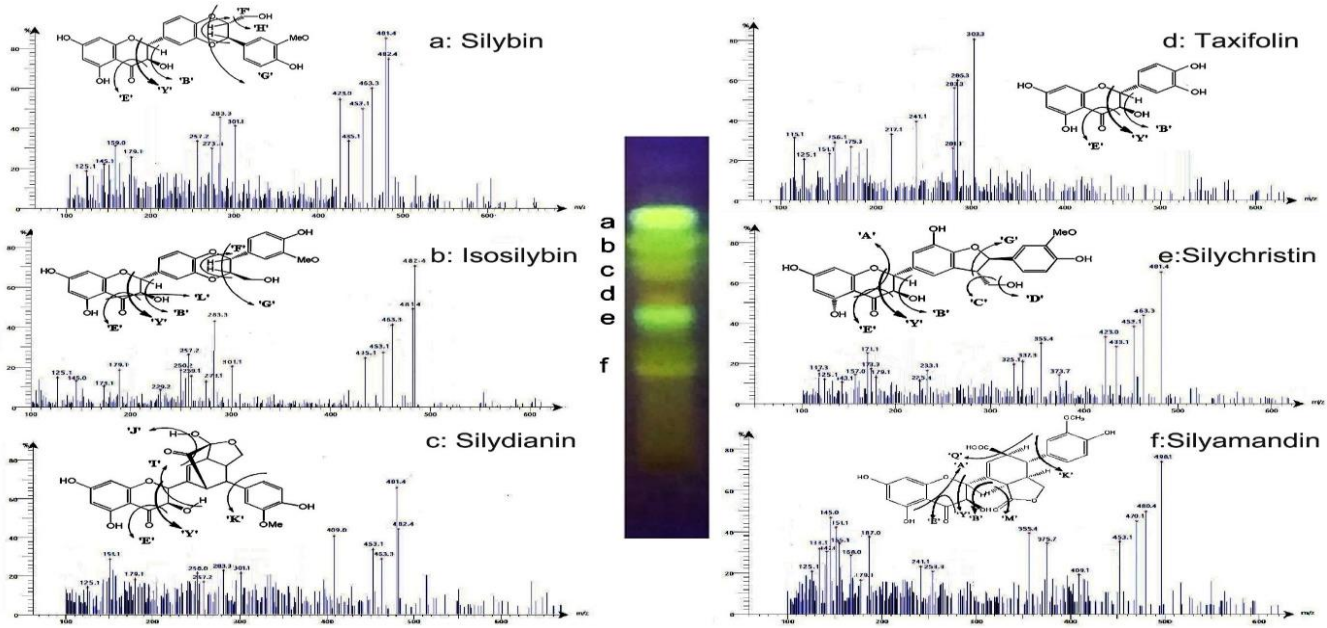

Fig.1: The identified flavonolignans; silybin, isosilybin, silydianin, taxifolin, silychristin and silyamandin with their relative arrangement in chromatogram under UV 366 nm after derivatization with NP/PEG spray reagent along with proposed fragmentation sites of major flavonolignans in silymarin mixture with arrows indicating proposed cleavage sites and the convex side of the curve indicating the leaving molecules by fragmentation to confirm their identity.

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Table 1: Validation parameters of the developed method for simultaneous estimation of total silymarin and its major flavonolignans.

Compound	Calibration curve	Correlation coefficient (R^2)	R_f	LOD (µg/band)	LOQ (µg/band)	Reproducibility (Intermediate Precision (RSD%)	Accuracy (Recovery±% RSD%)
Total silymarin	$y = 7800.3x + 512085$	0.9983		17.37	52.63	1.34	99.05±1.48
Silybin	$y = 8559.5x + 148664$	0.9812	0.71	5.91	17.93	1.61	97.82±1.41
Isosilybin	$y = 5952.9x + 123270$	0.982	0.62	3.55	10.77	1.52	97.89±1.67
Silydianin	$y = 7671.2x + 81191$	0.9839	0.51	2.47	7.49	1.64	98.32±1.71
Taxifolin	$y = 10630x + 25129$	0.9887	0.45	1.03	3.12	1.79	99.21±1.45
Silychristin	$y = 7789.5x + 101501$	0.9818	0.43	3.94	11.95	1.42	99.03±1.39
Silyamandin	$y = 8027.9x + 32330$	0.9841	0.34	1.22	3.72	1.83	98.21±1.57

Table 2: Quantitation values of total silymarin and its major flavonolignans in different fruit extracts of *Silybum marianum* var. marianum and var. albiflora.

	***Silybum marianum* var. albiflora**				***Silybum marianum* var. marianum**			
	Sample 1	**Sample 2**	**Sample 3**	**Average**	**Sample 1**	**Sample 2**	**Sample 3**	**Average**
Total silymarin (mg/g PM)	14.02±0.03	12.73±0.03	13.38±0.04	13.38±0.05	17.19±0.03	18.18±0.03	19.11±0.04	18.16±0.05
Silybin (mg/g PM)	5.87±0.03	5.33±0.03	5.67±0.04	5.62±0.05	6.82±0.03	7.13±0.03	7.32±0.04	7.09±0.05
Isosilybin (mg/g PM)	1.25±0.02	1.19±0.01	1.21±0.02	1.22±0.02	1.35±0.02	1.42±0.02	1.48±0.03	1.42±0.02
Silydianin (mg/g PM)	0.58±0.01	0.51±0.01	0.55±0.01	0.55±0.01	0.97±0.01	1.12±0.01	1.2±0.02	1.1±0.01
Taxifolin (mg/g PM)	0.88±0.01	0.79±0.01	0.83±0.02	0.83±0.01	1.73±0.01	1.85±0.01	2.1±0.02	1.89±0.01
Silychristin (mg/g PM)	4.95±0.03	4.47±0.02	4.65±0.02	4.69±0.02	5.64±0.03	5.87±0.03	6.2±0.03	5.9±0.03
Silyamandin (mg/g PM)	0.49±0.01	0.44±0.01	0.47±0.01	0.47±0.01	0.68±0.01	0.79±0.01	0.81±0.01	0.76±0.01

PM (Plant Material)

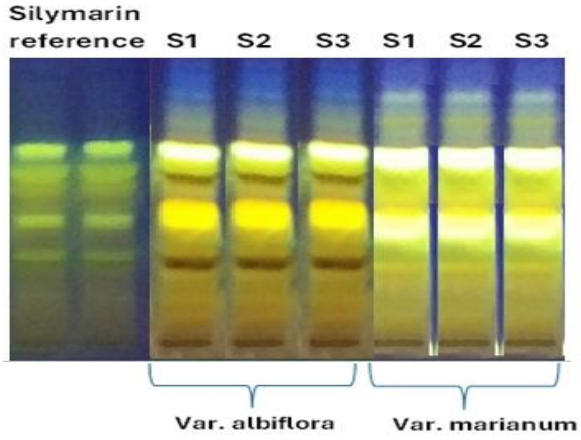

Fig.2: The developed plate of *Silybum marianum* representative samples under UV 366 nm after post-chromatographic derivatization with NP/PEG spray reagent. Samples (fruit extracts) of the variety albifora were applied followed by those of variety marianum against silymarin flavonolignans standards.

3.2. Fluctuation in total silymarin content and its major flavonolignans in the fruit extracts of *S. marianum* varieties

The amount of total silymarin and individual flavonolignans in the fruit extracts of the two studied varieties was tracked based on spot/band areas and constructed calibration curves. Studying **Fig. 2** and **Table 2** revealed relatively higher flavonolignans' accumulation in the fruits of the *S. marianum* var. marianum [with purple floral corolla], with average total silymarin content of 18.16±0.05 mg/g PM (Plant Material), compared to the fruits of variety albiflora [with white floral corolla] which found to be 13.38±0.05 mg/g PM. The results were found to be in accordance with previous reports for total silymarin content in fruits [21–23].

Considering the silymarin constituents in both varieties, predominance of silybin and

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silychristin was observed with average content of 5.62±0.05 & 4.69±0.02 mg/g PM in variety albiflora and of 7.09±0.05 & 5.9±0.03 mg/g PM in variety marianum respectively, followed by lower amounts/concentrations of other flavonolignans including isosilybin, silydianin and silyamandin, respectively. Significant levels of taxifolin were detected and accumulated to a relatively higher extent in the variety marianum (1.89±0.01 mg/g PM) compared to the variety albiflora (0.83±0.01 mg/g PM). **Table 2** and **Fig. 2**.

4. Conclusion

This paper is the first to report on the use of HPTLC-MS method for comparative profiling and simultaneous quantitation of the major silymarin flavonolignans in *Silybum marianum* fruits. The acquired results indicated that there was a little rise in the variety marianum as compared to that of albiflora with minor variation in the flavonolignan profiles of the tested varieties. The average total silymarin content in *S. marianum* var. albiflora and *S. marianum* var. marianum were 13.38±0.05 and 18.16±0.05 mg/g PM, respectively, with about 13% variation in favor of *S. marianum* var. marianum.

Declarations

Consent for publication

All authors have read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability

All data generated or analyzed during this study are available upon contacting the authors.

Ethical approval and consent to participate

Not applicable

Authors' contributions

Yasmin A. Mahgoub: Conceptualization, Methodology, Data Curation, Original Draft Preparation-Writing and Editing**.**
Fikria A. Darwish: Supervision, Original Draft Preparation and Visualization
Nadia A. El Sebakhy: Supervision, Original Draft Preparation and Visualization
Amr M. El-Hawiet: Conceptualization, Methodology, Data Curation, Supervision, Writing -Reviewing and Editing**.**
Eman Shawky: Conceptualization, Methodology, Data Curation, Supervision, Validation, Writing -Reviewing and Editing**.**

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