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## **CHROMATOGRAPHIC ANALYSIS OF SILYBUM MARIANUM (L.) GAERTN. FATTY OIL**

Alexandru Ciocarlan <sup>a\*</sup>, Ion Dragalin <sup>a</sup>, Aculina Aricu <sup>a</sup>, Nina Ciocarlan <sup>b</sup>,  
Cristina Stavarache <sup>c</sup>, Mariana Deleanu <sup>d</sup>

<sup>a</sup>*Institute of Chemistry of Academy of Sciences of Moldova, 3, Academiei str., Chisinau MD 2028, Republic of Moldova*

<sup>b</sup>*Botanical Garden (Institute) of Academy of Sciences of Moldova, 18, Padurii str., Chisinau MD-2002, Republic of Moldova*

<sup>c</sup>*"C.D. Nenitescu" Centre of Organic Chemistry, Romanian Academy, 202-B, Independentei spl., Bucharest RO-060023, Romania*

<sup>d</sup>*Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest, 59, Marasti blvd., Bucharest RO-011464, Romania*

\**e-mail: [algociocarlan@yahoo.com](mailto:algociocarlan@yahoo.com); phone: (+373 22) 739 769; fax: + (373 22) 739 775*

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## CHROMATOGRAPHIC ANALYSIS OF *SILYBUM MARIANUM* (L.) GAERTN. FATTY OIL

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Cristina Stavarache <sup>c</sup>, Mariana Deleanu <sup>d</sup>

<sup>a</sup>Institute of Chemistry of Academy of Sciences of Moldova, 3, Academiei str.,  
Chisinau MD 2028, Republic of Moldova

<sup>b</sup>Botanical Garden (Institute) of Academy of Sciences of Moldova, 18, Padurii str.,  
Chisinau MD-2002, Republic of Moldova

<sup>c</sup>"C.D. Nenitescu" Centre of Organic Chemistry, Romanian Academy, 202-B, Independentei spl.,  
Bucharest RO-060023, Romania

<sup>d</sup>Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest,  
59, Marasti Blvd., Bucharest RO-011464, Romania

\*e-mail: [algciocarlan@yahoo.com](mailto:algciocarlan@yahoo.com); phone: (+373 22) 739 769; fax: + (373 22) 739 775

**Abstract.** The present paper describes biochemical (fatty oil) composition of *Silybum marianum* (L.) Gaertn. of Moldovan origin. The oil content of the seeds was approximately 25%. Linoleic acid (C18:2), an essential polyunsaturated fatty acid, is the most abundant (48.88%), followed by monounsaturated oleic acid (C18:1, 31.94%) and saturated palmitic acid (C16:0, 7.61%). Also, saturated stearic (C18:0, 4.31%), arachidic (C20:0, 2.63% and behenic acid (C22:0, 2.30%) were identified. The minor fatty acids are represented by saturated myristic (14:0, 0.09%) and margaric acid, (17:0, 0.07%), monounsaturated eicosenoic (C20:1, 0.99%), palmitoleic (C16:1, 0.07%) and erucic acid (C22:1, 0.08%). The RP-HPLC analysis of tocopherols composition showed the main components:  $\alpha$ -tocopherol (23.45 mg/100g) and  $\gamma$ -tocopherol (5.60 mg/100g). Based on the obtained results, the extracted oil from milk thistle seeds is rich in essential fatty acids (about 50%) and tocopherols (29.09 mg/100g) and it can be used in food preparation.

**Keywords:** *Silybum marianum* fatty oil, GC analysis, fatty acids methyl ester, RP-HPLC analysis,  $\alpha$ -tocopherol.

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### Introduction

*Silybum marianum* (L.) Gaertn. is an important medicinal plant belonging to the *Asteraceae* Dumort family. Native of the Mediterranean region, it is cultivated now in many countries. In the Republic of Moldova, this species is cultured and does not grow in the spontaneous flora.

As a medicinal plant, milk thistle has been traditionally used for centuries to treat liver diseases and, presently, it is one of the most commonly used herbs worldwide. The active component of dried fruit extract of *S. marianum* is silymarin, an isomeric mixture of three flavonolignans: silybin, the main and most effective compound, silycristin and silydianin [1-4].

Silymarin possess hepatoprotective [5], antioxidant [6], anti-inflammatory [6,7], anticancer [8,9], antifibrotic [10], liver regenerating and immunomodulatory effects [6]. The hepatoprotective properties of *S. marianum*

flavonolignans have been proven clinically in the therapy of some connected with liver disorders, as alcohol poisoning [11], viral hepatitis [12], liver cirrhosis [13] and mushroom poisoning [14]. Pharmacological studies have demonstrated that the flavonolignans isolated from *S. marianum* seeds stimulate kidney cells, avoiding nephrotoxic effects [15]. They are proven to be useful in the treatment of type II diabetes [16].

The plant seeds also contain a high amount of oil [17,18]. Numerous studies have been conducted on these species, growing in different regions of the world, particularly on their fatty oil compounds [19,20].

The aim of this work is to reveal the biochemical composition of *S. marianum* oil, more exactly the content of fatty acids by means of gas-chromatography (GC) and the contents of tocopherols in the fatty oil using high-pressure liquid chromatography (HPLC).

## Experimental

Seeds samples of milk thistle (*S. marianum* (L.) Gaertn.) plant variety “Panaceaia”, 1<sup>st</sup> year of reproduction were collected from experimental fields of medicinal and aromatic collection, Botanical Garden (Institute) of Academy of Sciences of Moldova. The plants were cultivated at a density of 50 x 60 m<sup>2</sup> without soil fertilizer and seeds were collected manually at full maturity in the second decade of July. Their humidity was about 5.5%.

### Fatty oil (FO) extraction

A sample of grounded seeds (0.2-0.5 mm, 300 g), was extracted at reflux in a Soxhlet type extractor using light petroleum ether (*b.p.* 40-60°C) during 3 h. After filtration, the solvent was removed at reduced pressure. The obtained fatty oil (75.8 g, 25.27%) was further used for measurements and chromatographic analyses.

### Physical and chemical characteristics of fatty oil (FO)

Physical and chemical characteristics of *S. marianum* oil were established using approved methods. Fresh oil samples showed the following characteristics: relative density – 0.860-0.890 g/cm<sup>3</sup> (measured at 20°C); refractive index ( $n_D^{20}$ ) – 1.4705-1.4760; iodine value (Wijs) – 79-88; acidity index – 2.0-2.8% m/m and peroxide index – 3.5-4.0 (meqO<sub>2</sub>/kg oil).

### Fatty acid methyl esters (FAMES) preparation

The oil samples (200 mg) were dissolved in hexane (4 mL) in a conic tube and 200 µL of 2 M methanolic potassium hydroxide solution was added. After vigorous shaking in a vortex for 1 minute, the samples were neutralized with potassium hydrogen phosphate. The organic layer, which contains FAMES, was filtered and 1 µL was injected into the gas-chromatograph [21-23].

### Gas chromatography (GC) analysis of fatty oil

Qualitative and quantitative analysis of fatty acid composition was performed on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID). A fused-silica SP-2560poly(biscyanopropylsiloxane) capillary column (100 m x 0.25 mm *i.d.*; film thickness 0.20 µm) from SUPELCO was used for separation and operated under the following conditions: oven temperature program: from 120°C up to 240°C at a rate of 4°C/min and then kept at 240°C for 30 min; injector and detector temperatures, 250 and 260°C, respectively; carrier gas, helium at a flow rate of 2 mL/min; split ratio, 1:100; nitrogen at a flow rate of 30 mL/min, hydrogen at a flow rate of 30 mL/min and air at a flow rate of 300 mL/min [24]. Identification of

fatty acids methyl esters was made with a standard mixture of 37 esters of fatty acids (FAME MIX 37) from SUPELCO. The results are expressed % (w/w) FA.

### Sample preparation for tocopherols analysis

The oil samples (2.0 g) were dissolved in methanol (50 mL) and ascorbic acid (0.5 g) was added. After vigorous shaking, 50% aqueous potassium hydroxide solution (5 mL) was added and the resulted mixture was refluxed for 35 min under nitrogen. After saponification, the samples were let to cool down and diluted with water (55 mL). The extraction step was performed in a dark separation funnel with a mixture (50 mL) of petroleum ether:diethyl ether (80:20, v/v). After the separation of phases, the organic layer was transferred into another dark separation funnel and mother liquid was extracted additionally twice with the same mixture of solvents. The combined organic phase was washed with water (150 mL) to the neutral stage, evaporated to dryness under reduced pressure and re-dissolved in methanol (10 mL) for RP-HPLC analysis.

### RP-HPLC quantification of tocopherols from milk thistle oil

Chromatographic separation of tocopherols was performed using a high performance liquid chromatography (HPLC) equipped with a quaternary pump, column oven, autosampler and diode array detector (DAD) (Agilent 1200 series, USA). The analytic column was an RP Ascentis C<sub>18</sub> (250 mm; 4.6 mm; 5 µm; Supelco Analytical) equipped with a guard column and thermostated at 30°C. Tocopherols were separated isocratically within 16 min using a mobile phase containing MeOH:H<sub>2</sub>O (97:3, v/v), at a flow rate of 2 mL/min and detected at  $\lambda = 292$  nm. The concentrations of tocopherols were calculated with a 5-point calibration curve with external standards. The standard concentrations ranged from 1.12 to 66.5 µg/mL.

For analysis, 10 µL samples were injected into the HPLC system. Tocopherols were identified by comparing their retention times against commercially available standards (Sigma-Aldrich) [21,25]. The results were expressed as mg of  $\alpha$ -,  $\gamma$ -tocopherols/100g oil.

## Results and discussion

The gas-chromatographic analysis of *S. marianum* L. fatty oil has shown to consist of thirteen fatty acid of both, saturated and unsaturated groups (Figure 1, Table 1). The first group includes saturated fatty acids like palmitic (1) (16:0, retention time (RT)= 28.15 min), stearic (2) (18:0, RT= 31.26 min), arachidic (3)

(20:0, RT= 34.31 min), behenic (4) (22:0, RT= 37.43 min) and lignoceric acids (5) (24:0, RT= 40.82 min) with a total content of 17.59 %

(Figure 2). The most abundant saturated fatty acid was palmitic (7.61%) followed by stearic (4.31%) and arachidic (2.63%).

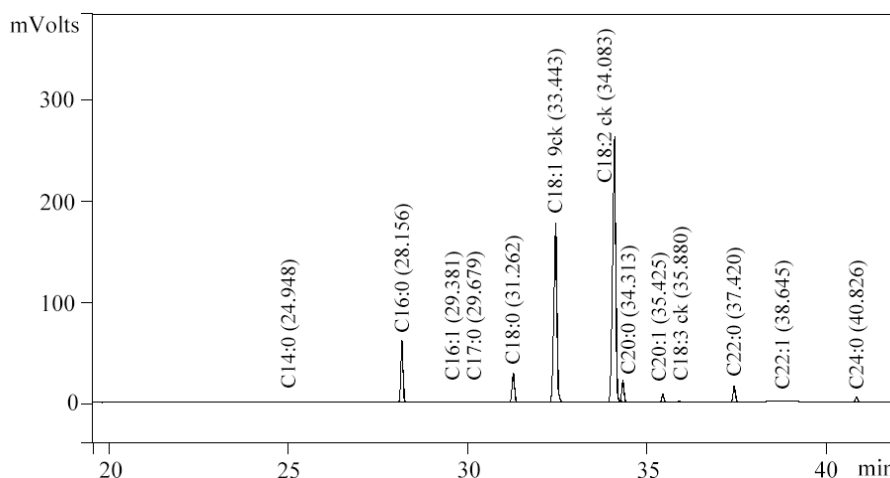


Figure 1. GC chromatogram of fatty acids methyl esters.

Table 1  
Fatty acids composition % (w/w) of *S. marianum* L. fatty oil.

Fatty acids	% (w/w)
C14:0 (myristic)	0.09
C16:0 (palmitic)	7.61
C16:1 (palmitoleic)	0.07
C17:0 (margaric)	0.07
C18:0 (stearic)	4.31
C18:1 (oleic)	31.94
C18:2 (linoleic)	48.88
C18:3 (linolenic)	0.19
C20:0 (arachidic)	2.63
C20:1 (eicosenoic)	0.99
C22:0 (behenic)	2.30
C22:1 (erucic)	0.08
C24:0 (lignoceric)	0.74
SFA*	17.75
MUFA**	33.08
PUFA***	49.07

\*SFA - total saturated FA;

\*\*MUFA - total monounsaturated FA;

\*\*\*PUFA - total polyunsaturated FA.

Unsaturated acids such as oleic (6) (18:1, RT= 32.44 min), linoleic (7) (18:2, RT= 34.08 min),  $\alpha$ -linolenic (8) (18:3, RT= 35.88 min) and eicosenoic (9) (20:1, RT= 35.42 min) quantitatively represent the absolute majority 82.0%. The main constituents are polyunsaturated linoleic  $\omega$ -6 (48.88%) and monounsaturated oleic (31.94%) acids (Figure 3). Unfortunately, linolenic  $\omega$ -3 acid represents only 0.19%, but the ratio  $\omega$ -6/ $\omega$ -3 is similar to sunflower oil [26]. It has been reported that  $\omega$ -3 PUFAs have effects on atherosclerosis, circulating lipid profile, cell membranes, cell proliferation,

and cardiac arrhythmias [27]. It is better to mix this oil with other oils very rich in  $\omega$ -3 PUFAs, such as flaxseed, rapeseed or soya oil in daily dietary intakes.

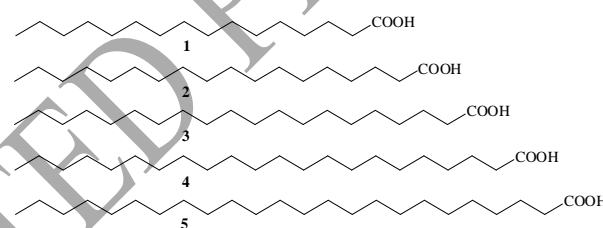


Figure 2. Saturated fatty acids from *S. marianum* L. oil. 1- Palmitic; 2- stearic; 3- arachidic; 4- behenic; 5- lignoceric.

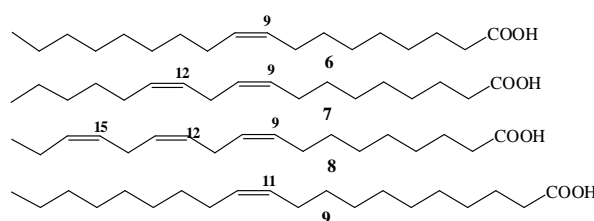
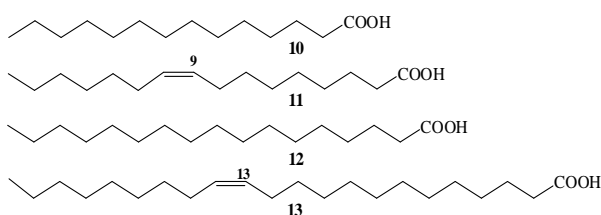


Figure 3. Unsaturated fatty acids from *S. marianum* L. oil. 6- Oleic; 7- linoleic; 8-  $\alpha$ -linolenic; 9- eicosenoic.

Also, there are traces of other fatty acids like myristic (10) (14:0, RT= 24.94 min), palmitoleic (11) (16:1, RT= 29.38 min), margaric (12) (17:0, RT= 29.67 min) and erucic (13) (22:1, RT= 38.64 min) (Figure 4). It should be mentioned that fatty acid composition of milk thistle seeds oil is similar to sunflower oil [26].

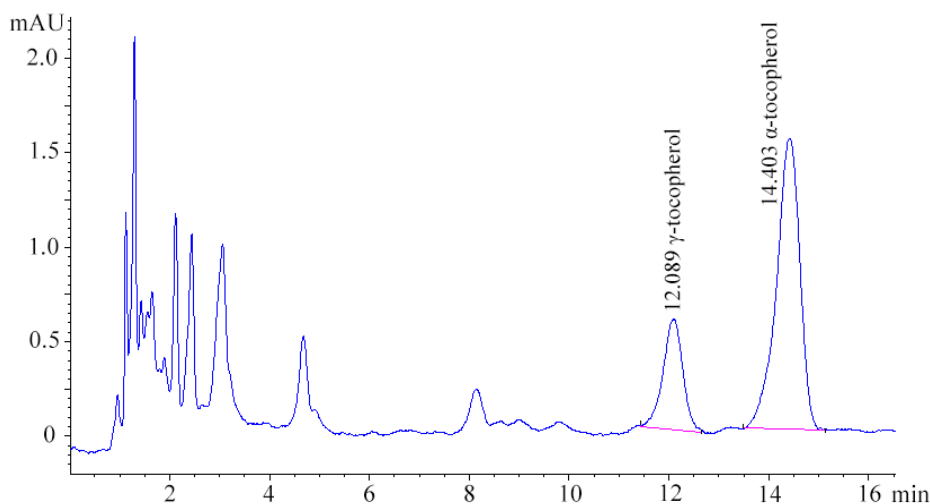


**Figure 4. Minor saturated and unsaturated fatty acids from *S. marianum* L. oil. 10-Myristic; 11-palmitoleic; 12- margaric; 13-erucic.**

Tocopherols are important compounds in vegetable oils and very important lipid oxidation inhibitors in food and biological systems. They are naturally occurring phenolic antioxidant constituents found in various amounts in vegetable oils [28]. Tocopherols are found in

oilseeds in four different forms:  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols. Among these tocopherols,  $\alpha$ -tocopherol is the most active form and  $\gamma$ - and  $\delta$ -tocopherols have shown better antioxidant activities than the others [29]. Tocopherols may reduce the risk of cardiovascular diseases because of their antioxidant properties and various functions at the molecular level [30].

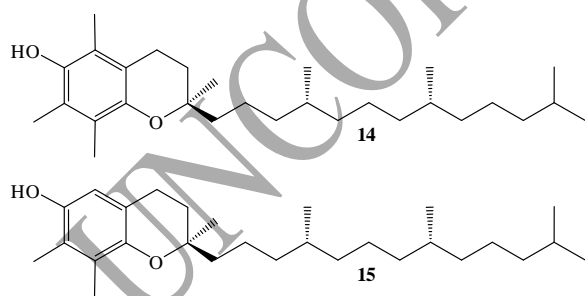
RP-HPLC chromatogram of the milk thistle oils revealed the presence of  $\alpha$ -tocopherol (**14**) (RT= 14.40 min) and  $\gamma$ -tocopherol (**15**) (RT= 12.08 min) (Figures 5 and 6). The analyzed milk thistle seed oils had a higher amount of  $\alpha$ -tocopherols (almost 4 times) compared with  $\gamma$ -tocopherols, while the  $\beta$ - and  $\delta$ -tocopherols are present in trace amounts (Table 2).



**Figure 5. RP-HPLC analysis of tocopherols in *S. marianum* L. fatty oil.**

**Table 2**  
**The content of tocopherols (mg/100 g) in *S. marianum* L. fatty oil.**

Milk thistle oil	mg/100 g oil
$\alpha$ -tocopherol	23.45
$\gamma$ -tocopherol	5.60
Total tocopherols	29.05



**Figure 6. The 14-  $\alpha$ -tocopherol and 15-  $\gamma$ -tocopherol.**

## Conclusions

The milk thistle fatty oil of Moldovan origin has a biochemical composition comparable with samples of other origins reported before and can be used for the same purposes.

This study revealed that the seeds of *S. marianum* are a rich source of  $\omega$ -6 polyunsaturated fatty acids (PUFAs) (almost 50%) and  $\alpha$ -tocopherol (23.45 mg/100g) which are very good antioxidants. The composition of extracted oil was similar to sunflower oil and might be used as cooking oil or salad dressing oil, alone or mixed with the other oils very rich in  $\omega$ -3 PUFAs.

## References

- Dixit, N.; Baboota, S.; Kohli, K.; Ahmad, S.; Ali, J. Silymarin: a review of pharmacological aspects and bioavailability enhancement approaches. Indian

- Journal of Pharmacology, 2007, 39(4), pp. 172-179. DOI: [10.4103/0253-7613.36534](https://doi.org/10.4103/0253-7613.36534)
2. Kurkin, V.A. Saint-Mary thistle: a source of medicinals (A review). *Pharmaceutical Chemistry Journal*, 2003, 37(4), pp. 189-202. DOI: <https://doi.org/10.1023/A:1024782728074>
  3. Kvasniska, F.; Biba, B.; Sevcik, R.; Voldrich, M.; Kratka, J. Analysis of the active components of silymarin. *Journal of Chromatography A*, 2003, 990(1-2), pp. 239-245. DOI: [https://doi.org/10.1016/S0021-9673\(02\)01971-4](https://doi.org/10.1016/S0021-9673(02)01971-4)
  4. Quaglia, M.G.; Bossu, E.; Donati, E.; Mazzanti, G.; Brandt, A. Determination of silymarin in the extract from the dried *Silybum marianum* fruits by high performance liquid chromatography and capillary electrophoreses. *Journal of Pharmaceutical and Biomedical Analysis*, 1999, 19(3-4), pp. 435-442. DOI: [https://doi.org/10.1016/S0731-7085\(98\)00231-3](https://doi.org/10.1016/S0731-7085(98)00231-3)
  5. Pradhan, S.C.; Girish, C. Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. *Indian Journal of Medical Research*, 2006, 124(5), pp. 491-504. [http://www.ijmr.org.in/temp/IndianJMedRes1245491-808193\\_021441.pdf](http://www.ijmr.org.in/temp/IndianJMedRes1245491-808193_021441.pdf)
  6. Katiyar, S.K. Silymarin and skin cancer prevention: Anti-inflammatory, antioxidant and immunomodulatory effects (Review). *International Journal of Oncology*, 2005, 26(1), pp. 169-176. DOI: <https://doi.org/10.3892/ijo.26.1.169>
  7. De la Puerta, R.; Martinez, E.; Bravo, L.; Ahumada, M.C. Effect of silymarin on different acute inflammation models and on leukocyte migration. *Journal of Pharmacy and Pharmacology*, 1996, 48(9), pp. 968-970. DOI: <https://doi.org/10.1111/j.2042-7158.1996.tb06014.x>
  8. Greenlee, H.; Abascal, K.; Yarnell, E.; Ladas, E. Clinical applications of *Silybum marianum* in oncology. *Integrative Cancer Therapies*, 2007, 6(2), pp. 158-165. DOI: <https://doi.org/10.1177/1534735407301727>
  9. Vue, B.; Zhang, S.; Zhang, X.; Parisis, K.; Zhang, Q.; Zheng, S.; Wang, G.; Chen, Q.H. Silibin in derivatives as anti-prostate cancer agents: synthesis and cell-based evaluations. *European Journal of Medicinal Chemistry*, 2016, 109, pp. 36-46. DOI: <http://doi.org/10.1016/j.ejmech.2015.12.041>
  10. Jia, J-D.; Bauer, M.; Cho, J.J.; Ruehl, M.; Milani, S.; Boigk, G.; Riecken, E.O.; Schuppan, D. Antifibrotic effect of silymarin in rat secondary biliary fibrosis is mediated by down regulation of procollagen  $\alpha 1(I)$  and TIMP-1. *Journal of Hepatology*, 2001, 35(3), pp. 392-398. DOI: [http://dx.doi.org/10.1016/S0168-8278\(01\)00148-9](http://dx.doi.org/10.1016/S0168-8278(01)00148-9)
  11. Ball, K.R.; Kowdley, K.V. A review of *Silybum marianum* (milk thistle) as a treatment for alcoholic liver disease. *Journal of Clinical Gastroenterology*, 2005, 39(6), pp. 520-528. DOI: [10.1097/01.mcg.0000165668.79530.a0](https://doi.org/10.1097/01.mcg.0000165668.79530.a0)
  12. Yang, Z.; Zhuang, L.; Lu, Y.; Xu, Q.; Chen, X. Effects and tolerance of Silymarin (milk thistle) in chronic hepatitis C virus infection patients: a meta-analysis of randomized controlled trials. *BioMed Research International*, 2014, pp. 1-9. DOI: <http://dx.doi.org/10.1155/2014/941085>
  13. Mulrow, C.; Lawrence, V.; Jacobs, B.; Dennehy, C.; Sapp, J.; Ramirez, G.; Aguilar, C.; Montgomery, K.; Morbidoni, L.; Arterburn, J.M.; Chiquette, E.; Harris, M.; Mullins, D.; Vickers, A.; Flora, K. Milk thistle: effects on liver disease and cirrhosis and clinical adverse effects. Evidence Report/Technology Assessment No. 21, AHRQ Publication No. 01-E025. Rockville, MD: Agency for Healthcare Research and Quality. 2000, 158 p. <http://www.pkids.org/files/milkthistle.pdf>
  14. Parish, R.C.; Doering, P.L. Treatment of amanita mushroom poisoning: a review. *Veterinary and Human Toxicology*, 1986, 28(4), pp. 318-322. <http://europepmc.org/abstract/med/3092435>
  15. Momeni, A.; Hajigholami, A.; Geshnizjani, S.; Kheiri, S. Effect of silymarin in the prevention of cisplatin nephrotoxicity, a clinical trial study. *Journal of Clinical and Diagnostic Research*, 2015, 9(4), pp. OC11-OC13. DOI: <http://doi.org/10.7860/JCDR/2015/12776.5789>
  16. Stolf, A.M.; Cardoso, C.C.; Acco, A. Effects of silymarin on diabetes mellitus complications: a review. *Phytotherapy Research*, 2017, 31(3), pp. 366-374. DOI: <https://doi.org/10.1002/ptr.5768>
  17. Hasanloo, T.; Bahmani, M.; Sepehrifar, R.; Kalantari, F. Determination of tocopherols and fatty acids in seeds of *Silybum marianum* (L.) Gaertn. *Journal of Medicinal Plants*, 2008, 7(4), pp. 69-76. <http://jmp.ir/article-1-568-en.html>
  18. Ahmad, T.; Atta, S.; Ullah, I.; Zeb, A.; Nagra, S.A.; Perveen, S. Characteristics of *Silybum marianum* as a potential source of dietary oil and protein. *Pakistan Journal of Scientific and Industrial Research*, 2007, 50(1), pp. 36-40. <http://www.pjsir.org/arc.php>
  19. Fadavi, A.; Barzegar, M.; Azizi M.H. Determination of fatty acids and total lipid content in oilseed of 25 pomegranates varieties grown in Iran. *Journal of Food Composition and Analyses*, 2006, 19(6-7), pp. 676-680. DOI: <https://doi.org/10.1016/j.jfca.2004.09.002>
  20. Kuhnlein, H.V.; Barthet, V.; Farren, A.; Falahi, E.; Leggee, D.; Receveur, O.; Berti, P. Vitamins A, D, and E in Canadian Arctic traditional food and adult diets. *Journal of Food Composition and Analysis*, 2006, 19(6-7), pp. 495-506. DOI: <https://doi.org/10.1016/j.jfca.2005.02.007>
  21. Meier, S.; Mjos, S.A.; Joensen, H.; Grahl-Nielsen, O. Validation of a one-step extraction/methylation method for determination of fatty acids and cholesterol in marine tissues. *Journal of Chromatography A*, 2006, 1104(1-2), pp. 291-298. DOI: <https://doi.org/10.1016/j.chroma.2005.11.045>
  22. SR EN ISO 5508/2002 Animal and vegetables fats and oils. Analysis by gas chromatography of methyl esters of fatty acids.

23. SR EN ISO 5509/2002 Animal and vegetables fats and oils. Preparation of methyl esters of fatty acids.
24. SR EN ISO 15304 /2003 Animal and vegetables fats and oils. Determination of the content of trans fatty acid isomers of vegetable fats and oils. Gas chromatographic method.
25. SR EN 12822-/2002 Foodstuffs. Determination of vitamin E by high performance liquid chromatography. Measurement of  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols.
26. Orsavova, J; Misurcova, L.; Ambrozova, J.V.; Vicha, R.; Mlcek, J. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *International Journal Molecular Sciences*, 2015, 16(6), pp. 12871-12890.  
DOI: <https://doi.org/10.3390/ijms160612871>
27. Ander, B.P.; Dupasquier, Ch.M.C.; Prociuk M.A.; Pierce, G.N. Polyunsaturated fatty acids and their effects on cardiovascular disease. *Experimental and Clinical Cardiology*, 2003, 8(4), pp. 164-172.  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2719153/>
28. Yoshida, H.; Hirakawa, Y.; Murakami, C.; Mizushima, Y.; Yamade, T. Variation in the content of tocopherols and distribution of fatty acids within soya bean seeds (*Glycine max* L). *Journal of Food Composition and Analysis*, 2003, 16(4), pp. 429-440. DOI: [https://doi.org/10.1016/S0889-1575\(03\)00028-0](https://doi.org/10.1016/S0889-1575(03)00028-0)
29. Gimeno, E.; Castellote, A.I.; Lamuela-Raventos, R.M.; de la Torre, M.C.; Lopez-Sabater, M.C. Rapid determination of vitamin E in vegetable oils by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A*, 2000, 881(1-2), pp. 251-254. DOI: [https://doi.org/10.1016/S0021-9673\(00\)00219-3](https://doi.org/10.1016/S0021-9673(00)00219-3)
30. Burton, G.W. Vitamin E: molecular and biological function. *The Proceeding of the Nutrition Society*, 1994, 53(2), pp. 251-262.  
DOI: <https://doi.org/10.1079/PNS19940030>

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