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LIPID COMPOSITION OF *CARDUUS THOERMERI* WEINM., *ONOPORDUM ACANTHIUM* L. AND *SILYBUM MARIANUM* L., GROWING IN BULGARIA

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Abstract

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Seed oil chemical composition of wild growing *Carduus thoermeri* Weinm., *Onopordum acanthium* L. and *Silybum marianum* L. were studied for the first time in Bulgaria, by using GC, HPLC, TLC and spectrophotometrical methods. The major components of fatty acids were oleic (342 - 530 $g.kg^{-1}$), linoleic (176 - 511 $g.kg^{-1}$) and palmitic (99 - 150 $g.kg^{-1}$). α-tocopherol was the main component in the tocopherol fraction of *O. acanthium* seeds (911 $g.kg^{-1}$). In the sterol fraction the main components were β-sitosterol (546 - 632 $g.kg^{-1}$) and campesterol (128 - 156 $g.kg^{-1}$). Phosphatidylinositol was with the highest concentration in the phospolipid fraction (317 $g.kg^{-1}$ in *C. thoermeri* and 320 $g.kg^{-1}$ in *O. acanthium*). Due to content of unsaturated fatty acids, tocopherols and phytosterols, the seeds of these widespread species (especially *O. acanthium)* could be utilized as a valuable oil source for human consumption.

Key words: Carduus thoermeri, *Onopordum acanthium, Silybum marianum*, seeds, lipid composition

Abrreviations: FAME (fatty acid methyl esters), GC (gas chromatography), HPLC (high performance liquid chromatography), TLC (thin layer chromatography)

Introduction

Silybum marianum L. (Milk thistle) is a biennial in nature or annual in cultivation medicinal plant that has been widely used in the European traditional medicine and belongs to the family Compositae (Khan et al., 2007). In addition to the fatty acids content (Marquard, 1982) the seeds have also about 0.1% essential oil, amines, flavonoids, trace elements (predominantly Se), vitamins (F, E, and B), and other components (Lovkova, 1989). Medicinal properties of *S. marianum* are based on silimarin in the seeds (Lavreneva and Lavrenev, 1997). Silimarin, derived from the milk thistle plant, has been used for centuries as a natural remedy for diseases of the liver and biliary tract (Flora et al., 1998; Kurkin, 2003). High pressure extraction of vitamine E from seeds of *S. marianum* was investigated (Hadolin et al., 2001). Fatty oil chemical composition of wild *S. marianum* was not studied in Bulgaria.

Onopordum acanthium L. (Cotton thistle, Scotch thistle) is a biennial plant belongs to Asteraceae (Compositae) family, native to South and Central Europe, naturalized or casual in the north (Tutin et al., 1976). In Bulgaria this species are commonly spread at dry stony ruderal places and disturbed fields up to 1500 m altitude. The species contain different class of biologically active substances as saponins, alkaloids, sesquiterpen lactones and inulin. According to the Bulgarian folk medicine this species have refreshing and invigorating effect on the body (Petkov, 1982). Oil from the seeds has been used for burning and cooking (Grieve, 1971). Chemical content of fatty oils in *O. acanthium* seeds was not studied in details (Tonguc and Erbas, 2012).

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Carduus thoermeri Weinm. (Musk thistle, Asteraceae) is a biennial ruderal plant, occurring in Bulgaria up to 1500 m above sea level in dry pastures and waste places (Delipavlov and Cheshmedzhiev, 2003). The lipid composition of *C. thoermeri* seeds has not been studied until now. Phytochemical surveys on *Carduus* species established the presence of different types of constituents - flavonoids, phenolic acids, steroids (Jordan-Thaden and Louda, 2003). Previous research on some Bulgarian *Carduus* species found that they (especially *C. thoermeri)* could be evaluated as rich sources of antioxidants (Zheleva-Dimitrova et al., 2011; Mihaylova et al, 2013).

Fatty oils composition of *C. thoermeri, O. acanthium* and *S. marianum* seeds in Bulgarian flora are not investigated to date. This issue and the growing interest of searching new sources of healthy foods set the aim of the present study: to determine the chemical composition of fatty oils of *C. thoermeri* and *O. acanthium* and compare it with the components of the lipid fraction *Silybum marianum,* which is a medicinal herb and it is systematically close to the abovementioned two species.

Materials and Methods

Plant material: Seeds (achenes) of *Carduus thoermeri*, *Onopordum acanthium* and *Silybum marianum* were collected from natural habitats of Northeast Bulgaria during the vegetation season of 2012. The plant materials were identified and morphologically described at the Botany department of Plovdiv University "Paisii Hilendarski".Voucher specimens of the species (059724, 059726, 059782) were deposited in the herbarium of the Agricultural University of Plovdiv (SOA).

Methods:

Moisture determination: With the help of laboratory grinding mill the seeds were finely ground under 0.5 mm size. The moisture was determinated according to State Pharmacopoeia in the Union of Soviet Socialist Republics X (1970).

Isolation of lipid fraction: The seeds were extracted with n-hexane in Soxhlet for 8 h. The solvent was partly removed in a rotary vacuum evaporator, the residue was transferred to a pre-weighed glass vessel and the rest of the solvent was removed under stream of nitrogen to a constant weight, in order to determine the oil content (ISO 659, 2009).

Fatty acid: The total fatty acid composition of the oil was determined by GC after transmethylation of the representative sample with 2N methanolic KOH at 50°C according to Christie (2003). Fatty acid methyl esters (FAME) were purified by TLC on 20 cm x 20 cm glass plates covered with 0.2 mm Silica gel 60 G layer (Merck, Darmstadt, Germany) with mobile phase n-hexane: acetone, 100 : 8 (v/v). Determination was performed on a gas chromatograph equipped with a 30 m x 0.25 mm x 25 µm (I.D.) capillary EC 30-Wax column (Hewlett Packard GmbH, Vienna, Austria) and a flame ionization detector. The column temperature was programmed from 130°C (hold 4 min), at 15°C/min to 240°C (hold 5 min); injector and detector temperatures were 250°C. Hydrogen was the carrier gas at a flow rate 0.8 ml/min; split was 50:1. Identification was performed by comparison of retention times with those of a standard mixture of FAME subjected to GC under identical experimental conditions (ISO 5508, 1990).

Sterols*:* Unsaponifiables were determined by weight after saponification of the glycerides oil and extraction with hexane (ISO 18609, 2000). The unsaponifiable matters (100 mg) was applied on 20 cm x 20 cm glass plates (ca. 1 mm thick Silica gel 60 G layer) and developed with n-hexane: acetone, 100: 8 (by volume). Free sterols (R_f = 0.4) were detected under UV light by spraying the edges of each plate with 2´,7´-dichlorofluorescein, they were then scraped, transferred to small glass columns and eluted with diethyl ether. The solvent was evaporated under a stream of nitrogen and the residue was weighed in small glass containers to a constant weight. Sterol composition was determined by GC using HP 5890 gas chromatograph (Hewlett Packard GmbH, Vienna, Austria) equipped with a 25 m x 0.25 mm DB – 5 capillary column (Agilent Technologies, Santa Clara CA, USA) and a flame ionization detector. Temperature gradient was from 90°C (hold 2 min) up to 290°C at a rate 15°C/min and then up to 310°C at a rate of 4°C/min (hold 10 min); the injector temperature was 300°C and the detector temperature was 320°C. Hydrogen was used as carrier gas at a flow rate 0.8 ml/min; split 50:1. Identification was confirmed by comparison of retention times with those of a standard mixture of sterols (ISO 12228, 1999).

Tocopherols: Tocopherols were determined directly in the oil by high performance liquid chromatography (HPLC) by a Merck-Hitachi (Merck, Darmstadt, Germany) unit equipped with a 250 mm x 4 mm Nucleosil Si 50-5 column (Merck, Darmstadt, Germany) and a fluorescent detector Merck-Hitachi F 1000. The operating conditions were as follows: mobile phase n-hexane: dioxan, 96: 4 (v/v), flow rate 1.0 ml/min, excitation 295 nm, and emission 330 nm. 20 µl 1% solution of crude oil were injected. Tocopherols were identified by comparing the retention times to those of authentic individual pure tocopherols. The tocopherol content was calculated on the base of tocopherol peak areas in the sample vs. tocopherol peak area of the standard tocopherol solution (ISO 9936, 2006).

Phospholipids: Another part (10 g) of air-dried seeds was subjected to Folch extraction according to Christie, 2003. Polar lipids were isolated from the total lipids by column chromatography. Briefly, the sample (100 mg) was applied on 40

cm x 2 cm glass column packed with Silica gel Unisil 100-200 mesh (Clarkson Chemicals Co., USA) and eluted in sequence with chloroform (for neutral lipids, sterols and sterol esters), acetone (sterol glycosides) and with methanol to isolate phospholipids. The phospholipid classes were isolated by a variety of the two-dimensional thin-layer chromatography on 20 cm x 20 cm glass plates with 0.2 mm Silica gel 60 G layer (Merck) impregnated with aqueous $(NH_4)_2SO_4$ (10 $g.kg^{-1}$). In the first direction the plate was developed with chloroform: methanol : ammonia, 65 : 25 : 5 (by volume) and in the second – with chloroform : acetone : methanol : acetic acid : water, 50 : 20 : 10 : 10 : 5 (v/v). The individual phospholipids were detected and identified by spraying with specific reagents according to Christie (2003) - Dragendorff test (detection of choline-containing phospholipids), ninhydrin spray (for phospholipids with free amino groups), and Shiff's reagent (for inositol containing phospholipids). Additional identification was performed by comparing the respective R_f values with those of authentic commercial standards subjected to Silica gel TLC under identical experimental conditions. The quantification was carried out spectrophotometrically against a standard curve by measuring the phosphorous content at 700 nm after scrapping the respective phospholipid spot and mineralization of the substance with a mixture of perchloric acid and sulphuric acid, 1:1 (v/v). The calibration curve was obtained by using a standard solution of KH_2PO_4. It was linear in the concentration range 1 – 130 μg ml^{-1} (as phosphorus). In each series of measurements a standard solution of KH_2PO_4 (10 μl ml^{-1} in water) was used to confirm the validity of calibration (ISO 10540-1, 2003).

Results and Discussions

The collected seeds (achenes) were compressed, with coloration, as follows - golden brown at *C.thoermeri,* brown, marbled greyish-black at *O. acanthium* and shiny, dark brown to black with yellow basal ring at *S. marianum.* Achenes differed in terms of size – those of *C.thoermeri* were the smallest (3-5 mm x 1 mm), followed by the seeds of *O. acanthium (*4-5 mm *x* 2 mm*)* and the largest were achenes of *S. marianum* (6-7 mm x 3 mm). The morphological data are based on measurements of a hundred seeds for every species. The moisture of the seeds was 6.20% for *C. thoermeri*, 6.43% for *O. acanthium* and 5.47% for *S. marianum*.

The total content of lipids was 304 $g.kg^{-1}$ for *C. thoermeri*, 212 $g.kg^{-1}$ for *O. acanthium* and 232 $g.kg^{-1}$ for *S. marianum*. Thirteen fatty acids were determined by GC, constituting 100% (Table 1). The main fatty acids were oleic, linoleic and palmitic. Distribution of fatty acids is presented in Figures 1, 2 and 3.The correlation unsaturated: saturated fatty acids were 74.2: 25.8 for *C. thoermeri* (Figure 1), 88.5: 11.5 for *O. acanthium* (Figure 2) and 80.3: 19.7 for *S. marianum* (Figure 3). Palmitic acid ($C_{16:0}$) predominated in the fraction of saturated fatty acids. Oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids were predominant among the unsaturated acids.

Similar data about correlation between unsaturated: saturated fatty acids for *S. marianum* (75.1: 24.9 respectively) established El-Mallah et al. (2003). These and other authors (Khan et al., 2007; Mirzaeva et al., 2011) found major components in seed oil as linoleic acid (43.5 - 64.4%), oleic acid (20.8 - 29.8%), palmitic acid (7.2% - 9.7%) and stearic acid (2% - 6.6%).

Table 1
Fatty acid composition of seed oils

№	Fatty acids		Content, $g.kg^{-1}$		
			Carduus thoermeri	*Onopordum acanthium*	*Silybum marianum*
1	$C_{12:0}$	Lauric	21	-	-
2	$C_{12:1}$	Lauricoleic	5	11	1
3	$C_{14:0}$	Myristic	41	2	1
4	$C_{14:1}$	Miristicoleic	23	19	18
5	$C_{16:0}$	Palmitic	150	99	142
6	$C_{16:1}$	Palmitoleic	7	1	1
7	$C_{17:0}$	Margaric	1	1	1
8	$C_{18:0}$	Stearic	7	9	20
9	$C_{18:1}$	Oleic	530	342	503
10	$C_{18:2}$	Linoleic	176	511	277
11	$C_{20:0}$	Arachidic	36	1	30
12	$C_{20:1}$	Gadoleic	1	1	3
13	$C_{22:0}$	Behenic	2	3	3

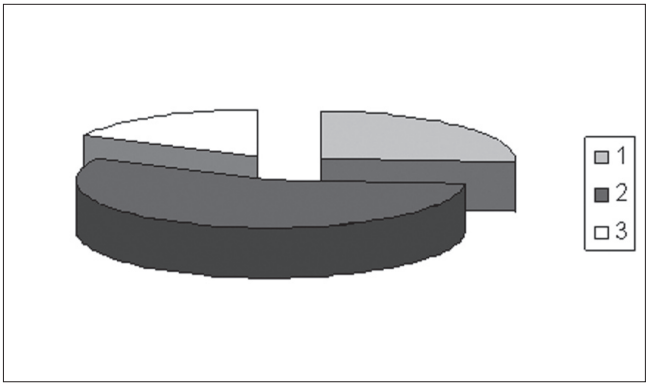

Fig. 1. Distribution of fatty acids in *Carduus thoermeri*
1-saturated fatty acids (258 g.kg^{-1})
2-monounsaturated fatty acids (566 g.kg^{-1})
3-polyunsaturated fatty acids (176 g.kg^{-1})

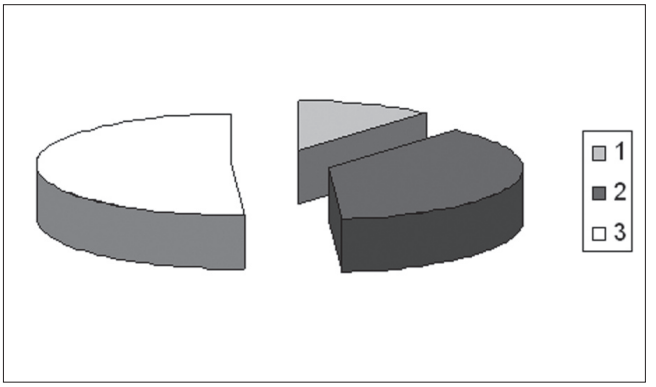

Fig. 2. Distribution of fatty acids in *Onopordum acanthium*
1-saturated fatty acids (115 g.kg^{-1})
2-monounsaturated fatty acids (374 g.kg^{-1})
3-polyunsaturated fatty acids (511 g.kg^{-1})

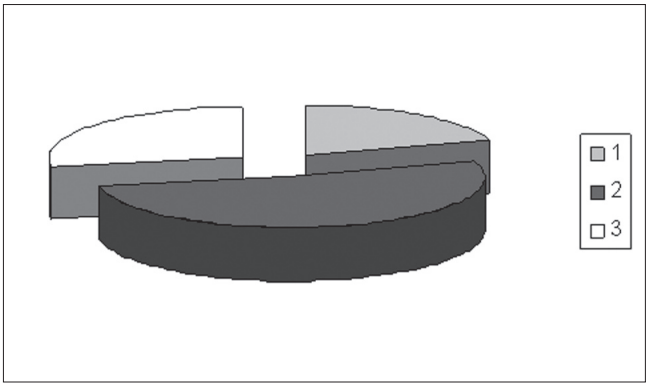

Fig. 3. Distribution of fatty acids in *Silybum marianum*
1-saturated fatty acids (197 g.kg^{-1})
2-monounsaturated fatty acids (526 g.kg^{-1})
3-polyunsaturated fatty acids (277 g.kg^{-1})

The fatty oil amound in *O. acanthium* seeds found by other authors (Tonguc and Erbas, 2012) is 144 g.kg^{-1}, which is less than what we determined for this species. They found the main components in fatty oil linoleic acid (576.5 g.kg^{-1}), oleic acid (287.9 g.kg^{-1}), palmitic acid (88.1 g.kg^{-1}) and stearic acid (39.5 g.kg^{-1}). Our study confirmed the presence of linoleic, oleic and palmitic acids as main components.

For *C. thoermeri* lacks of date for the seed oil constituents and this is investigation for the first time. Obtained results showed that *C. thoermeri* seeds maintained higher total lipid content then those of *S. marianum*, and were equally rich in unsaturated fatty acids. Madrigal et al. (1975) find out methyl oleanolat, triterpens (α- and β-amyrin) and triterpene alcohols, diglycerides and triglycerides for other *Carduus* species *(C. nigrescens).* Al-Shammari et al. (2012) determine saturated and unsaturated fatty acids and their esters in the aerial part of *Carduus pycnocephalus.*

Sterols were presented in the so called non-saponificated part (72 g.kg^{-1} in *C. thoermeri,* 25 g.kg^{-1} in *O. acanthium* and 27 g.kg^{-1} in *S. marianum*) and their total content in the oils was found to be 3 g.kg^{-1}, 5 g.kg^{-1} and 6 g.kg^{-1}, respectively. The individual sterol composition is presented in Table 2, where it is obvious that β-Sitosterol predominated in the sterol fraction with 546 g.kg^{-1}, 632 g.kg^{-1} and 595 g.kg^{-1}, followed by campesterol with 156 g.kg^{-1}, 128 g.kg^{-1} and 131 g.kg^{-1} respectively for the studied species. El-Mallah et al. (2003) found β-sitosterol (574 g.kg^{-1}) and stigmasterol (204 g.kg^{-1}) as valuable components in *S. marianum* oil.

The total content of tocopherols in the studied oils was comparatively low – 0.246 g.kg^{-1} for *O. acanthium* oil and 0.602 g.kg^{-1} for *S. marianum* oil. Tocopherols were not determinated in *C. thoermeri* oil. In *O. acanthium* oil only α-tocopherol (911 g.kg^{-1}) and α-tocotrienol were detected (Table 3).

Table 2
Sterol composition of seed oils

Sterols	Content, g.kg^{-1}		
	Carduus thoermeri	*Onopordum acanthium*	*Silybum marianum*
Cholesterol	25	11	35
Brassisterol	25	16	52
Campesterol	156	128	131
Δ^7-Campesterol	29	48	29
Stigmasterol	15	33	6
β-Sitosterol	546	632	595
Δ^5-Avenasterol	39	36	41
Δ^7-Stigmasterol	78	57	88
Δ^7-Avenasterol	87	39	23

α-tocopherol predominated also in *S. marianum* oil (720 $g.kg^{-1}$), followed by γ-tocopherol and β-tocopherol (Table 3). El-Mallah et al. (2003) established 0,260 $g.kg^{-1}$ total tocopherols in *S. marianum* seed oil. The authors confirmed that α-tocopherol is the component with highest amount in the tocopherol fraction (845 $g.kg^{-1}$).

The total content of phospholipids in the oils was 4 $g.kg^{-1}$ in *C. thoermeri*, 19 $g.kg^{-1}$ in *O. acanthium* and 3 $g.kg^{-1}$ in *S. marianum*. Phospholipid composition is presented in Table 4, which illustrates that the phosphadilinositol, phosphadilholin and phosphatidic acids were predominant in the oils.

Table 3
Tocopherol composition of seed oils

Tocopherols and tocotrienol	Content, $g.kg^{-1}$		
	Carduus thoermeri	*Onopordum acanthium*	*Silybum marianum*
α - Tocopherol	-	911	720
α - Tocotrienol	-	89	-
β - Tocopherol	-	-	125
γ - Tocopherol	-	-	155

Table 4
Phospoholipid composition of seed oils

Phospoholipids	Contents, $g.kg^{-1}$		
	Carduus thoermeri	*Onopordum acanthium*	*Silybum marianum*
Phosphatidylcholine	244	183	280
Phosphatidylethanolamine	98	188	200
Phosphatidylinositol	317	320	240
Phosphatidic acids	122	147	120
Phosphatidilserine	-	-	80
Diphosphatidylglycerol	122	162	80
Lysophosphatidyethan olamine	97	-	-

Conclusion

The present study established similar compounds in *Carduus thoermeri, Onopordum acanthium* and *Silybum marianum* seed oils, some of them have valuable applications as a food and pharmaceuticals. We found that the main components in fatty acids were oleic, linoleic and palmitic acids, with a predominance of unsaturated fatty acids. In the sterol fraction the main components were β-sitosterol and campesterol. Phosphatidylinositol was with the highest concentration in the phospolipid fraction. Due to content of unsaturated fatty acids, tocopherols and phytosterols, the seeds of the studied species (especially *O. acanthium)* could be utilized as a source of valuable oil for human consumption.

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