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Separation of high-value extracts from *Silybum marianum* seeds: Influence of extraction technique and storage on composition and bioactivity

Ivana Lukic [a], Stoja Milovanovic [a,b,*], Milica Pantic [c], Ivana Srbljak [d], Ana Djuric [d], Vanja Tadic [e], Katarzyna Tyśkiewicz [b]

[a] University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11120, Belgrade, Serbia
[b] Łukasiewicz Research Network- New Chemical Syntheses Institute, Al. Tysiąclecia Państwa Polskiego 13a, 24-110, Puławy, Poland
[c] University of Maribor, Faculty of Chemistry and Chemical Engineering, Smetanova 17, 2000, Maribor, Slovenia
[d] Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000, Belgrade, Serbia
[e] Institute for Medical Plant Research "Dr Josif Pancic", Tadeusa Koscuska 1, 11000, Belgrade, Serbia

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ABSTRACT

Extracts from *Silybum marianum* seeds have high industrial potential due to their application as phytopharmaceuticals and food. In order to optimize its production, conventional extraction (in Soxhlet apparatus using solvents ethanol and *n*-hexane) and supercritical fluid extraction (using CO_2 at the pressure of 30 MPa and temperature of 40 °C with and without co-solvent ethanol, from oil-rich and defatted seeds) were compared. In addition, the effect of plant cultivation (years 2011 and 2019) and the effect of seeds and extract storage on the yield and quality of extracts were assessed. It was shown that unsaturated fatty acids (linoleic and oleic acids being most dominant) constituted 64–87% of obtained extracts. Content of α-tocopherol varied from 0.01 to 13 g/100 g, while total phenolic content was in the range from 13.2 to 104.2 g_{GAE}/100 $g_{extract}$. In vitro cytotoxic activity analysis confirmed that extracts obtained from defatted seeds showed activity against cancer cells. This study pointed out the important role of the cultivation year, selection of extraction technique and solvent, as well as storage on the extraction yield, chemical profile, and cytotoxic activity of extracts.

1. Introduction

Silybum marianum (Asteraceae) has been used in traditional medicine since ancient times mostly for treatment of liver disorders and protection of the liver from toxins (Çelik & Gürü, 2015; Chambers et al., 2017; Đorđević et al., 2018; Elateeq et al., 2020a; Hadolin et al., 2001). It has been also reported that the *S. marianum* extracts show numerous beneficial pharmacological effects such as anti-inflammatory, antioxidant, cardiovascular protective, anti-cancer, neuroprotective, and anti-diabetic (Ben Rahal et al., 2015; Elateeq et al., 2020b; Z. S. Zhang et al., 2020). The extracts from the *S. marianum* seeds can slow down the aging of the skin by radical-inhibiting activity and can reduce UVA-induced skin damage (Chambers et al., 2017). The seeds contain a high amount of oil rich in unsaturated fatty acids such as linoleic (polyunsaturated omega-6 essential fatty acid) and oleic (monounsaturated omega-9 fatty acid) (Ben Rahal et al., 2015; Çelik & Gürü, 2015; Růžičková et al., 2011). Both fatty acids have been associated with health and aesthetical benefits (Z. S. Zhang et al., 2020). Furthermore, this oil is a natural source of vitamin E, which represents the complex mixture of four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol) and four tocotrienols (Hadolin et al., 2001; Z. S. Zhang et al., 2020). In addition, the major group of biologically active phytochemicals present in the seeds is a complex mixture of a polyphenolic molecule called silymarin (Çelik & Gürü, 2015; Elateeq et al., 2020a; Hadolin et al., 2001; Szentmihályi et al., 1998). Silymarin is composed of flavonolignans (silybin, isosilybin, silychristin, silydianin) and minor fractions of other flavonoids (toxifolin) (Ben Rahal et al., 2015; Chambers et al., 2017; Elateeq et al., 2020b; Lucini et al., 2016; Marmouzi et al., 2021; Yang et al., 2017; Z. S. Zhang et al., 2020). Evidently, *S. marianum* seeds contain valuable bioactive constituents that can be potentially used in various industries such as food, pharmaceutical, and cosmetic. Therefore, there is considerable interest in studying different parameters that affect the amount and composition of the separated extract such as storage and extraction method. The quantity and quality of the extract can be significantly influenced by the seeds' storage before extraction especially when storing over an

* Corresponding author. University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11120, Belgrade, Serbia.
E-mail address: smilovanovic@tmf.bg.ac.rs (S. Milovanovic).

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extended period. Changes in physical and chemical characteristics of both stored extract and extract separated from stored seeds were attributed to hydrolysis and oxidation (Fotouo et al., 2016).

Usually, conventional methods that employ organic solvents are used for the separation of extracts from *S. marianum* seeds (Đorđević et al., 2018; Lucini et al., 2016; Růžičková et al., 2011). Although these methods provide high extraction yields, organic solvents are non-selective, can be toxic, and remain in the final product (Çelik & Gürü, 2015). In addition, conventional processes are long, performed at high temperatures, and obtained extracts have low purity (Ben Rahal et al., 2015). Thus, there is a need to establish new processes in correspondence with the concept of green chemistry, which can reduce solvent consumption, waste accumulation, extraction time, and extract toxicity (Ben Rahal et al., 2015). In these sense, application of supercritical fluid extraction (SFE) has been recognized as a promising 'green' alternative to conventional processes (Reverchon & De Marco, 2006). It allows the production of pure, solvent-free, and highly valuable extracts. The SFE process can be conducted at relatively low temperatures, decreasing the energy demand and avoiding thermal degradation of heat-sensitive compounds (Yang et al., 2017). Supercritical carbon dioxide ($scCO_2$) is a commonly used fluid for the SFE process since it is safe, widely available, easily recyclable, and possesses a unique and easily adjustable properties (Ben Rahal et al., 2015). Several studies have reported the extraction from *S. marianum* seeds using $scCO_2$ (Ben Rahal et al., 2015; Hadolin et al., 2001; Ivanovic et al., 2014; Szentmihályi et al., 1998; Çelik; Gürü, 2015) concluding that yield and composition of extracts significantly differ depending on cultivation region, material pretreatment, pressure (10–40 MPa) and temperature (25–80 °C) applied. However, the information on selection of solvent and extraction method as well as seeds storage on yield and composition of extracts is lacking. Furthermore, there is no information on the composition of the supercritical extract from *S. marianum* seeds grown in Serbia. Additionally, this manuscript describes for the first time the effect of storage on extract composition and activity of extracts obtained by SFE from defatted seeds. For this purpose, a comprehensive study on extraction by conventional technique (in Soxhlet apparatus using ethanol and *n*-hexane) and SFE technique (using $scCO_2$ with and without ethanol for separation of extract from oil-rich and defatted seeds) was performed. The extraction process was conducted on 1-year-old seeds produced in 2011 and 2019, as well as 8-years-old seeds produced in 2011. In addition, the composition and activity of extracts after 8 years of storage were determined. Obtained extracts were analyzed by GC/MS, GC/FID, and HPLC methods. Due to the high potential of extracts for oral and topical applications, cytotoxic activity and total phenolic content was also evaluated.

2. Material and methods

2.1. Materials

The seeds of *S. marianum* were produced in 2011 and 2019 (Institute for Medical Plant Research "Dr Josif Pancic", Serbia). The seeds were stored in a dark and dry place at room temperature (20 °C) prior to use. Commercial CO_2 (purity 99.9%) was purchased from Messer-Tehnogas (Serbia), ethanol (96 vol%) was purchased from Zorka Pharma (Serbia), *n*-hexane, and petroleum ether from Sigma-Aldrich (Germany). Additionally, sodium sulfate (Centrohem, Serbia), methylene chloride (Fisher Scientific UK Ltd, UK), phosphoric acid (Sigma, USA), and DL-all-*rac*-α-tocopherol (Sigma, USA) were used.

2.2. Moisture content and pre-treatment of seeds

Moisture content in *S. marianum* seeds was determined to be 5.15% using a laboratory moisture analyzer (MAC 50/1/WH, RADWAG®, Poland).

The seeds were milled using a basic coffee mill and sieved using a laboratory sets of sieves. Obtained material with an average particle size of 0.4 mm was further used in extraction processes.

2.3. Extraction techniques

Conventional extraction processes were performed in a Soxhlet apparatus (100 mL). Plant material (10.00 g) was packed in a filter bag, placed in a Soxhlet, and soaked with 250 mL of solvent. The average extraction temperature was 78 °C, 69 °C, and 40–60 °C when ethanol, *n*-hexane, and petroleum ether were used as solvents, respectively. The extraction lasted for 4 h, after which solvents were removed using a rotary vacuum evaporator (Devarot, Elektromedicina, Slovenia). Extracts were stored at 8 °C until analysis.

Supercritical fluid extraction (SFE) processes were performed in an Autoclave Engineers Screening System (Autoclave Engineers Group, USA) previously described in detail (Milovanovic et al., 2013) with some variations. *Method I*: Plant material (10.00 g) was closed in the extractor and heated to 40 °C. CO_2 was introduced and pressure was raised to 30 MPa. *Method II*: Ethanol (5 wt%) was added to plant material placed in the extractor and the SFE process was performed in a manner described in Method I. *Method III*: Defatted plant material (using petroleum ether) was transferred to the extractor and processed as described in Method I. *Method IV*: Defatted plant material was transferred to the extractor and processed as described in Method II. Extracts were stored at 8 °C until analysis.

2.4. Analytical procedures

Gas chromatography analysis was carried using an HP-5890 Series II GC apparatus (Hewlett-Packard, Germany) equipped with HP-5 column (25 m × 0.32 mm, 0.52 μm) and fitted to a flame ionization detector. The carrier gas was helium with a flow rate of 1 mL/min, split ratio 1:30, injector temperature 250 °C, and detector temperature 300 °C. The column temperature was linearly increased from 40 °C to 260 °C at a rate of 4 °C/min and then kept isothermally at 260 °C for 10 min. Area percent reports were used as a base for the quantification analysis.

The same analytical conditions were employed for GC/MS analysis using a HP G1800C Series II GCD system (Hewlett-Packard, USA) and column HP-5MS (30 m × 0.25 mm, 0.25 μm). Mass spectra were acquired in EI mode (70 eV) in m/z range 40–450. The components of oil were identified by comparison of their spectra to those from Wiley 275 and NIST/NBS libraries. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System Software (Software, 2005), compared to those from available literature (Adams, 2007) and used as an additional tool to approve MS findings. A DL-all-*rac*-α-tocopherol standard was used for the analyses of α-tocopherol content in extracts.

Extracts were additionally analyzed after converting the fatty acids to methyl esters using AOAC procedure 965.49 (Method, 1998) and a Shimadzu GCMSQP2010 ultra mass spectrometer (Japan) previously described (Tadic et al., 2021). The derivatized samples were dissolved in the methylene chloride and injected in an amount of 1 μL. The content of compounds was determined based on area of chromatograms and defined as content according to the GC area. The identification of the constituents was performed by comparing their mass spectra and retention indices with those obtained from authentic samples and/or listed in the NIST/Wiley mass-spectra libraries (PBM/NIST/AMDIS) and available literature data (Adams, 2007).

High-performance liquid chromatography (HPLC) analysis was performed using Agilent Technologies 1200 HPLC (USA) with a column 150 × 4.6 mm, 5 μm. Separation of components was achieved using a Phenomenex Syringe Hydro RP C18 at 35 °C with a flow rate of 1 mL/min and a mobile phase of solvent A (0.1 mol/L phosphoric acid) and solvent B (acetonitrile). Elution was the combination of gradient and isocratic mode: 5–30% A, 0–20 min; 30% A, 5 min; 30–35% A, 25–30 min. The samples were prepared by dissolving 32.00 mg of extract in 1

mL ethanol and filtering through 0.22 μm PTFE filters. The identification was based on retention time and spectra matching.

Total phenolic content was estimated by the Folin–Ciocalteu method as previously described (Makanjuola, 2017; Parry et al., 2008). Extracts (100 mg) were diluted with acetone (Poch, Poland) to 5 mL. Distilled water (1.5 mL) was added to 100 μL of obtained solution and 100 μL of the Folin–Ciocalteu reagent (Sigma, USA). After 8 min, 300 μL of sodium carbonate (20 g/100 mL, Sigma, USA) was added and intensively mixed. After 40 min at room temperature in a dark place, absorbance was recorded at 765 nm using a spectrophotometer (V-650 JASCO Deutschland GmbH, Germany). A calibration curve was prepared using solutions of gallic acid (97.5–102.5%, Sigma, Poland) in acetone. Results are expressed as mass (mg) of gallic acid equivalents (GAE) per mass (g) of extract.

2.5. Cytotoxic activity

In vitro study by MTT colorimetric assay was performed using cell lines: normal, human embryonic lung fibroblast (MRC-5), human cervical adenocarcinoma (HeLa), human prostate cancer (DU145), and human colonic adenocarcinoma (LS174T). Cells were grown in RPMI-1640 medium with 3 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10% fetal bovine serum, and 25 mM Hepes adjusted to pH 7.2, at 37 °C in an atmosphere of 5% CO_2 and humidified air. Cancer cell lines were purchased from the American Type Culture (USA) while Hepes, L-glutamine, and RPMI 1640 were purchased from PAA (Austria). Cells were seeded into 96-well microtiter plates in following densities: 3000 per well for HeLa, 5000 per well for MRC-5 and LS174T, and 7000 per well for DU145 cells. Upon adhesion, 24 h later, cells were treated with extracts (concentrations 12.5–200 μg/mL). After 72 h incubation, 10 μL of MTT solution (5 mg/mL of phosphate-buffered saline) was added. Samples were then incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Subsequently, 100 μL of 100 g/L sodium dodecyl sulfate was added. The absorbance was measured 24 h later at 570 nm, using a Multiskan EX reader (Thermo Labsystems Beverly, USA).

2.6. Statistical analysis

Quantitative data were reported as mean ± standard deviation. A one-way ANOVA (analysis of variance) method followed by post hoc Tukey's HSD test was used to evaluate the significant difference between results. Statistical analyses were performed using Astatsa online statistical calculator (Vasavada, n.d.). Different letters were used to indicate that the means difference is significant at a level $p < 0.05$.

3. Results and discussion

3.1. Conventional vs. supercritical fluid extraction technique

The results of the conventional extraction process using ethanol and *n*-hexane (Table 1) show that the year of *S. marianum* cultivation and the seeds storage had a significant effect ($p < 0.05$) on an amount of extractible compound. The 80% yield increase in process with ethanol (from 17.3 g/100 g for SXT_E^{2012} to 31.0 g/100 g for SXT_E^{2019}) and 10% decrease in process with *n*-hexane (from 25.3 g/100 g for SXT_H^{2012} to 22.4 g/100 g for SXT_H^{2019}) after 8 years of seeds storage can lead to the conclusion that the amount of non-polar compounds decreased while the amount of polar compounds increased with storage. Similarly, Fotouo et al. (2016) reported a decrease of extraction yield from 39 to 36 g/100 g for moringa seeds upon storage at room temperature (15–25 °C) in dark glass bottles for two years. Additionally, it can be seen that seeds produced in 2019 had a higher yield (25.3 g/100 g) in process with ethanol (SXT_E^{2020}) and lower extraction yield (22.3 g/100 g) in process with *n*-hexane (SXT_H^{2020}) compared to seeds produced in 2011. Similar to our study, Růžičková et al. (2011) found out the change in yield from 20.3 to 19.6 g/100 g for *S. marianum* seeds

Table 1
Nomenclature of the samples and its visual appearance.

Year of seeds growing	Year of extraction	Extraction technique	Extraction solvent	Abbreviation	Yield (g/100 g)	Year of analysis	State (at 22 °C)	Color	Color (Pantone scale)
2011	2012	Conventional	E	SXT_E^{2012}	17.3 ± 0.9 ^{d}	/	Semi solid	Orange-brown	Pantone 138
		Conventional	H	SXT_H^{2012}	25.3 ± 0.6 ^{b}		Liquid	Light brown	Patone 131
		SFE (Method I)	C	SFE_C^{2012}	21.4 ± 0.1 ^{c}		Liquid	Light yellow	Pantone 586
		SFE (Method II)	C + E	SFE_C + E^{2012}	18.2 ± 0.4 ^{d}		Liquid	Light yellow	Pantone 585
						2019	Semi-solid	Dark brown	Pantone 732
							Liquid	Dark yellow	Pantone 108
							Tick liquid	no color, transparent	x
							Tick liquid	no color, transparent	x
2011	2019	Conventional	E	SXT_E^{2019}	31.0 ± 0.9 ^{a}	2020	Semi-solid	Brown	Patone 154
		Conventional	H	SXT_H^{2019}	22.4 ± 0.6 ^{c}		Liquid	Light brown	Patone 131
		SFE (Method I)	C	SFE_C^{2019}	10.3 ± 0.1 ^{f}		Liquid	Milky white-green	Pantone 611
		SFE(Method II)	C + E	SFE_C + E^{2019}	10.7 ± 0.2 ^{f}		Liquid	White-yelow-green	Pantone 610
		SFE (Method III)	P + C	SFE_P + C^{2019}	0.7 ± 0.1 ^{h}		Semi-solid	Dark brown-green	Pantone 581
		SFE (Method IV)	P + C + E	SFE_P + C + E^{2019}	1.3 ± 0.1 ^{g}		Semi-solid	Dark green	Pantone 5815
2019	2020	Conventional	E	SXT_E^{2020}	25.3 ± 0.9 ^{b}	2021	Semi-solid	Orange-brown	Pantone 138
		Conventional	H	SXT_H^{2020}	22.3 ± 0.7 ^{c}		Liquid	Light brown	Patone 131
		SFE (Method I)	C	SFE_C^{2020}	16.7 ± 0.3 ^{d,e}		Liquid	Light yellow	Pantone 586
		SFE (Method II)	C + E	SFE_C + E^{2020}	15.3 ± 0.4 ^{e}		Liquid	Light yellow	Pantone 585
		SFE (Method III)	P + C	SFE_P + C^{2020}	0.7 ± 0.0 ^{h}		Semi-solid	Dark brown-green	Pantone 581
		SFE (Method IV)	P + C + E	SFE_P + C + E^{2020}	1.4 ± 0.1 ^{g}		Semi-solid	Dark green	Pantone 5815

E−ethanol; H- *n*-hexane; C- $scCO_2$; P- petroleum ether.
Different letters indicate significantly different values ($p < 0.05$) among the results.

produced in 2007 and 2008, respectively.

Kinetics of SFE from oil-rich seeds as well as defatted seeds are shown in Fig. 1. The extraction yields ranged from around 10 g/100 g (sample SFE_C^{2019}) to around 21 g/100 g (sample SFE_C^{2012}), which is in accordance with the results reported in literature where yield varied from 5 to 26 g/100 g (Çelik & Gürü, 2015; Hadolin et al., 2001; Ivanovic et al., 2014; Szentmihályi et al., 1998). Çelik and Gürü (2015) achieved 13 g/100 g yield at 20 MPa and 40 °C from seeds grown in Turkey while Hadolin et al. (2001) reported yield at 30 MPa and 40 °C to be around 17 g/100 g from seeds grown in Slovenia. The reason for such variation can be found in different plant origins, material pretreatment and/or pressure and temperature conditions. Our findings confirmed that the year of *S. marianum* cultivation also had a significant effect ($p < 0.05$) on extraction yield (Table 1). Namely, the seeds produced in 2011 allowed a yield of 21.4 g/100 g while the seeds produced in 2019 enabled a significantly lower yield of 16.7 g/100 g (Fig. 1a,c). In addition, seeds storage significantly affected the efficiency of the SFE process, as indicated by the yield of 21.4 g/100 g achieved when extraction was performed using 1-year-old seeds, and twice as low (10.3 g/100 g) when extraction was performed using 8-years-old seeds (Fig. 1a and b). Obviously, the storage caused the loss of compounds that can be extracted using non-polar CO_2. The same behavior was noticed for conventional extraction using non-polar solvent *n*-hexane (Table 1). The reduced amount of extractible oil has been reported for the argan kernel, with the 7% lower yield after 1-year storage independently of the storage temperature (room temperature or 4 °C) (Harhar et al., 2010), as well as for the moringa seed (the yield decreased up to 11% after 2-years storage at room temperature) (Fotouo et al., 2016).

Addition of ethanol as co-solvent to 1-year-old seeds (samples SFE_C + E^{2012} and SFE_C + E^{2020}) led to the slight decrease of the extraction yields (Fig. 1a,c) probably due to the low affinity of ethanol towards oil components. Namely, it appears that ethanol, as a polar solvent, does not achieve efficient solvation of the components at tested extraction conditions and decreases its extractability. While yield for 1-year-old seeds decreased to 18.2 g/100 g upon addition of ethanol (SFE_C + E^{2012}), extraction yield for 8-years-old seeds was unaffected with the addition of ethanol. Only difference was a higher initial rate of extraction for this sample.

The SFE process was also studied for the separation of highly-valuable compounds from defatted seeds (Csupor et al., 2016; Wianowska & Wiśniewski, 2014). For this purpose, a pretreatment step involving Soxhlet extraction with petroleum ether was performed separating 23.0 and 21.8 g/100 g of oil from 8-years-old and 1-year-old seeds, respectively. After drying of defatted seeds, SFE was performed resulting in yields between 0.6 and 1.4 g/100 g (Fig. 1d). Similarly, 1.1 g/100 g yield was reported for SFE from *S. marianum* seeds using $scCO_2$ with dimethyl sulfoxide at 35 MPa and 60 °C after seeds defatting using dimethyl sulfoxide (Momenkiaei & Raofie, 2018). It is interesting to notice that after the oil was removed from the seeds, a higher yield was obtained for SFE with ethanol. This could be explained by the fact that more polar components, with higher solubility in ethanol, were left in the seeds after defatting.

According to the statistical analysis (Table 1), resulting extraction yields for conventional and SFE techniques were significantly different for seeds produced in 2011 and 2019. Besides, the year of seeds production and its storage had a significant effect on extraction yield.

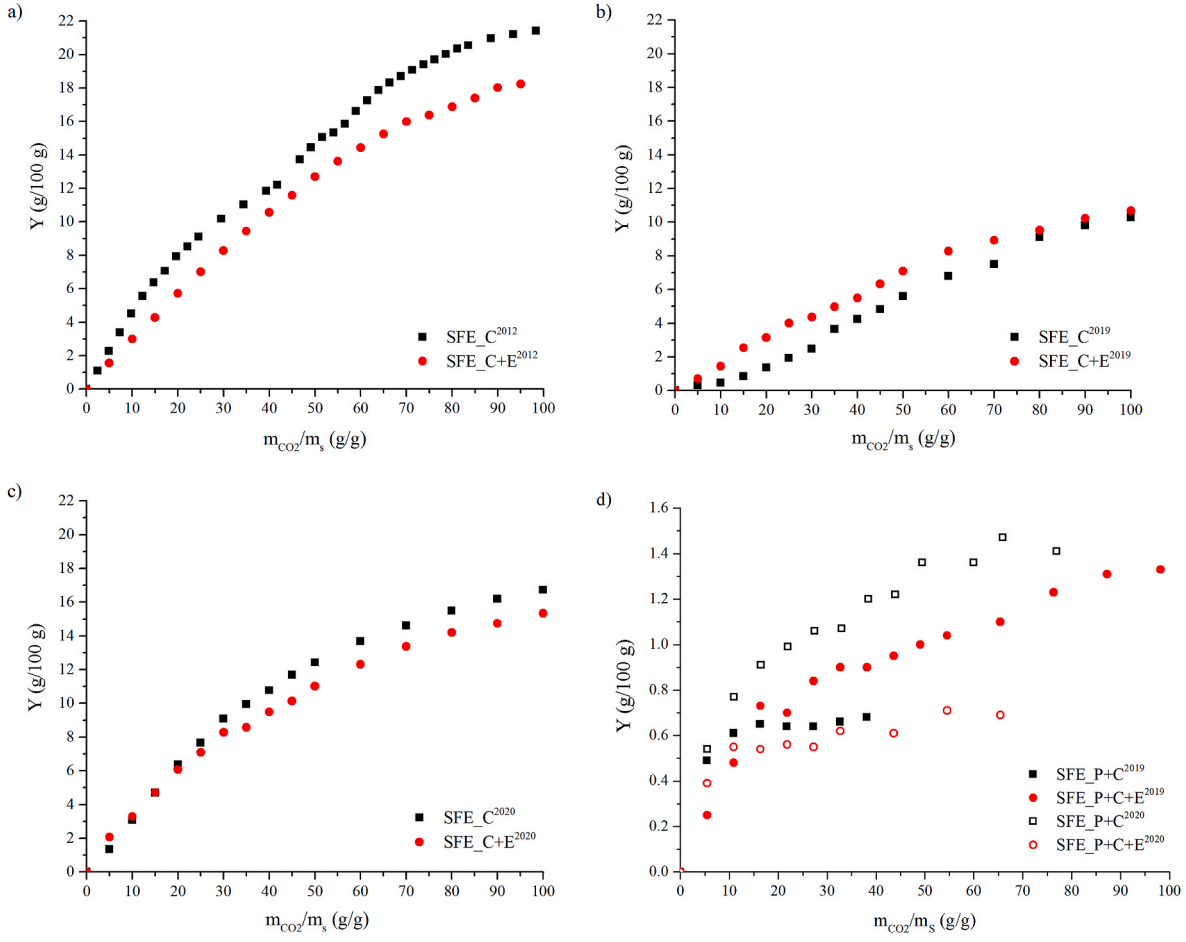

Fig. 1. Kinetic of SFE from *S. marianum* seeds at 30 MPa and 40 °C without and with co-solvent for: a) 1-year-old seeds produced in 2011, b) 8-years-old seeds produced in 2011, c) 1-year-old seeds produced in 2019, and d) defatted seeds produced in 2011 and 2019.

Although slightly higher yields were obtained by conventional technique, it is not a prerequisite for labeling this technique as superior. The conventional technique requires high temperatures, use of a relatively large amount of organic solvents, an additional step of solvent removal that is time and energy-consuming, and finally the risk of organic solvent residue in the extract. In addition, besides oil (21–27 g/100 g), *S. marianum* seeds are composed of protein (15–18 g/100 g), fiber (25–27 g/100 g), ash (4–5 g/100 g), moisture (4–8 g/100 g), and total carbohydrate (20–24 g/100 g) (Z. S. Zhang et al., 2020), which can also be extracted by conventional techniques making extracts less valuable.

The appearance of obtained extracts significantly differs (Fig. 2). All supercritical extracts were oily and in a liquid state at room temperature (Table 1). On the other hand, extracts obtained by the conventional process were semi-liquid (containing both oily and solid phases). During storage at 8 °C, all extracts become solid. While extracts obtained by $scCO_2$ were light yellow (SFE_C^{2020} and SFE_C + E^{2020}), the extracts after the 8-years storage lost color (SFE_C^{2012} and SFE_C + E^{2012}). Extracts obtained by Soxhlet extraction using *n*-hexane were brown, but after the 8-years storage, they become dark yellow (SXT_H^{2012}). It was reported that the yellow color of *S. marianum* extracts comes from carotenoids (mainly xanthophyll derivatives) and that the brown color comes from porphyrin derivatives (Meddeb et al., 2017; Szentmihályi et al., 1998).

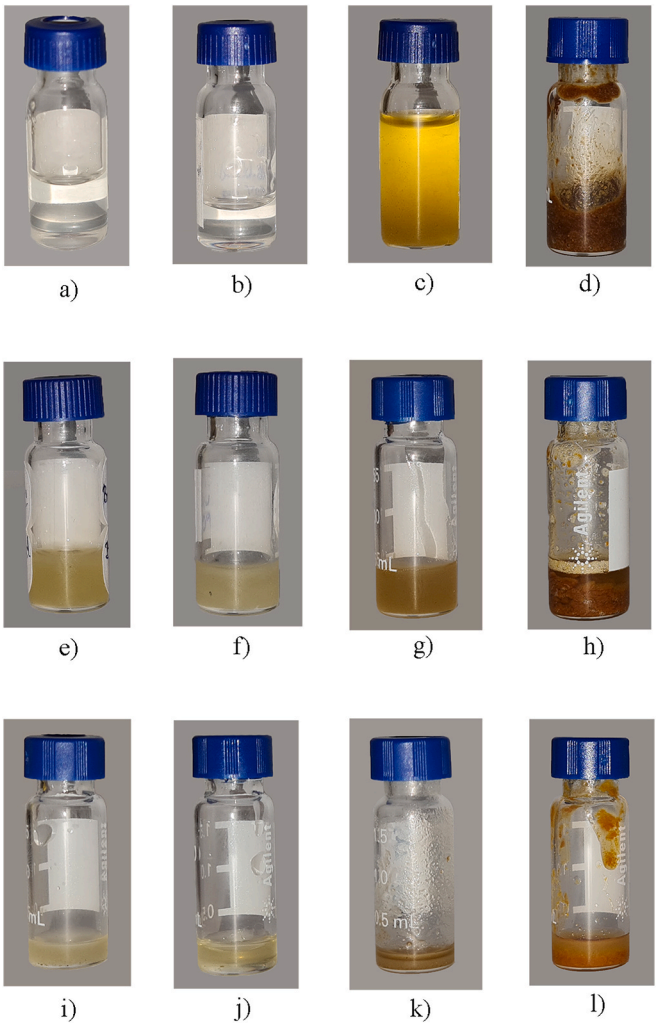

Fig. 2. Images of vials containing *S. marianum* extracts: a) SFE_C^{2012}, b) SFE_C + E^{2012}, c) SXT_H^{2012}, d) SXT_E^{2012}, e) SFE_C^{2019}, f) SFE_C + E^{2019}, g) SXT_H^{2019}, h) SXT_E^{2019}, i) SFE_C^{2020}, j) SFE_C + E^{2020}, k) SXT_H^{2020}, l) SXT_E^{2020}, m) SFE_P + C^{2020}, and n) SFE_P + C + E^{2020}.

Table 2
Fatty acid content in extracts obtained from of *S. marianum* seed determined by GC/FID as area percentage.

Name	KI	SXT_E^{2012}	SXT_H^{2012}	SFE_C^{2012}	SFE_C + E^{2012}	SXT_E^{2019}	SXT_H^{2019}	SFE_C^{2019}	SFE_C + E^{2019}	SXT_E^{2020}	SXT_H^{2020}	SFE_C^{2020}	SFE_C + E^{2020}
Saturated Fatty Acids													
Palmitic acid	**1921**	8.9 ± 0.2^{c}	10.7 ± 0.2^{b}	15.0 ± 0.2^{a}	9.5 ± 0.2^{c}	8.9 ± 0.2^{c}	8.7 ± 0.2^{c}	9.1 ± 0.2^{c}	5.2 ± 0.2^{d}	8.4 ± 0.1^{c,e}	8.1 ± 0.1^{c,e}	8.0 ± 0.2^{c,e}	8.6 ± 0.2^{c,e}
Stearic acid	**2124**	1.5 ± 0.2^{c}	4.4 ± 0.2^{d}	7.2 ± 0.3^{a}	5.4 ± 0.2^{b,d}	5.8 ± 0.2^{b,c}	5.4 ± 0.2^{c,d}	5.5 ± 0.2^{c,d}	2.5 ± 0.2^{e}	5.0 ± 0.2^{c,d}	5.1 ± 0.2^{c,d}	5.1 ± 0.1^{c,d}	5.1 ± 0.1^{c,d}
Arachidic acid	**2311**	3.0 ± 0.1^{b}	1.8 ± 0.1^{c}	4.7 ± 0.1^{a}	2.7 ± 0.1^{b}	2.9 ± 0.1^{b}	3 ± 0.1^{b}	2.8 ± 0.1^{b}	0.7 ± 0.1^{d}	2.7 ± 0.0^{b}	0.1 ± 0.0^{d}	2.8 ± 0.1^{b}	2.7 ± 0.1^{b}
9,10,12-trimethyl stearic acid	**2411**	/	0.1 ± 0.0^{b}	/	1.3 ± 0.0^{a}	1.3 ± 0.0^{a}	1.4 ± 0.0^{a}	1.4 ± 0.0^{a}	/	0.1 ± 0.0^{b}	/	*t*	*t*
9,10-dihydroxy stearic acid	**2611**	/	0.1 ± 0.0^{a}	/	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	0.9 ± 0.0^{a}	0.9 ± 0.0^{a}	/	/	/	/	/
Behenoic acid	**2530**	2.0 ± 0.0^{b}	1.1 ± 0.0^{c}	2.9 ± 0.0^{a}	1.8 ± 0.0^{b,c}	2.0 ± 0.0^{b}	2.0 ± 0.0^{b}	1.9 ± 0.0^{b,c}	1.0 ± 0.0^{b}	1.8 ± 0.0^{b,c}	2.9 ± 0.1^{a}	1.8 ± 0.1^{b,c}	1.7 ± 0.1^{b,c}
Lignoceric acid	**2712**	0.5 ± 0.0^{b}	0.3 ± 0.0^{b}	0.7 ± 0.0^{b}	0.4 ± 0.0^{b}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.6 ± 0.0^{b}	0.2 ± 0.0^{b}	0.5 ± 0.1^{b}	2.4 ± 0.1^{a}	0.5 ± 0.1^{b}	0.4 ± 0.0^{b}
Unsaturated Fatty Acids													
γ-Linolenic acid	**2091**	/	/	/	/	/	/	/	2.6 ± 0.1^{a}	/	/	/	/
Linoleic acid	**2095**	55.4 ± 0.3^{a}	52.1 ± 0.3^{b}	25.6 ± 0.3^{e}	47.7 ± 0.3^{c}	47.4 ± 0.3^{c}	47.4 ± 0.3^{c}	47.9 ± 0.3^{c}	56.0 ± 0.3^{a}	49.7 ± 0.2^{c,d}	49.4 ± 0.2^{c}	48.0 ± 0.2^{c}	48.4 ± 0.3^{c}
Oleic acid	**2101**	26.0 ± 0.3^{c}	23.1 ± 0.3^{c}	37.1 ± 0.3^{a}	24.9 ± 0.3^{c}	24.9 ± 0.3^{c}	23.9 ± 0.3^{c}	25.2 ± 0.3^{c}	11.3 ± 0.3^{d}	30.6 ± 0.2^{b,c}	30.5 ± 0.3^{b,c}	31.1 ± 0.2^{b,c}	31.0 ± 0.2^{b,c}
α-Linolenic acid	**2104**	/	0.2 ± 0.0^{a}	/	0.5 ± 0.0^{a}	*t*	/	0.1 ± 0.0^{a}	16.7 ± 0.3^{b}	/	/	0.2 ± 0.1^{a}	/
Methyl-linolenate	**2200**	/	/	/	/	1.3 ± 0.1^{a}	1.4 ± 0.1^{a}	1.0 ± 0.0^{a}	*t*	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
α-Eleostearic acid	**2212**	/	0.2 ± 0.0^{b}	/	0.5 ± 0.0^{b}	0.9 ± 0.0^{a}	0.9 ± 0.0^{a}	0.9 ± 0.0^{a}	/	0.1 ± 0.0^{b}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.3 ± 0.0^{b}
(6*Z*,9*Z*,11*E*)-octadecatrienoic acid	**2216**	0.1 ± 0.0^{c}	0.3 ± 0.0^{b,c}	/	0.8 ± 0.0^{a,b}	1.0 ± 0.0^{a}	0.9 ± 0.0^{b}	0.8 ± 0.0^{a,b}	/	/	/	0.3 ± 0.1^{b,c}	0.3 ± 0.0^{b,c}
Ricinoleic acid	**2247**	0.1 ± 0.0^{b}	0.3 ± 0.0^{a,b}	/	0.7 ± 0.0^{a}	/	0.1 ± 0.0^{b}	/	0.2 ± 0.0^{a,b}	/	/	/	/
Eicosaenoic acid	**2284**	0.9 ± 0.0^{a}	0.7 ± 0.0^{a}	1.3 ± 0.1^{a}	0.9 ± 0.0^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	1.0 ± 0.0^{a}	0.3 ± 0.0^{a,b}	0.8 ± 0.0^{a}	0.1 ± 0.0^{b}	0.6 ± 0.1^{a,b}	0.8 ± 0.2^{a}
Total content													
SFA		*15.9*	*18.5*	*30.5*	*21.9*	*22.2*	*21.9*	*22.2*	*9.6*	*18.5*	*18.6*	*18.2*	*18.5*
UFA		*82.5*	*76.9*	*64.0*	*76.0*	*76.4*	*75.4*	*76.9*	*87.1*	*81.3*	*80.2*	*80.4*	*80.9*

KI – Kovats Index; *t*-in traces; *SFA* – saturated fatty acids; *UFA* – unsaturated fatty acids.
Different letters in the same row indicate significantly different values ($p < 0.05$) among the results.

Szentmihályi et al. (1998) reported that the coloring contents of $scCO_2$ extracts, obtained at 30 MPa and 35 °C, expressed in pheophytin was 71.6 μg/g and in total carotenoids was 21.1 μg/g. They also reported that *n*-hexane extracts contain a higher amount of coloring contents (expressed in pheophytin 146 μg/g and expressed in total carotenoids 21.6 μg/g). Presented results indicate that the amount of coloring compounds significantly decreased in extracts upon storage. On the other hand, coloring compounds were preserved in seeds upon 8-years storage.

3.2. Chemical profile of extracts

All obtained extracts have a high content of unsaturated fatty acids (UFA) and low content of saturated fatty acids (SFA) (Table 2). UFA constitute 64.0–87.1%, while the content of SFA ranges from 9.6 to 30.5%. This information is especially important from a nutritional point of view considering the role of UFA in the prevention of cardiovascular diseases and its effect on a number of different metabolic pathways (Lunn & Theobald, 2006). Extracts obtained from the 8-years-old seeds had higher content of UFA compared to 8-years-old extracts. Linoleic (25.6–56.0%) and oleic (11.3–37.1%) acids were the predominant fatty acids in the separated extracts. Linoleic acid is known as a nutrient that is not produced in the human body and thus must be acquired through the consumption of food. α-linolenic acid was present in small amounts (0.1–0.5%) in several samples and in the amount of 16.7% in sample SFE_C + E^{2019}. This sample was also the only one that contained γ-linolenic acid (2.6%). Linolenic acids are especially favorable as they significantly decrease blood triglycerides (Szentmihályi et al., 1998).

Extract that was stored for 8 years had a higher content of palmitic (8.9–15.0%) and oleic acid (24.9–26.0%) compared to extract obtained from 8-years-old seeds (5.2–9.1% and 11.3–25.2%, respectively). On the other hand, higher content of linoleic acid can be seen only for extracts obtained by conventional technique stored for 8 years. Supercritical extracts that were stored for 8 years have a lower content of linoleic acid (25.6–47.7%) than supercritical extracts obtained from 8-years-old seeds (47.9–56.0%). Harhar et al. (2010) reported a decrease in palmitic acid content and an increase in oleic acid content after plant material storage. Also, Canavar (2015) reported an increase in linoleic acid content upon one year of peanut seeds storage at 5 °C and 60% relative humidity. Namely, seeds oil is prone to hydrolysis due to moisture as triglycerides are decomposed and fatty acids are released. Both hydrolysis and oxidation can affect the amount of separated oil and quality (Fotouo et al., 2016).

The addition of co-solvent for SFE led to the increase of UFA. More specifically, it led to a decrease in oleic acid content and an increase in linoleic acid content. Similarly, Ben Rahal et al. (2015) reported higher content of linoleic acid compared to oleic acid present in extract from *S. marianum* seeds grown in Tunisia obtained by use of $scCO_2$ with ethanol.

There was no significant difference in the type of fatty acids present in supercritical extracts and extract obtained by conventional technique although the quantities of fatty acids significantly changed depending on the parameters of extraction. Zhang et al. (2020) compared the extraction with solvents (*n*-hexane and ethanol) and by cold press from *S. marianum* seeds and reported that selection of the extraction method had no significant effect on the fatty acid profile.

3.3. Content of α-tocopherol

The selection of solvent for extract separation had a dominant effect on α-tocopherol content (Fig. 3). The highest content was present in extracts separated using *n*-hexane, while the lowest was in extracts separated using $scCO_2$. The exception was the sample SXT_E^{2019}, which had the lowest value of 0.08 g/100 g. Zhang et al. (2020) also determined a lower amount of α-tocopherol in the extract from *S. marianum* seeds obtained using ethanol compared to the using *n*-hexane. Ethanol as a co-solvent was used in SFE to modify the selectivity of $scCO_2$. Indeed, the addition of ethanol to $scCO_2$ enabled an increase in α-tocopherol content by around 30%. Also, defatting of seeds prior to SFE significantly decreased α-tocopherol content (to 0.62–1.64 g/100 g) (Fig. 3d) which could be ascribed to fat-soluble nature (Meddeb et al., 2017) that allows its separation from plant material with oil. In addition,

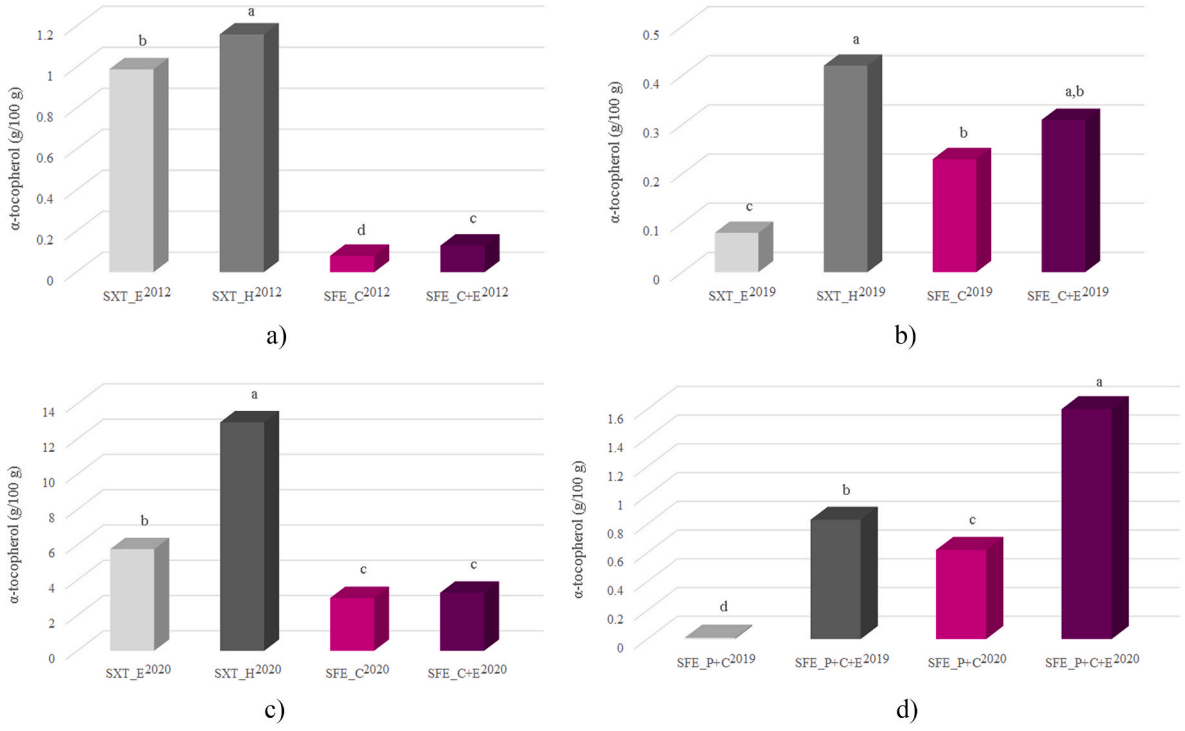

Fig. 3. Content of α-tocopherol in *S. marianum* extracts separated: a) from 1-year-old seeds produced 2011 and stored for 8 years, b) from 8-years-old seeds produced in 2011, c) from 1-year-old seeds produced in 2019, and d) from defatted seeds produced in 2011 and 2019.

it can be seen that α-tocopherol content in obtained extracts is also significantly affected by the storage (both seeds and extracts). Canavar (2015) reported that α-tocopherol content in peanut seed extract decreases around 1% upon 1-year storage.

Considering that the content of α-tocopherol in extracts from 1-year-old seeds ranges from 3.0 to 13.0 g/100 g and that the prescribed oral dose of vitamin E for human health is 15 mg/day (Federal Express, 2018), it can be concluded that fresh *S. marianum* seeds are a valuable source of this bioactive compound. The mixture of four different forms of tocopherols and four different forms of tocotrienols with α-tocopherol being the most effective, represent Vitamin E. This vitamin is known for antioxidant, immune-enhancing, and anti-inflammatory activity (Canavar, 2015) and is used clinically for the prevention of cardiovascular disease, cancer, cataract, and complications of diabetes. It is also responsible for extract long durability due to inhibition of the lipid peroxidation and conversion of lipid radicals into more stable products (Z. S. Zhang et al., 2020).

3.4. Flavonolignans analysis

Even though all extracts were analyzed, silybin was only detected in extracts obtained by Soxhlet extraction using ethanol (Table 3). The amount of detected silybin ranged from 3.5 to 10.6 g/100 g being the highest in extracts obtained from 1-year-old seeds. The content of flavonolignans was in the range from 20.5 to 34.4 g/100 g. It was previously reported that flavonolignans in *S. marianum* seeds can vary from 0.1 to 14.7 g/100 g for dry weight depending on the country of plant growing as well as the stage of plant development (Chambers et al., 2017). Đorđević et al. (2018) reported flavonolignans content in the ethanolic extract to be 0.2–2.0 g/100 g.

It is interesting to hypothesize that extracts separated using $scCO_2$ with and without co-solvent did not contain a detectable amount of flavonolignans especially considering previous reports which testify otherwise (Ben Rahal et al., 2015; Çelik & Gürü, 2015; Yang et al., 2017). On the other hand, similar to our study, Meddeb et al. (2017) also did not identify the presence of silybin in extracts from *S. marianum* seeds grown in Tunisia obtained by cold pressing. Difference between results may be attributed to variation in extraction method, pre-treatment of seeds, seeds origin (including the influence of climate, soil type, irrigation, spaces between/within rows, fertilization, harvesting, maturity, and genotypes) (Elateeq et al., 2020a; Marmouzi et al., 2021; Růžičková et al., 2011). For example, a higher temperature of 78 °C for extract separation with ethanol, compared to 69 °C in process with *n*-hexane and 40 °C in process with $scCO_2$, could be the reason for higher flavonolignans content in ethanolic extracts. Namely, it was reported that flavonolignans content can be significantly increased with temperature increase from 50 to 100 °C (Chambers et al., 2017). Variation in results can be also ascribed to different extract preparation methods for specific analysis as well as parameters of analysis.

3.5. Total phenolic content

As polyphenols are one of the major groups of compounds acting as primary antioxidants or free radical terminators, it is important to determine their presence in the different extracts (Lucini et al., 2016; Parry et al., 2008). Therefore, total phenolic content (TPC) was analyzed and presented in Fig. 4. It can be seen that the highest TPC was detected in ethanolic extracts regardless of seeds or extract storage. The value of TPC in these extracts was significantly different from others, according to Tukey's HSD test ($p < 0.05$). The highest value of 104.2 $g_{GAE}/100\ g_{extract}$ was detected in the sample SXT_E^{2012}. Around six times lower TPC value was determined in *n*-hexane and $scCO_2$ extracts ($scCO_2$ extracts obtained using co-solvent have slightly higher TPC values). Similarly, it was reported that *n*-hexane extract from *S. marianum* seeds has TPC of 2.5 $g_{GAE}/100\ g_{extract}$ (Parry et al., 2008). Additionally, it can be seen that extracts from 1-year-old seeds produced in 2019 have lower values of TPC compared to 8-years-old extract and extract obtained from 8-years-old seeds. An increase in TPC by 2-fold upon storage of 15 months was previously reported by Bolling et al. (2010) for extracts obtained from almonds. The reason for the increase of TPC upon storage can be explained by an increase in polyphenol extractability, an increase in polyphenol content due to degradation of polymeric polyphenols, and an increase in soluble phenolic (Bolling et al., 2010; Y. Zhang et al., 2021).

TPC analysis showed that extract obtained from 1-year-old defatted seeds contained 15.9 $g_{GAE}/100\ g_{extract}$ (sample SFE_P + C^{2020}) and 22.0 $g_{GAE}/100\ g_{extract}$ (sample SFE_P + C + E^{2020}). It can be concluded that the amounts of TPC detected in supercritical extracts from oil-rich seeds and defatted seeds were comparable.

3.6. Cytotoxic activity

Due to the potential oral and topical application of *S. marianum* seed extracts, the cytotoxic activity of extracts obtained from 1-year-old *S. marianum* seeds (produced in 2020) was further tested. Extracts from oil-rich seeds did not show cytotoxic effects on either cancer or normal cell lines (Table 4) regardless of the high flavonolignans content present in SXT_E^{2020} (Table 3). On the other hand, Parry et al. (2008) reported that extract obtained using *n*-hexane had antiproliferative effects against HT-29 human colon cancer cells. Ben Rahal et al. (2015) found that the extract from *S. marianum* seeds obtained after SFE with ethanol as co-solvent showed a significant decrease in the proliferative activities of Caco-2 cancer cells from 43 to 71% for extract concentrations 50–100 μg/mL. Based on the presented results it could be noticed that the biological activity of *S. marianum* seeds extracts may vary considerably, probably due to the different amount of potentially active compounds, mainly flavonolignans, depending on the seed origin.

Further analysis indicated supercritical extracts from 1-year-old defatted seeds as active against certain cancer cells. Therefore, it can be concluded that defatting of seeds as a pretreatment subsequently enabled the separation of extracts with higher content of bioactive compounds. Besides mentioned flavonolignans, *S. marianum* extracts can also contain polyphenolic compounds identified as hydroxycinnamic acids (i.e. caffeic, chlorogenic, ferulic, and cynarin) and flavonoids (apigenin, catechin, luteolin, luteolin-7-*O*-glucoside, quercetin, and myricetin) (Lucini et al., 2016; Marmouzi et al., 2021; Meddeb et al., 2017) which can be respon for its bioactivity.

Additional HPLC analysis of extracts that revealed cytotoxic activity was performed to screen out the nature of compounds responsible for that potential effect (Table 5). It can be seen that the sample with the strongest cytotoxic activity (SFE_P + C^{2020}) has a high content of *t*-cinnamic acid. It was reported that cinnamic acid has anti-tumor activities against a broad spectrum of human solid tumors, such as glioblastoma, melanoma, prostate, and lung carcinoma cells, at doses that have no significant effect on normal cells (Liu et al., 1995). Its potential use as an adjuvant in melanoma therapy was suggested due to the antiproliferative activity of cinnamic acid in melanoma cells (Niero & MacHado-Santelli, 2013). Besides, in both extracts, chlorogenic and ferulic acid, and pyrogallol derivatives were detected, and for all of them anticancer activity has been previously reported in the literature (Ahn et al., 2019; Rocha et al., 2012).

Table 3
Silybin and flavolignans content in *S. marianum* extracts.

Sample	Silybin (g/100 g)	Flavonolignans (g/100 g)
SXT_E^{2012}	10.57 ± 0.52 ^{a}	26.17 ± 0.71 ^{b}
SXT_E^{2019}	9.86 ± 0.33 ^{b}	20.47 ± 0.70 ^{c}
SXT_E^{2020}	3.46 ± 0.03 ^{c}	34.41 ± 0.83 ^{a}

Different letters in the same column indicate significantly different values ($p < 0.05$) among the results.

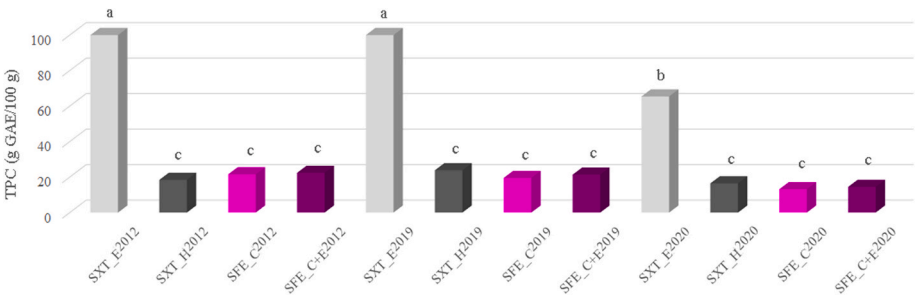

Fig. 4. Total phenolic content in *S. marianum* extracts as gallic acid equivalents (GAE).

Table 4
Cytotoxic activity of *S. marianum* extracts against human cell lines (normal and cancer).

Samples	MRC-5	HeLa	DU145	LS174T
	IC50 (μg/mL)			
SXT_E^{2020}	>200	>200	>200	>200
SXT_H^{2020}	>200	>200	>200	>200
SFE_C^{2020}	>200	>200	>200	>200
SFE_C + E^{2020}	>200	>200	>200	>200
SFE_P + C^{2020}	184.4 ± 0.5 [a]	165.1 ± 14.5 [a]	>200	154.5 ± 7.4 [a]
SFE_P + C + E^{2020}	>200	>200	>200	152.8 ± 17.2 [a]

Different letters in the same column indicate significantly different values ($p < 0.05$) among the results.

Table 5
Content of phenolic compounds in *S. marianum* extracts obtained from defatted seeds by the SFE process.

Samples	Chlorogenic acid	Ferulic acid	t-cinnamic acid	Pyrogallol derivatives
	$mg/g_{extract}$			
SFE_P + C^{2020}	0.005 ± 0.002 [b]	0.002 ± 0.001 [a]	14.990 ± 1.000 [a]	3.874 ± 0.060 [a]
SFE_P + C + E^{2020}	0.009 ± 0.003 [a]	0.002 ± 0.001 [a]	/	2.294 ± 0.040 [b]

Different letters in the same column indicate significantly different values ($p < 0.05$) among the results.

4. Conclusions

Considering that *Silybum marianum* is a highly valuable industrial raw material, every aspect of its processing and storage is important. This study pointed out the significant effect of the year of plant cultivation, selection of extraction technique and solvent, as well as the extract and seed storage on the amount, chemical profile, and cytotoxic activity of extract separated from *S. marianum* seeds. By comparing conventional and supercritical fluid extraction techniques, it was shown that the yield of extract from fresh seed ranged from 16.7 to 25.3 g/100 g, while after 8-years storage difference between techniques was more pronounced, with the yield of 10.3 g/100 g using SFE and 31.0 g/100 g in conventional extraction process using ethanol. The addition of co-solvent ethanol to $scCO_2$ in the SFE technique from oil-rich seeds led to the decrease of extraction yield, however, it enabled higher yield when defatted seeds were used. Chemical analysis revealed that all tested solvents allowed separation of extracts rich in unsaturated fatty acids, which constituted 64–87%. Content of α-tocopherol and total phenolic compounds was significantly affected by the selection of extraction method, solvent, and storage and it ranged from 0.01 to 13.0 g/100 g and 13.2–104.2 $g_{GAE}/100\ g_{extract}$, respectively. In vitro cytotoxic activity analysis revealed that only extracts obtained by SFE technique from defatted seeds, exhibited activity against cancer cells. Presented results suggested that all *S. marianum* seed extracts are nutritionally valuable and might serve as dietary sources of unsaturated fatty acids and natural antioxidants. Although slightly higher yield as well as α-tocopherol and total phenolic contents were determined in the extracts obtained using conventional extraction technique, SFE being environmentally friendly technique can be also considered as applicable for production of high-value extracts from *S. marianum* seeds at lower operating cost and relatively low temperatures.

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Data availability statement

"The data presented in this study are available on request from the corresponding author."

CRediT authorship contribution statement

Ivana Lukic: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Stoja Milovanovic:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Resources, Writing – original draft, Writing – review & editing, Supervision. **Milica Pantic:** Methodology, Investigation, Writing – review & editing. **Ivana Srbljak:** Methodology, Formal analysis, Investigation, Resources. **Ana Djuric:** Methodology, Formal analysis, Investigation, Resources. **Vanja Tadic:** Methodology, Formal analysis, Investigation, Resources. **Katarzyna Tyśkiewicz:** Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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