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AUTHORS: Marija Radojković, Zoran Zeković, Pavle Mašković, Senka Vidović, Anamarija Mandić, Aleksandra Mišan, Saša Đurović

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Biological activities and chemical composition of *Morus* leaves extracts obtained by maceration and supercritical fluid extraction

Marija Radojković[a, *], Zoran Zeković[a], Pavle Mašković[c], Senka Vidović[a], Anamarija Mandić[b], Aleksandra Mišan[b], Saša Đurović[a]

[a] *Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia*
[b] *Institute of Food Technology in Novi Sad, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia*
[c] *Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, 32000 Čačak, Serbia*

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ABSTRACT

Supercritical CO_2 extraction of *Morus* species (*Morus alba* and *Morus nigra*) was performed and compared with maceration as conventional extraction technique. Composition and biological activity of extracts were evaluated. The thirteen fatty acids were quantified in obtained extracts by GC-FID analysis. Their percentages varying from 0.33% for palmitoleic acid (C16:1) to 37.57% for α-linolenic acid (C18:3 n3). The main fatty acids were palmitic acid (C16:0) (26.38 and 25.99%), α-linolenic acid (C18:3 n3) (34.97 and 37.57%) and linoleic acid (C18:2 n6c) (14.76 and 16.05%). Total phenolic and flavonoid contents were determined using spectrophotometric methods, while phenolic profile was determined using HPLC-DAD analysis. Antioxidant and cytotoxic activities were also determined. Main phenolic compound was caffeic acid. Rutin, derivatives of caffeic acid and quercetin were also presented in high amounts. Our findings demonstrated that *Morus* CO_2 extracts possess significant characteristics and may be suggested as a new potential source of natural compounds.

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1. Introduction

Maceration is widely employed technique for the extraction of nonvolatile plant compounds for the purpose of pharmaceutical industry. However, maceration does exhibits some limitations such as time-consuming, labor-intensive procedures, extended concentration steps, and the involvement of large volumes of hazardous solvents, which may restrict the use of this method in food, cosmetic and pharmaceutical industries [1,2]. The conventional extraction process exploited mainly different non-polar organic solvents for the purpose of isolation of non-polar compounds. In comparison with conventional techniques of extraction, supercritical fluid extraction (SFE) possess numerous advantages. Carbon dioxide (CO_2) represents the most widely used compressed fluid, especially for the purpose of extraction of natural products. Its widely application in this field is mainly due to its several advantages, such as non-toxic and non-explosive properties, readily-available, easy removable from the products and possesses convenient critical properties ($T_c = 31.1$ °C and $p_c = 73.8$ bar) [3]. It represents the convenient solvent for extracting valuable components which may be of importance for both the food and the pharmaceutical industries. CO_2 is generally recognized as safe (GRAS) solvent thus the extract obtained using this solvent are considered as safe with respect to human health [4].

* Corresponding author.
Email address: ramarija@uns.ac.rs, marijalveb@yahoo.com (M. Radojković)

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Mulberry (*Morus* L., *Moraceae*) plant was originally growth in China and has been widely used in both agricultural and medicinal fields. Extensive traditional use and modern medical application of several *Morus* species is due to their remarkable variety of curative properties: antimicrobial, astringent, expectorant, emetic, diuretic and demulcent [5–9]. Additional studies confirmed that certain *Morus* species reveal considerable bioactivities such as cytotoxic effects on cancer cell lines [7,10,11], reduce risk of atherosclerosis [12–15], anti-inflammatory effect [16,17], hypolipidemic effect [18], neuroprotective effect [19] and antioxidant effect [20–23]. *Morus alba* (*M. alba*) and *Morus nigra* (*M. nigra*) are two species of the genus which can be located in Serbia.

To the best of our knowledge subcritical and maceration extracts of *M. alba.* and *M. nigra.* have not been evaluated in terms of chemical composition or biological activity. This was the first such study regarding the *M. alba* and *M. nigra* species in Serbia. In this study subcritical extraction and maceration of *M. alba* and *M. nigra* leaves were performed in order to compare the techniques efficiency. Obtained extracts were analyzed using GC-FID and HPLC-DAD in order to obtain lipid profile, fatty acid content, and polyphenolic content. Antioxidant capacity was also evaluated using four different assays: DPPH assay, reducing power assay, β-carotene bleaching method and deoxyribose assay. Finally, influence of obtained extracts on cell growth was estimated *in vitro* using the panel of three different cell lines: cell line derived from human rhabdomyosarcoma (RD), cell line derived from human cervix carcinoma (Hep2c) and cell line derived from murine fibroblast (L2OB).

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2. Materials and methods

2.1. Chemicals and reagents

Commercial carbon dioxide (Messer, Novi Sad, Serbia), methanol, ethyl acetate and *n*-hexane (J.T. Baker, The Nederlands), 1.1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), potassium hydroxide (KOH), methylene chloride, sodium chloride, sodium sulfate, and sodium hydrogen carbonate (Sigma-Aldrich, Germany) were used. Linoleic acid and β-carotene were purchased from Fluka (Germany). All other chemicals were of analytical reagent grade. All standards for HPLC analysis were of analytical grade and purchased from Sigma Chemicals Co. (Ct. Louis, USA) and Alfa Aesar (Karlsruhe, Germany). Acetonitrile and phosphoric acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade and purchased from Aldrich Chemical Co. (Steinheim, Germany).

2.2. Plant material

In this research dried plant material (leaves) was used. Voucher specimens (*Morus alba* L. N° 2-1794, Kać, UTM 34TDR211, and *Morus nigra* L. N° 2-1753, Novi Sad, Rimski šančevi, in 2010 year, UTM 34TDR2 01, det.: Goran Anačkov) were confirmed and deposited by the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Serbia [24]. The samples of mulberry leaves were dried naturally (in the shade, on draft) during one month and grounded in a blender before the extraction. Mean particle size was determined using sieve sets (Erweka, Germany). The mean particle size of the plant samples was 0.3092 ± 0.0198 mm for *M. alba* and 0.3070 ± 0.03266 mm for *M. nigra*.

2.3. Extraction processes

2.3.1. Supercritical CO_2 extraction

The extraction process was carried out on a laboratory scale high pressure extraction plant (HPEP, NOVA Swiss 565.0156, Effertikon, Switzerland). The main parts and properties of the plant according to manufacturer specification, were: gas cylinder with CO_2, the diaphragm type compressor (with pressure range up to 1000 bar), extractor (with internal volume 200 mL, internal diameter of 40 mm, maximum operating pressure of 700 bar), separator (with internal volume 200 mL, internal diameter 40 mm, maximum operating pressure of 250 bar), pressure control valve (self-contained, direct-acting, spring-loaded reducing valve with self-relieving and venting, Series 26–1000, Tescom corporation), temperature regulation system made using thermostats and liquid water, regulation valves and control and operating panel (with two pressure digital indicator and three digital temperature indicator). Maximum carbon dioxide mass flow rate was 5.7 kg/h.

The mulberry leaves sample (100.00 g) was placed in an extractor vessel. Operational parametars were: pressure 300 bar, temperature 40 °C and extraction time 17 h. The flow rate of carbon dioxide, expressed under normal conditions, was 0.194 kg/h. Separator conditions were 15 bar and 23 °C. The extraction process was carried out and the extraction yield was measured. After extraction, the obtained extracts were transferred into the glass bottles, sealed, and stored at 4 °C to prevent any possible degradation.

2.3.2. Maceration

Morus leaves and exhausted leaves obtained after subcritical extraction were extracted by maceration with 70% aqueous ethanol, at a temperature of 30 °C accompanied by liquid-solid ratio of 15 mL/g (expressed as solvent volume per g of sample). The extraction process was carried out in the shaker (3015 GFL, Germany) during the period of 24 h. Extractions were performed under the same conditions and repeated three times. After filtration, 50 mL of liquid extract was used for extraction yield determination by removing the solvent drying to the constant mass and measuring the mass of dry residue. The solvent was also removed from the rest of the extract by rotary vacuum evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) and dried at 60 °C until the constant mass was achieved. Dry extracts were stored in glass bottles at 4 °C until the analysis in order to prevent eventual oxidative damage.

2.3.3. Soxhlet extraction

To determine the total extraction yield, grounded *M. alba* and *M. nigra* leaves were extracted by a Soxhlet device for 10 h using hexane as the solvent. Solvent was evaporated after the discoloration. Obtained extracts were further dried at 60 °C until the constant mass was achieved. Experiments were conducted in triplicate.

2.4. Determination of biologically active compounds

Total phenolics content (TPC) in ethanolic extracts were determined using the Folin–Ciocalteu reagent [25]. The total phenolic content, expressed as mg of chlorogenic acid equivalents per g dry mulberry extract (mg CAE/g), was recalculated using calibration curve of chlorogenic acid as standard.

Total flavonoids content (TFC) in ethanolic extracts were estimated according to previously described method [26]. Total flavonoid content, expressed as mg of rutin equivalents per g dry extract (mg RE/g), was calculated from a calibration curve using rutin as standard.

To determine the total carotenoids content (TCC) supercritical mulberry extracts were estimated according to previously described method [27]. β-carotene was used as a standard and TCC was determined in triplicate and expressed as mg/g of dry extract.

2.5. GC-FID analysis of fatty acids

The dry supercritical extract sample (200 mg) was extracted by 1 mL of petroleum ether for 1 h using ultrasonic bath. After filtration, extraction solvent was removed by rotary evaporator under vacuum and samples were prepared for GC-FID analysis. Esterification of fatty acids were carried out by dissolving of each sample (60 mg) with isooctane (4 mL). The vials with reaction mixtures were sealed and heated in ultrasonic bath. After cooling, the mixture was mixed and shook for 30 s with 200 μL of KOH (2.3 mol/L) in methanol. The organic layer was dried over $NaHSO_4xH_2O$. After separation of the layers, 1 mL of isooctane layer was taken for analysis and injected in GC system.

GC-FID analysis was run on Agilent 6890 GC system. For mulberry extract analysis, the GC was fitted with a capillary HP-88 column (0.20 μm film thickness, 100 m length and i.d. 0.25 mm). The initial oven temperature was 120 °C (held for 1 min), and then increased to 175 °C at a rate of 10 °C/min (held for 10 min), then increased to 210 °C at a rate of 5 °C/min (held for 5 min) and finally increased to 230 °C at 5 °C/min (held for 12 min). The operating conditions were as follows: injector temperature, 250 °C; detector temperature, 300 °C; carrier gas, H_2 with constant flow on pressure 28.32 psi. Total analysis time was 44.5 min. The fatty acid methyl esters (FAME) peaks were identified using FAME standards. The concentrations of the fatty acids were calculated from their peak areas.

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2.6. HPLC-DAD analysis of phenolic compounds

Quantification of individual phenolic compounds was performed by reversed phase HPLC analysis, using a method modified by Mišan et al. [28]. HPLC analysis was performed by using a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies).

2.7. Determination of antioxidant activity

2.7.1. Determination of DPPH free radical scavenging activity
The free radical scavenging activity of mulberry extracts was determined according the previously described method [29]. Butylated hydroxytoluene (BHT) was used as standard. Radical scavenging capacity (%RSC) was calculated using the Eq. (1), where A_s represents the absorbance of sample solution, while A_b is the absorbance of a blank sample.

$$RSC\ (\%) = 100 - (A_s/A_b) \times 100 \quad (1)$$

This activity was expressed as the inhibition concentration at 50% (IC_{50}) i.e. the concentration of the tested solution (mg/mL) required for obtaining 50% of radical scavenging capacity.

2.7.2. Reducing power assay
Reducing power of the samples was determined according to assay based on the reduction of Fe^{3+} by presented antioxidants [30]. Antioxidant activity was further expressed as EC_{50} value (mg/mL), which causes reduction of 50% Fe^{3+} ions in reaction mixture. All experiments were performed in triplicate, and results were expressed as mean values.

2.7.3. β-carotene bleaching method
The procedure is based on a previously reported method [31] with BHT as a standard. The antioxidant activity (AOA) was calculated in terms of percentage of inhibition relative to the control using the following Eq. (2), where R_s is the value of sample solution and R_c is the value of a control. A_0 is the absorbance measured immediately (t = 0 min) and A_{180} is the absorbance measured after 180 min (t = 180 min). The results were expressed in mg/mL.

$$AOA\ (\%) = [(R_c - R_s)/R_c] \times 100 \quad (2)$$

Where $R_{c,s} = [\ln (A_0/A_{180})] \times 1/180$

2.7.4. Hydroxyl radical scavenger capacity
The deoxyribose assay [32] was applied to measure HO• scavenger capacity. BHT was used as a positive control. All experiments were performed in triplicate and results were expressed as mean values. Capacity of scavenging the hydroxyl radicals was calculated from Eqs. (3) (4) and (5). Scavenging activity was expressed as the inhibition concentration at 50% (IC_{50}) i.e. the concentration of the tested solution (mg/mL) required for obtaining 50% of radical scavenging capacity

$$RSC\ (\%) = [1 - (A/A_{con})] \times 100 \quad (3)$$

$$A = A_{ws} - A_{bp} \quad (4)$$

$$A_{con} = A_{cs} - A_{bp} \quad (5)$$

where A represents absorbance for every solution individually, A_{con} is absorbance of control solution A_{bp} is absorbance of blank, A_{ws} is absorbance of working solutions, and A_{cs} is absorbance of control solution.

2.8. Determination of cytotoxic activity

Obtained *Morus* extracts were evaluated for their cytotoxic activity through their influence on growth of malignantly transformed cell lines using the MTT assay. Malignantly cell lines used it this assay were cell line derived from human rhabdomyosarcoma (RD cell line), cell line derived from human cervix carcinoma (Hep2c cell line) and cell line derived from murine fibroblast (L2OB cell line). Cells were seeded (10^4 cell/mL; 100 µL/well) in 96-well cell culture plates (NUNC) in nutrient medium (MEM Eagle supplemented with 5% (for Hep2c) or 10% (for RD and L2OB) and grown at 37 °C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100 µL/well) and cells were incubated at 37 °C in humidified atmosphere for 48 h. Pure nutrient medium (100 µL) represented positive control for each cell line. After incubation period, supernatants were discarded and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500 µg/mL) was added in each well (100 µL/well). After addition all wells were incubated at 37 °C in humidified atmosphere for 4 h. Reactions were halted by addition of 100 µL of sodium dodecyl sulfate (SDS) (10% in 10 mM HCl). After overnight incubation at 37 °C, absorbance was measured at 580 nm using a spectrophotometer. The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against absorbance at 580 nm. Corresponding cells (grown in flasks), after cell counting using haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements are expressed as the percentage of positive control growth taking the *cis*-diamminedichloroplatinum (*cis*-DDP) determined in positive control wells as 100% growth [33,34]. All experiments were done in triplicate.

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2.9. Statistical analysis

Statistical analysis was carried out using Statistica 6.0. (StatSoft Inc, Tulsa, OK, US). All experiments were performed at least in triplicate unless specified otherwise. Results are presented as value ± standard deviation (SD). Significant levels were defined at $p < 0.05$.

3. Results and discussion

3.1. Total extraction yield

Total extraction yields of previously performed Soxhlet extraction with hexane, SFE, maceration, as well as of maceration after SFE were presented in Table 1. Results were expressed as grams of extract per 100 g of leaves (g/100 g).

Comparing the yields of Soxhlet and SFE extraction techniques it could be easily concluded that, in this case, SFE was more efficient technique for isolation of non-polar compound than Soxhlet extraction with non-polar solvent such as hexane. It could also be noticed that yield of *M. nigra* was higher than of *M. alba*, as it was the case with yield for Soxhlet extraction, what was also observed examining the kinetic curves of the SFE extraction (Fig. 1).

On the other hand, yields of maceration process were higher in the case of fresh leaves in both *M. alba* and *M. nigra* cases. This small difference between yields for both maceration extractions could imply that some of the compounds were extracted by carbon dioxide during SFE process thereby causing the lower yield in the followed maceration. Comparing the result of SFE extraction with those of maceration could be noted that polar compounds are in majority in leaves of *Morus* species. In the case of *M. alba* yield of polar compounds was about 8-fold higher, whereas that difference was lower in the case of *M. nigra*, about 6-fold higher.

Previously conducted study dialed with extracts of *M. alba* obtained using Soxhlet extraction and SFE [35]. Achieved yield for Soxhlet extraction was 8.86 g/100 g. SFE process was examined at 40 °C and 0.280-0.300 mL/min of CO_2, three different pressure values: 200, 300 and 400 bar, while the extraction time was 60 and 90 min, respectively. Obtained yields of SFE process were as following: 0.30 (200 bar, 60 min), 0.28 (200 bar, 90 min), 0.32 (300 bar, 60 min), 0.36 (300 bar, 90 min), 0.53 (400 bar, 60 min) and 0.65 g/100 g (400 bar, 90 min). Comparing obtained results in this study with the previously obtained ones, it might be noticed that yield of SFE process in this study were similar in the given period of time (Fig. 1a), while the yield of Soxhlet extraction technique, achieved in this study, was lower.

Table 1
Extraction yield (g/100 g) of *M. alba* and *M. nigra* obtained by Soxhlet extraction, SFE and maceration.

Species	Total extraction yield (g/100 g)[a]			
	Soxhlet[b]	SFE	Maceration	Maceration[c]
M. alba.	2.60 ± 0.07	2.96 ± 0.03	23.40 ± 0.09	22.58 ± 0.07
M. nigra.	3.00 ± 0.02	3.46 ± 0.05	22.42 ± 0.08	22.0 ± 0.1

[a] Expressed as value ± SD from three replicates.

[b] Hexane was used as a solvent.

[c] Maceration of the leaves sample after SFE extraction.

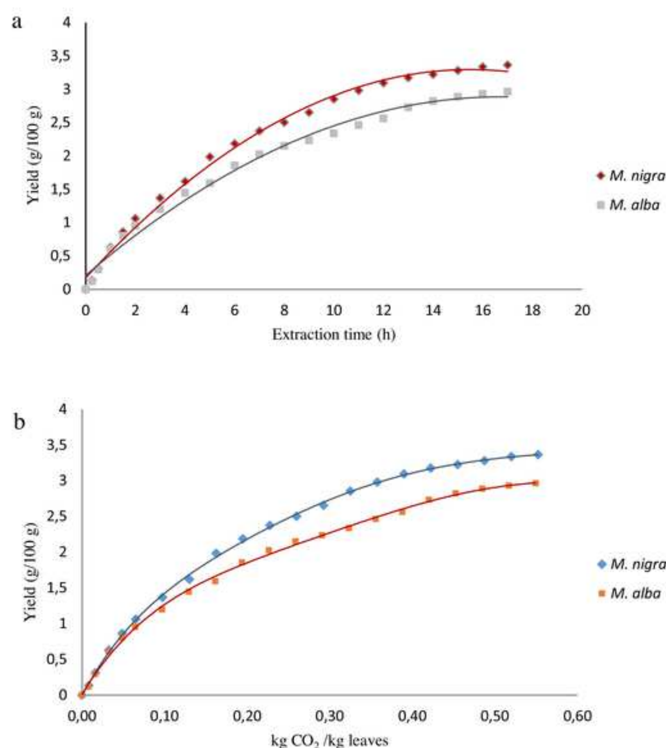

Fig. 1. Kinetics curves for SFE extraction of *M. alba* and *M. nigra*. leaves (300 bar, 40 °C, 0.194 kg/h).

3.2. Total carotenoids content and GC-FID analysis of fatty acids

Carotenoids had attracted a lot of attention as a result of a great number of epidemiological studies which revealed that a diet rich in the carotenoids is closely related with a diminished risk of several degenerative disorders including various types of cancer, cardiovascular and ophthalmological diseases. Their preventive effects have been associated with their antioxidant potential which is in connection with ability to protect cells and tissue from oxidative damage. Carotenoid also affect cellular signaling pathway and may trigger redox-sensitive regulatory pathways [36,37]. TCC of *M. alba* and *M. nigra* leaves extracts were 65.08 and 52.84 mg/g.

Comparing the TCCs for two *Morus* species, it could be concluded that *M. alba* possessed higher content of carotenoids than *M. nigra*. Carotenoids content had been previously reported [38–41]. The TCC of 5.6% of the total in *Morus* leaves was reported [40], while another study reported the TCC of 0.9793 and 0.7086 mg/g in *M. alba* and *M. nigra* respectively [41]. This results were in agreement with our conclusion that *M. alba* contained higher amount of carotenoids than *M. nigra*. Detailed analysis resulted in detection of nine different carotenoids in *Morus* leaves of which major were β-carotene and lutein (41.4 and 22.4% respectively) [39]. Domination of β-carotene and lutein in *Morus* extracts was confirmed in other study, but in this case lutein was present in the higher content than β-carotene (19.9 and 6.36 μg/g) [38].

In addition to the TCC, fatty acids (FAs) content in SFE extract of *M. alba* and *M. nigra* was determined. Summarized results were presented in Table 2, while obtained chromatograms were presented in Fig. 2. Of 36 investigated FAs 13 were quantified. Detected FAs were divided into two groups: saturated FAs (SFA) and unsaturated FAs (UFA) which is further divided into monounsaturated FAs (MUFA) and polyunsaturated FAs (PUFA). Obtained results showed that *M.*

Table 2
Fatty acid profile of *Morus* SFE extracts.

Fatty acid	Species	
	M. alba	*M. nigra*
	% (g/100 g extracts)	
Myristic acid (C14:0)	2.36	2.24
Pentadecanoic acid (C15:0)	0.88	0.48
Palmitic acid (C16:0)	26.38	25.99
Palmitoleic acid (C16:1)	0.67	0.33
Heptadecanoic acid (C17:0)	2.66	1.93
Stearic acid (C18:0)	4.91	5.32
cis-9-Oleic acid (C18:1 n-9)	2.86	2.30
cis-9,12-Linoleic acid (C18:2 *n*-6)	14.76	16.05
Arachidic acid (C20:0)	4.45	4.13
cis-11-Eicosenoic acid (C20:1)	1.08	1.15
α-Linolenic acid (C18:3 n-3)	34.97	37.57
Behenic acid (C22:0)	2.81	1.90
Lignoceric acid (C24:0)	1.22	0.61
Saturated fatty acids (SFAs)	45.67	42.60
Unsaturated fatty acids (UFAs)	54.33	57.40
Monounsaturated fatty acids (MUFAs)	4.60	3.78
Polyunsaturated fatty acids (PUFAs)	49.73	53.62
UFA: SFA ratio	1.19	1.35

alba extract was richer in SFAs than *M. nigra*. (45.67 and 42.60% respectively), while in the case of UFAs was vice versa (54.33 and 57.40% respectively). In the case of UFAs, *M. alba* was richer in MUFAs than *M. nigra* (4.60 and 3.78% respectively), while in the case of PUFAs was vice versa (49.73 and 53.62% respectively). It could also be noticed that both *Morus* species possessed higher amount of UFAs than SFAs.

Presence of n-3 and n-6 FAs could be very significant because those compounds expressed anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties [42]. The beneficial effects of n-3 FAs were also showed in the secondary prevention of several diseases and disorders: coronary heart disease, hypertension, type 2 diabetes, in some patients with renal disease, rheumatoid arthritis, ulcerative colitis, Crohn disease, and chronic obstructive pulmonary disease [42] and in decreasing the risk of heart disease and cancer [43,44]. High percentage of FAs in both *Morus* extracts indicate that could be safely used as a dietary supplement as the result of previously mentioned beneficial effects of FAs.

Previous conducted study confirmed the presence of 10 FAs of which 6 were SFAs and 3 were UFAs. Obtained results showed that linolenic, linoleic, oleic, and palmitic acids predominated in *Morus* leaves [40]. This was in consistent with the results presented in Table 3. There were also several studies concerning the FA content in *Morus* fruits and seeds [6,45–48]. Conducted investigations reported the C18:2 FA as the major in *M. nigra* followed by C16:0, C18:1, C18:0, C19:1 and C14:0 acids [6,47]. Another authors reported the C16:0 and C18:1 FAs as the dominant in fruits of three *M. nigra* genotypes [46], while C18:2, C16:0, C18:1 and C18:0 were reported as the major FAs in *Morus* seed oil [45]. The most current study demonstrated that C18:2 FA was the major in four clones of *M. alba* and *M. nigra* fruits, followed by C16:0, C18:1 and C18:0 acids [48].

The major PUFAs in *M. alba* and *M. nigra* samples were α-linolenic acid (34.97 and 37.57% respectively) and linoleic acid (14.76 and 16.05% respectively), while major SFA was palmitic acid, (26.38 and 25.99% respectively). In addition to previously mentioned FAs, the following SFAs were presented: myristic acid, pentadecanoic acid, heptadecanoic acid, arachidic acid, behenic acid and lignoceric acid, while UFAs were: palmitoleic acid, oleic acid and eicosenoic acid. Linoleic acid belongs to the group of essential FAs and cannot be synthetized in the human organism. In other word, this acid must be supplied externally through the diet where the *Morus* extracts could be used as a diet supplement due to high content of this compound. Linoleic acid was showed as a potential suppressor of tumor growth and metastasis [49], as well as mentioned secondary prevention of coronary heart diseases, hypertension, rheumatoid arthritis, ulcerative colitis, Crohn disease, and chronic obstructive pulmonary diseases [42]. It was also showed that linolenic acid is very important for human health. The importance of linolenic acid was demonstrated by a large number of clinical studies which connected the diet rich with this acid and human health [50,51]. Higher dietary intake of linolenic acid was associated with a lower prevalence of insulin resistance in normal weight individuals of middle-aged Japanese men and women [50].

3.3. Total phenolics, flavonoids contents and HPLC analysis of phenolic profile

Total phenolics (TP) and flavonoids (TF) contents were presented in Table 3. Obtained results showed that *M. nigra* contained significantly higher amount of TP and TF than *M. alba*. In all cases leaves contained somewhat lower amount of TP and TF (about 2-fold lower) after the SFE extraction. Although leaves mostly retained its polar components it could be concluded that certain amount of those compounds was extracted during the SFE process. Obtained results in previously performed studies showed that TP content varied in range of 8.33-16.21 mg GAE/g of dry weight for *M. alba* and 9.94-24.27 mg GAE/g of dry weight for *M. nigra* [41,52,53]. On the other hand, TF content were in range of 6.11-26.41 mg RE/g for *M. alba* and 4.40 to about 29 mg RE/g for *M. nigra* [41,52]. Wang et al. [54] were investigated TP and TF contents in *M. alba* leaves. They obtained TP content of 13.35 and 21.75 mg GAE/g in aqueous and ethanolic extracts, respectively, while TF content in those extracts were 11.59 and 14.38 mg RE/g, respectively.

In order to determine phenolic profile more precisely, HPLC-DAD analysis was performed, while the results were presented in Table 4. Content of this compound was 16.0656 mg/g in *M. alba* and 85.2824 in *M. nigra*. The minor compound in *M. alba* was chlorogenic acid with content of 0.1316 mg/g, while in *M. nigra* was gallic acid (0.1532 mg/g).

Caffeic acid derivatives, quercetin derivatives and rutin were also presented in significant amount in all extracts. Vanillic acid was detected only in *M. alba.* leaves in amount of 0.9123 mg/g. Comparing the total quantity of detected phenolic compounds, it could be noticed that *M. nigra* contained significantly higher amount of those compounds than *M. alba* (about 4.5-fold higer). It is also notable that both macerate contained lower amount of total phenolic compounds after SFE extraction (about 4.2-fold). Lower amount of phenolic compounds in macerate after SFE extraction is in accordance with results obtained using Folin–Ciocalteu's method (Table 4) and the result of total extraction yield (Table 1). Obtained results regarding the TP and TF contents, and the absence of vanillic acid in macerate after SFE extraction, supports previous conclusion that some of the phenolic compounds were extracted during the SFE process. Rutin, quercetin and derivatives of quercetin were confirmed in *Morus* leaves [55–58], as well as gallic acid, protocatechuic acid, caffeic acid, catehin, epicatehin in *Morus* leaf extracts [55].

Caffeic acid was quantified as the major compound in Tunisian *M. alba* (15.8401 mg/g) while rutin was abundant in lower concentration (0.1942 mg/g) comparing to our result [59]. Absence of the gallic acid and protocatechuic acid was reported in the leaves of Pakistani *M alba* and *M. nigra*, as well as the presence of vanillic acid in *M. nigra* The most abundant compound it this case was chlorogenic acid in

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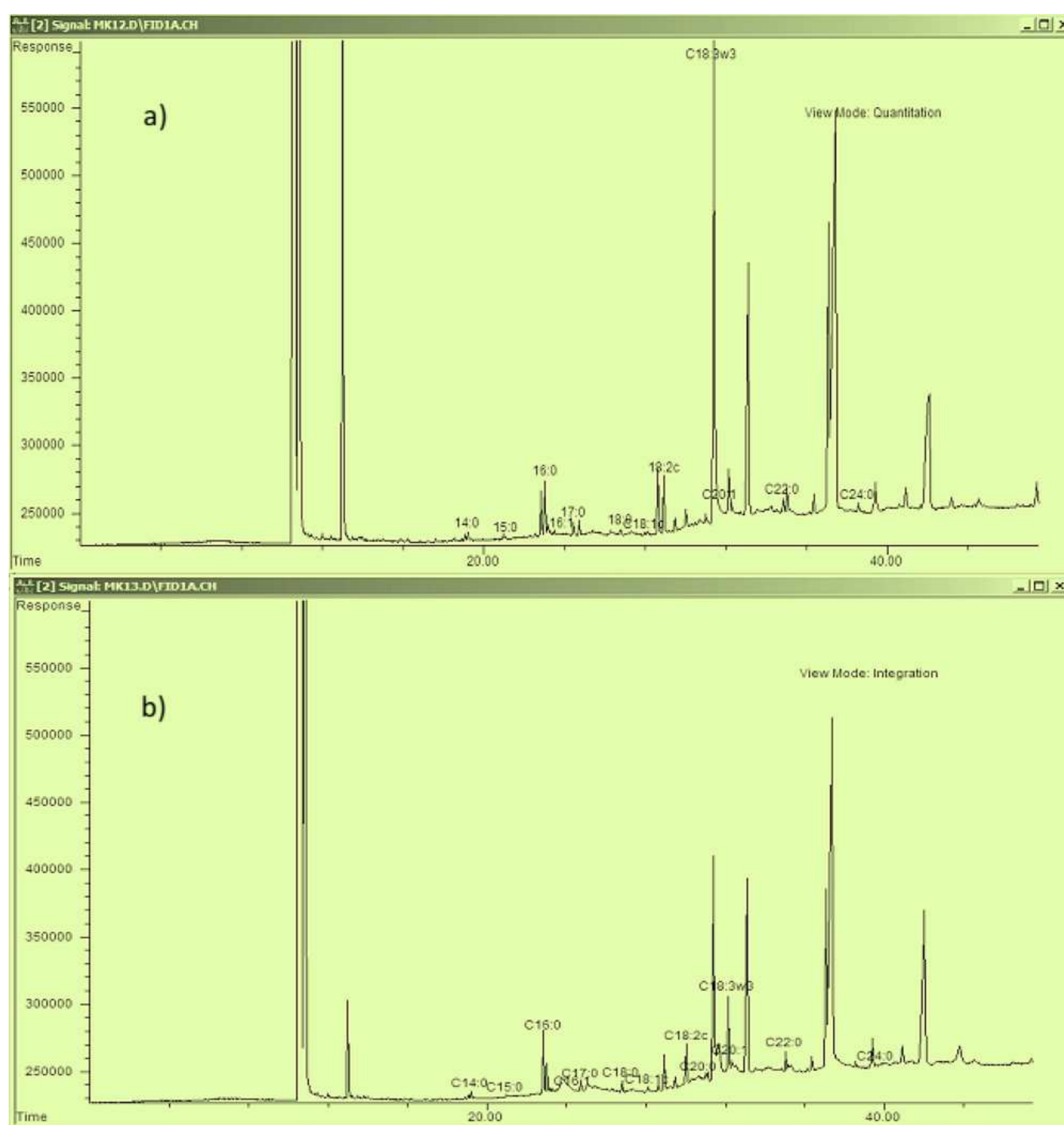

Fig. 2. Fatty acid profile of: (a) SFE extract of *M. alba* (b) SFE extract of *M. nigra* leaves.

Table 3
Total phenolics (TP) and flavonoids (TF) contents in *M. alba* and *M. nigra* extracts[a].

Test	*M. alba*		*M. nigra*	
	Maceration	Maceration[b]	Maceration	Maceration[b]
TP (mg CAE/g)	66.8 ± 0.8	47.0 ± 1	145.2 ± 0.5	122.1 ± 0.6
TF (mg RE/g)	33.3 ± 0.1	22.5 ± 0.7	67.4 ± 0.4	43.5 ± 0.1

[a] Mean value ± SD from three replicates.

[b] Maceration of the leaves sample after the SFE extraction.

amount of 0.6473 and 1.0439 mg/g in *M. alba* and *M. nigra* respectively [22]. Higher amount of chlorogenic acid was detected in Spanish *Morus* species (7.18-5.29 and 5.44-8.39 mg/g for *M. alba* and *M. nigra,* respectively) [53].

The presence of phenolic compounds in *Morus* species is very important. Previous studies showed that this class of compounds delay aging and regulate fat metabolism in *Caenorhabditis elegans* which was used as a model [60], and lower hepatic lipid accumulation [55].

Table 4
Polyphenolic profile of *Morus* species.

Polyphenolic compound	*M. alba*	*M. alba.*	*M. nigra*	*M. nigra*
	Macerate	Macerate[a]	Macerate	Macerate[a]
	(mg/g of extract)			
Gallic acid	0.4011	0.4723	0.2054	0.1532
Protocatechuic acid	0.4365	0.5209	0.6711	0.7369
Catehin	0.6844	0.3533	0.8418	0.5418
Caffeic acid	16.0656	14.5480	85.2824	79.7763
Caffeic acid derivatives	4.7207	4.1701	15.8793	14.8130
Vanillic acid	0.9123	–	–	–
Chlorogenic acid	0.1316	0.2720	0.5808	0.4697
Epicatechin	0.6123	0.5017	0.3386	0.3103
Quercetin derivatives	4.0190	3.7027	22.9412	7.2491
Rutin	2.1212	2.0994	10.5695	9.8730
Summary	30.1048	26.8472	137.3100	113.9232

[a] Maceration of the leaves sample after the SFE extraction.

It was established that quercetin derivatives improve lipid and glucose

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