Phytoconstituents of leaf extracts of *Ziziphus jujuba* Mill. plants harvested in Tunisia

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ABSTRACT

The present study aimed to determine the phytoconstituent compositions of the leaves of four *Ziziphus jujuba* ecotypes (Choutrana, Mahdia, Mahres and Sfax). The chromatographic peaks of 18 compounds, including nine major fatty acids, five sterols, two triterpene alcohols and two methysterols, were quantified by the capillary gaseous chromatography method. The major fatty acids identified were linolenic (42.04%) and palmitic (23.04%). Unsaturated fatty acids ranged between 53% and 60%. The predominant sterols (mg/100 g) were β-sitosterol (40.36) and stigmasterol (24.18). Cycloartenol (68.55 mg/100 g) and citrostadienol (12.27 mg/100 g) were the major metylsterols. Methylene cycloartanol ranged between 1.2 mg/100 g (Sfax) and 1.5 mg/100 g (Mahdia). Total phenolic content measured by Folin–ciocalteux ranged from 3.97 mg GAE/g to 6.04 mg GAE/g. The predominant flavonoids identified by HPLC were apigenin (6.1 mg/g) and rutin (1.91 mg/g).

The fatty acids and flavonoids in the *Z. jujuba* leaves were responsible for their therapeutic and pharmaceutical effects. This could explain why Tunisian people traditionally use it as medicine to treat several pathologies.

1. Introduction

The *Ziziphus* genus is known for its widespread use in modern ethnomedecine, especially in arid and semi-arid areas (Borgi et al., 2008). *Ziziphus jujuba* was introduced in Tunisia a long time ago and is now well acclimated (Laamouri et al., 2008). Its various effects include antimicrobial, antioxidant, immuno-stimulant, antidiabetic, hyperglycemic and anticancer (Preeti and Shalini, 2014; Sirajunnisa et al., 2014). The leaves have been used for herbal tea as a folk medicine for hemorrhaging and diarrhea (Mahajan and Chobda, 2009). They have also been used to improve sleep, nourish the heart and soothe the nerves (Zhang et al., 2014). Preeti and Shalini (2014) reported that extracts of *Ziziphus* leaves were used to treat children suffering from typhoid fever, furuncle and ecthyma; they were also used as an antipyretic and to reduce obesity. Other authors (Sirajunnisa et al., 2014) have confirmed that extracts of *Z. jujuba* leaves are an ecofriendly green inhibitor of aluminum corrosion in an NaOH solution. Oils extracted from different *Z. jujuba* organs (pulp, leaves and seeds) seem to be rich in fatty acids, sterols and triterpens (Croueour et al., 2002; Lee et al., 2003). Most studies mainly focused on the pulp and seed oil composition. These oils, used for medicinal and pharmaceutical applications (hypotensive, antihypoxia and hypothermic), make up 12.35% and 37.5% of the dry weight of the pulp and seeds, respectively (Elaloui et al., 2011, 2014a,b). Others works have shown that the compounds extracted from leaves are limited and fragmented (Zhang et al., 2014). The richness of the leaf oil in omega-3, a compound responsible for many health benefits, explained their use to treat some allergic and inflammatory reactions (Bell et al., 2009).

Phytosterols, the main components that regulate fluidity and permeability of the membrane (Darnet and Rahier, 2004), have been used as intermediates to synthesize hormones (Hamama et al., 2003). Scientists have subdivided sterols into three groups: free sterols, steryl esters and steryl glucosides (Benveniste, 2004). In *Z. jujuba* oils, sterols ranged from 14 mg/100 g to 182 mg/100 g in the pulp and seeds (Elaloui et al., 2011, 2014a,b).

Triterpens have anti-inflammatory (Manez et al., 1997), antimicrobial (Suksamrarn et al., 2006) and antioxidant effects

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(Bowen-forbes et al., 2009; Liu et al., 2010). Triterpenic acids were isolated from *Ziziphus* leaves by Guo et al. (2011).

Polyphenols have been shown to have antimicrobial, anti-inflammatory, antitumor and anti-sweet effects (Selloum et al., 2003; Guo et al., 2011; Morado-Castillo et al., 2014). Flavonoids were identified in *Z. jujuba* leaf extracts (Guo et al., 2011; Pei et al., 2011). In Tunisia, the chemical composition of *Z. jujuba* leaves used as an herbal tea have hardly been studied. Hence, there was a strong need to thoroughly analyze the bioactive components of *Z. jujuba* leaves.

The present study was undertaken to investigate the fatty acids, sterols, metylsterols, triterpene alcohols and the polyphenol composition of the leaves of four *Z. jujuba* ecotypes (Sfax, Choutrana, Mahres and Mahdia) harvested in Tunisia.

2. Materials and methods

2.1. Plant material

Z. jujuba leaves (Fig. 1) were collected in August 2010 (the maximum foliation period) from 5-year-old plants cultivated in the experimental station of "Rouhia" in northwestern Tunisia (35°40′–15.39″N; longitude 9°0.3′–15.29″E; altitude 636 m). Plant botanical identification was carried out by a Professor Mohamed Boussaid and a voucher sample was deposited at the Herbario of the National Institute for Research in Rural Engineering, Water and Forests (INRGREF) in Tunisia. Dried leaves were first ground using a mill equipped with a grid with holes 1.00 mm in diameter and then stored in plastic bags until chemical analysis could be done in the ENSCIACET Laboratory, France.

2.2. Methods

2.2.1. Reagents and standards

All solvents used in this experiment were purchased from Sigma–Aldrich (Steinheim, Germany) and used as received, namely: *tert*-butyl-methyl ether (TBME), cyclohexane; KOH; *N*-methyl-*N*-trimethylethylsilyl-heptafluorobutyramide (MSHFBA); dihydrocholesterol; chloroform; homologous fatty acids and sterols, rutin and apigenin standards.

2.2.2. Lipid extractions

The dried and powdered leaves were Soxhlet-extracted with cylohexane for 6 h. The extract, concentrated under reduced pressure using a rotary evaporator at 60 °C, was kept in darkness at 4 °C until analysis.

2.2.2.1. Fatty acids extraction. Fatty acids (FAs) were extracted in duplicate according the procedure used by Macherey Nagel of dissolving 20 mg of oils in 1 ml of TBME (Trimethylsulfonium hydroxide) solvent. Next, 50 μL of reagents were added to 100 μL of this solution. This methylation with TMSH was recommended for free acids, chlorophenoxycarboxylic acids, their salts and derivatives as well as for phenols and chlorophenols (Butte, 1983) in order to simplify the sample preparation. Lipids or triglycerides could be converted to the corresponding fatty acid methyl esters (FAMEs) by a simple transesterification. This reaction was very convenient, because only the reagent (0.2 M of methanol) was added to the sample solution. No excess reagent had to be removed since pyrolysis to volatilize methanol and dimethylsulfide will occur in the gas chromatograph injector at 250 °C. Due to the high reactivity, complete derivatization was often obtained at ambient temperature. However, heating (10 min at 100 °C) in a closed sample vial may be necessary.

2.2.2.2. Sterol extraction. Unsaponifiable compounds and sterols were extracted according to the method used by Elaloui et al. (2014a,b). 100 μg of dihydrocholesterol (internal standard dissolved in chloroform) was added to 140 mg of oil and mixed with 3 mL of a KOH solution (1 M in ethanol). After heating at 75 °C for 30 min, 1 mL of distilled water and 6 mL of isohexane were added to the mixture. The unsaponifiable fraction, separated by the isohexane, was analyzed by GC (Sriti et al., 2011). 160 μL of the organic phase containing the sample was added to 40 μL of silylation reagent (1 mL *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA)) and 50 μL of 1-methylimidazole. After mixing, the total extract was heated for 5 min at 103 °C before GC analysis. The extractions were carried out in duplicate.

2.2.3. Polyphenol compounds

The contents of total phenolic compounds of extracts from four ecotypes of *Z. jujuba* leaves were assayed following the Singleton's method, slightly modified by Dewanto et al. (2002). Levels were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW), using gallic acid as a standard. So, we prepared dilutions (0–100 mg/L) from gallic acid (2 g/L) solution. Then we added an aliquot (0.125 mL) of a suitable diluted methanolic leaf extract to 0.5 mL of deionized water and 0.125 mL of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then adjusted with deionized water to a final volume of 3 mL and mixed thoroughly. The absorption was carried out in triplicate using a SHIMADZU UV-1800 spectrophotometer at 760 nm after incubation for 90 min at 23 °C.

Dilutions were made in duplicate and phenolic levels were measured after a calibration curve.

2.2.4. HPLC analysis

The powdered leaves (2.5 mg) were submitted to maceration for 6 h with acetone–water (4:1, v/v) at room temperature. The

Fig. 1. *Z. jujuba* leaves cultivated in the experimental station in Rouhia, Tunisia.

acetonic extract was then filtered and a second maceration was done. The supernatants were combined and the acetone was evaporated at 40 °C in a vacuum. The solution was filtrated with 0.45 mm pore-sized membrane filters (Vivascience AG, Hanover, Germany) before HPLC injection. Investigated components were identified by comparing their retention times with those obtained from injecting standards under the same conditions. The extractions were carried out in duplicate.

2.2.5. Analysis conditions

FA was analyzed, in triplicate, using a capillary gaseous chromatography Varian (CPG). The mixture was injected directly. The temperature of the injector had to be at least 250 °C. The column used was Select CB for FAME fused silica WCOT (50 m × 0.25 mm, 0.25 μm film thickness). The temperature gradient was 185 °C for 40 min, then 15 °C/min to 250 °C, and 250 °C for 10 min. The analysis time was 55 min. The FID detector was set up at 250 °C. The Helium was the carrier gas at 1.2 mL/min.

Sterol was analyzed by GC using a flame ionization detector (FID) PerkinElmer (Waltham, MA, USA) chromatograph. A CP-SIL 8CB capillary column (30 m; 0.25 mm; 0.25 μm) was used. The column oven temperature was set at a rate of 20 °C/min from 160 to 260 °C, 2 °C min^{-1} up to 300 °C and 45 °C min^{-1} up to 350 °C. The quantity of injected sample was 1 μL. Helium was the carrier gas with a flow of 1 mL/min (using on column injection). The detector was set at 360 °C with an injection volume of 1 μL. The compounds were identified by comparing them to the commercial standards. Sterols were quantified by internal calibration after adding cholestanol. The sterols analyzed and cholestanol were observed to respond in the same way.

Rutin and apigenin analyses were carried out via HPLC DIONEX (P680-ASI 8100-Agilent). They were separated using a C_{18} column (5 μm, 4.6 mm × 150 mm).

The eluent was a mixture of 3% acetic acid (in water) (solvent A) and 100% methanol (solvent B). The first time, HPLC was stabilized to 20% (B) in 10 min, then increased from 60% at 28 min to 80% at 3 min. Elution was done at a flow rate of 0.8 ml/min. Compounds were identified by comparing them to the commercial standards. The column was washed between two successive injections with methanol. Results were expressed in mg/g dry weight.

2.2.6. Statistical analysis

Results were statistically evaluated using STATISTICA (Statsoft, 1998). Data from three samples was reported as means ± standard deviation. Differences were tested for significance with the ANOVA procedure using the Duncan test with a significance level of $p < 0.05$.

3. Results

As shown in Fig. 2, the oil yields obtained from *Z. jujuba* leaves ranged from 8.61% to 10.31% based on dry weight. Sfax was the richest ecotype and Choutrana had the poorest oil content.

3.1. Free fatty acids

The typical GC profile (Fig. 5) showed a large variation in fatty acid content between the four ecotypes (Sfax, Choutrana, Mahres and Mahdia).

In order of decreasing values, the major FAs in these oils (Table 1) were: linolenic acid = omega-3 (42.04%), palmitic acid (23.04%), oleic acid (13.08%), linoleic acid (12.19%), stearic acid (9.8%), behenic acid (3.47%) and myristic acid (2.5%). This large qualitative and quantitative oil composition of *Z. jujuba* leaves enhances its use in pharmacology and cosmetics.

The large proportion of linolenic acid (omega-3) illustrates the high levels of unsaturated FAs (the sum of monounsaturated and poly-unsaturated FAs), varying from 53% to 60%. The richness of unsaturated FAs, which have a multitude of uses in pharmacological activities, could reduce the incidence of human illnesses such as coronary heart disease, other heart diseases, diabetes, and certain types of cancer. This richness also means that leaf extracts have a high nutritional value. Some authors have confirmed this evidence (Berra, 1998; Lunn and Theobald, 2006).

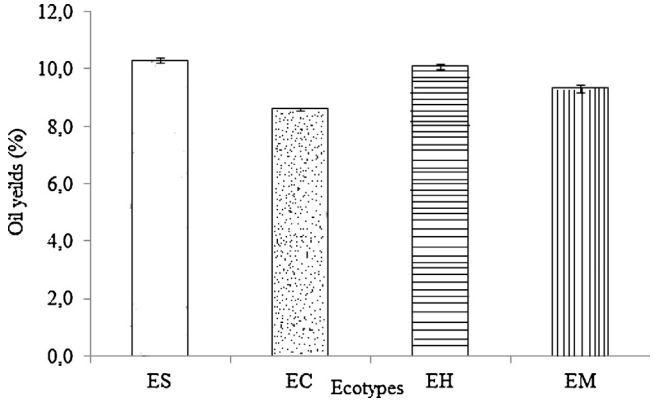

Fig. 2. Oil yields (%) of four Tunisian *Ziziphus jujuba* provenance (EC; EM; EH; ES). EC: Choutrana; EM: Mahdia; EH: Mahres; ES: Sfax. The data are mean values of three measurements. Confidence intervals were calculated at the threshold of 5%.

3.2. Sterols

The sterol content of *Z. jujuba* leaves is presented in Table 2. Sterol levels reached up to around 64 mg/100 g. β-sitosterol and stigmasterol were among the major components in all ecotypes (Fig. 6).

β-sitosterol was high for all ecotypes, with the maximum in the Mahdia ecotype (40.36 mg/100 g oil). This sterol was down to 37.32 mg/100 g oil in Mahres ecotype. It was detected at levels of 32.25 mg/100 g oil and 33.77 mg/100 g oil in Choutrana and Sfax, respectively. The stigmasterol varied from 18.06 (Sfax) to 24.18 mg/100 g oil (Mahdia).

Others sterols such as delta-5-avenasterol were present in the *Z. jujuba* leaves at levels of 6.91, 6.19, 5.2 and 3.88 mg/100 g oil in Mahdia, Sfax, Mahres and Choutrana, respectively. The campesterol was detected only in Sfax, Mahdia and Choutrana with 1.1, 0.8 and 0.6 mg/100 g oil, respectively.

Stigmastanol was present in *Z. jujuba* leaves at a level of 2.29 mg/100 g oil but only in the Sfax ecotype.

This information on the phytosterol composition of *Z. jujuba* leaves confirms the use of this plant to heal many ailments and incites further investigation for natural cosmetic products (Chebouat et al., 2013). In fact, plant sterols are very important to human health as well and can reduce dietary and biliary cholesterol absorption in the intestine (Harrabi et al., 2007).

Table 2 also shows the approximate composition of triterpene alcohols and methyl sterol fractions of the four ecotype leaf extracts. Of these compounds, squalene, cycloartenol and citrostadienol were the main components of these fractions. Methylene cycloartenol was only detected in Mahdia and Sfax ecotypes at levels of 1.47 and 1.18 mg/100 g oil, respectively. This richness of triterpene alcohols and methyl sterols, especially citrostadienol, is important because they may be used as antioxidants (Malecka, 2002).

Table 1
Fatty acid composition (%) of *Z. jujuba* leaves according to provenance (Sfax, Choutrana, Mahres and Mahdia).

Fatty acid	Saturation	Sfax	Choutrana	Mahres	Mahdia
Lauric	C 12:0	0.40 ± 0.43a	0.74 ± 0.07a	0.69 ± 0.02a	0.69 ± 0.03a
Myristic	C 14:0	2.50 ± 0,12a	2.14 ± 0,1b	2.39 ± 0,04a	2.48 ± 0,02a
Palmitic	C 16:0	23.04 ± 0,39a	20 ± 1,67a	21.12 ± 0,32a	21.17 ± 0,5a
Stearic	C18:0	9.82 ± 0,26a	9.80 ± 1a	9.5 ± 0,23a	9.5 ± 0,35a
Oleic (omega-9)	C 18:1n9	13.08 ± 1.29a	8.88 ± 1.23b	7 ± 0.31b	7.76 ± 0.67b
Linoleic (omega-6)	C 18:2n6	12.19 ± 0.15a	9.79 ± 0.81c	10.93 ± 0.05b	12.05 ± 0.13a
Arachidic	C20:0	2.38 ± 0.07b	2.47 ± 0.15b	3.13 ± 0.08a	3.24 ± 0.08a
Linolenic (omega-3)	C18:3n3	34.38 ± 0.1b	34.4 ± 1.58b	42.04 ± 0.83a	39.55 ± 1.3a
Behenic	C22:0	1.98 ± 0.18d	2.61 ± 0.06c	3.22 ± 0.03b	3.47 ± 0.07a
Omega-6/omega-3		0.35	0.28	0.26	0.30
$\sum$Poly-unsaturated		46.57	44.19	52.97	51.60
$\sum$Mono-unsaturated		13.08	8.88	7.00	7.76
$\sum$Saturated		40.12	37.76	40.05	40.55
$\sum$Unsaturated		59.65	53.07	59.97	59.36
I/S		1.48	1.41	1.50	1.46

The first number indicates the length of the fatty acid chain and the second indicates double bonds (all *cis*) showing the location of the double bond(s). $\sum$Saturated = C12:0 + C14:0 + C16:0 + C18:0 + C20:0 + C22:0. $\sum$Mono-unsaturated = C18:1. $\sum$Poly-unsaturated = C18:2 + C18:3. $\sum$Unsaturated: $\sum$mono-unsaturated + $\sum$poly-unsaturated. The data are the mean values of three measurements ± SD (standard deviation). For each column, values with the same alphabetical letter indicate no significant difference at 5% by the Duncan test.

Table 2
Sterol composition (mg/100 g oil) of four Tunisian *Ziziphus jujuba* provenance (Sfax, Choutrana, Mahres and Mahdia).

Ecotype Compound	Sfax	Choutrana	Mahres	Mahdia
β-Sitosterol	33.77 ± 0.1c	32.25 ± 0.07d	37.32 ± 0.8b	40.36 ± 0.66a
Stigmasterol	18.06 ± 1a	18.55 ± 0.01b	22.94 ± 0.03a	24.18 ± 1.21a
d-5-Avenasterol	6.19 ± 0.7a	3.88 ± 0.08c	5.2 ± 0.12b	6.91 ± 0.05a
Campesterol	1.11 ± 0.24a	0.55 ± 0.1b	*	0.81 ± 0.11a,b
Stigmastanol	2.29 ± 0.16b	*	*	*
Squalene	142.03 ± 7.16a	71.76 ± 1.46c	101.82 ± 4.1b	99.28 ± 1.47b
Cycloartenol	56.18 ± 2.42c	64.99 ± 0.76b	68.55 ± 0.06a	8.78 ± 1.42d
Citrostadienol	10.46 ± 2.16a	6.61 ± 1.87b	12.27 ± 0.7a	10.76 ± 0.56a
Methylene cycloartenol	1.18 ± 0.32a	*	*	1.47 ± 0.79a

*Not identified.
The data are mean values of three measurements ± SD (standard deviation). For each column, values with the same letter indicate no significant difference at 5%.

3.3. Total polyphenol contents

As shown in Fig. 3, the relative amounts of polyphenols in *Z. jujuba* leaves varied between 3.97 mg GAE/g (Sfax ecotype) and 6.04 mg GAE/g (Mahres ecotype).

The typical GC profile showed a large variation between the four ecotypes (Sfax, Choutrana, Mahres and Mahdia). The major flavonoid observed was Apigenin with a rate of about 6.1 mg/g of dried material in the Sfax ecotype. Mahres leaves exhibited an intermediate rate of about 0.9 mg/g. The lowest value (0.2 mg/g) was observed in the Choutrana ecotype.

Rutin was the second highest polyphenol from the leaf extracts at levels of 1.9 mg/g and 0.9 mg/g of the total polyphenols in the Sfax and Mahdia ecotypes, respectively (Fig. 4). Rutin rates decreased slightly to 0.45 mg/g and 2 mg/g in leaves from the Mahres and Choutrana ecotypes, respectively.

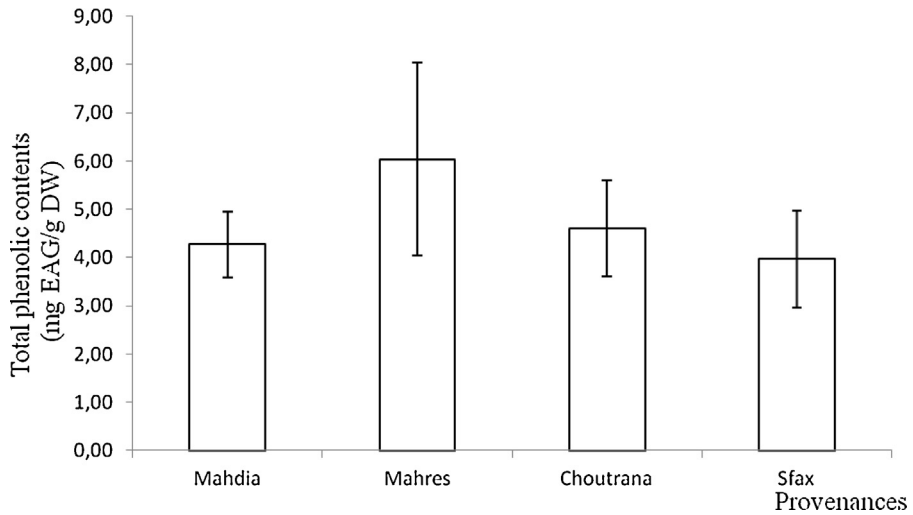

Fig. 3. Total phenolic contents (mg GAE/g) of four provenances of *Ziziphus jujuba* leaf extracts. The data are mean values of three measurements. The confidence intervals were calculated at the threshold of 5%.

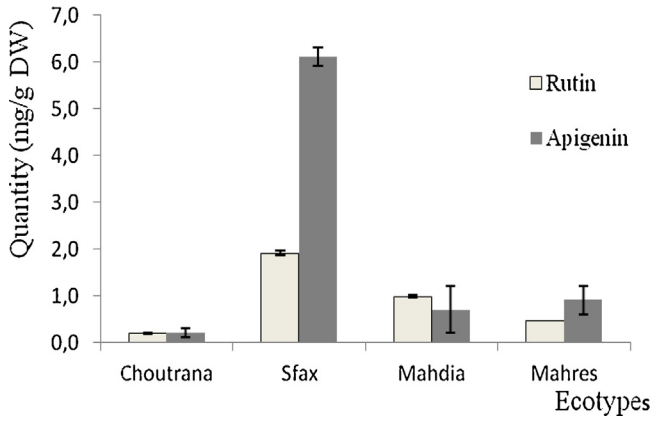

Fig. 4. Rutin and apigenin amounts of *Ziziphus jujuba* leaf extracts. The data are mean values of three measurements. The confidence intervals were calculated at the threshold of 5%.

Knowing the rutin and apigenin content in *Z. jujuba* leaves can be useful in order to implement them in pharmaceutical drugs and food supplements. In fact, polyphenols have attracted high interest from scientists due to their wide range of effects such as antimicrobial, anti-inflammatory, antitumor and anti-sweet (Selloum et al., 2003; Guo et al., 2011).

4. Discussion

The results of the free fatty acids of *Z. jujuba*, especially linolenic acid (42.04%) and palmitic acid (23.04%), were quite different from those reported for *Z. lotus*, where the main compounds were oleic acid (88.12%) and elaidic acid (7.88%) (Ghazghazi et al., 2014). Wei et al. (2014) noted a higher value of linoleic acid (62.04%) in *Averrhoa carambola* leaves.

Unsaturated FA levels found in *Z. jujuba* leaves grown in Tunisia were compared to those obtained by Abdeddaim et al. (2014) for

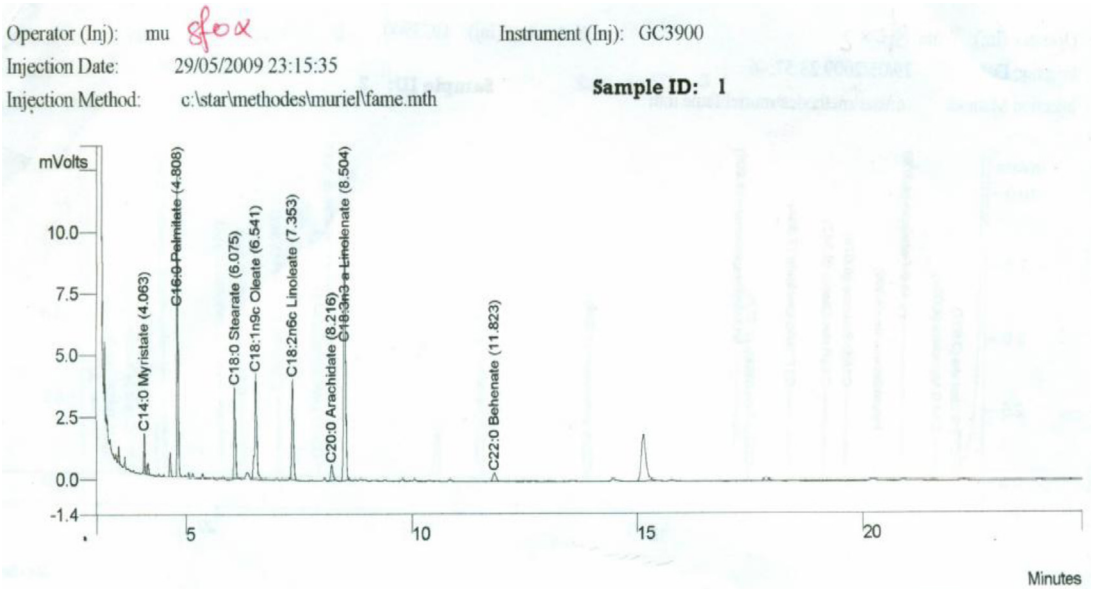

Fig. 5. Fatty acid composition of *Ziziphus jujuba* leaves (Chromatogram).

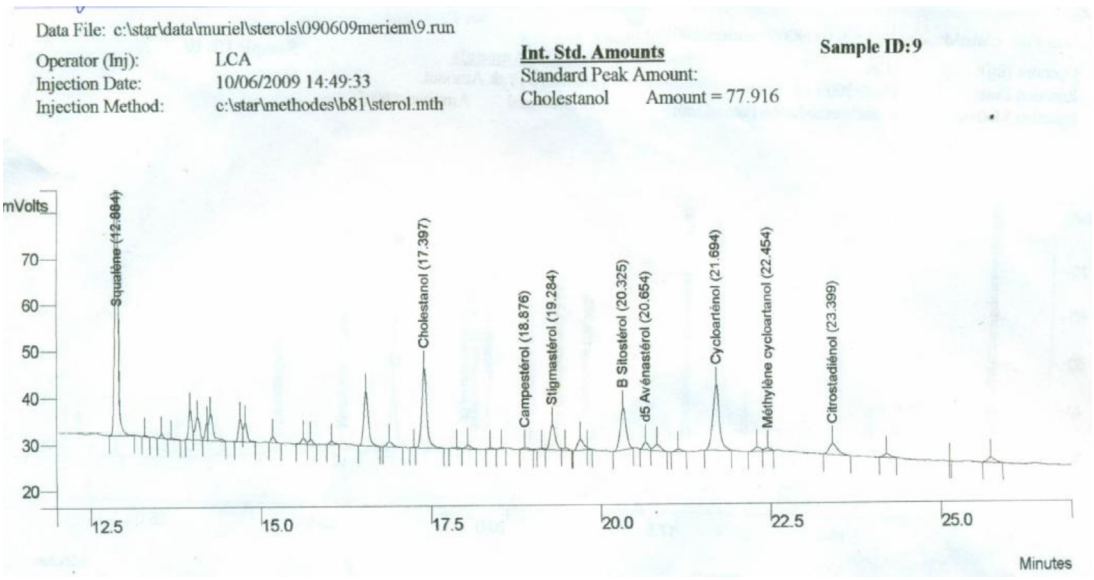

Fig. 6. Sterol composition of *Ziziphus jujuba* leaves (Chromatogram).

Ziziphus lotus fruits (71%). Wei et al. (2014) once again noted higher values for unsaturated fatty acid levels with 77% in *A. carambola*.

Stigmasterol and β-sitosterol were the predominant sterols in leaf extracts. This was confirmed by Elaloui et al. (2011, 2014a,b) in Z. *jujuba* pulp and seed extracts. She obtained 27.32 mg/100 g and 16.12 mg/100 g for stigmasterol and 214.32 mg/100 g and 10.65 mg/100 g for β-sitosterol in seeds and pulp, respectively. Other sterols such as stigmast-5-en-3β, 7α-diol and stigmast-5, 22-ene-3β, 7α-diol have been isolated from the *Z. jujuba* pulp (Wu et al., 2014a,b).

Suttisri et al. (1995) mentioned the existence of triterpenoids in leaf extracts of the *Zizyphus* species but no study found cycloartenol and citrostadienol in such products.

However, focusing on Z. *jujuba* pulp, Lee et al. (2003) showed that they were rich in triterpenoic acids such as colubrinic, alphitolic oleanolic, betulonic, zizyberenalic and betulinic. Wu et al. (2014a,b) detected three other triterpenoids (ilexsaponin B1, ilexsaponin B2 and mussaendoside) in the *Ilex urceolatus* species.

The polyphenol contents of Tunisian *Z. jujuba* leaves were less abundant (6.04 mg/L) than those found by Li et al. (2007) (8.53 mg/L). This difference could be explained by the richness of Tunisian *Z. jujuba* in the proanthocyanin which causes decreased levels of phenolic contents (Saleh et al., 2011). Others studies have shown that Tunisian *Z. jujuba* pulp (extracted under the same conditions) contained between 10.43 mg/L and 15.85 mg/L of polyphenols (Elaloui et al., 2014a,b).

The rutin ratio found in this study was greater (0.9–1.9 mg/g) than that obtained (0.3 mg/g) from *Heteropterys tomentosa* leaves (Paula-Freire et al., 2013). This work confirmed *Z. jujuba* leaves' richness in rutin compared to that found in the pulp (Elaloui et al., 2014a,b). In fact, according to that author, the pulp only contained 0.01 mg/g of rutin.

The variation in polyphenol levels between species could be explained by many genetic and environmental conditions (light, ripeness, maturity...). This idea has been confirmed by other authors (Bravo, 1988; Duthie et al., 2000; Chu et al., 2000).

The extraction efficiency (solvents, methods and times) and tissue drying techniques could influence polyphenol levels in the leaf extracts (Guo et al., 2011; Lee et al., 2003).

5. Conclusion

The biochemical characterization showed that *Z. jujuba* leaf extracts were distinct due to their richness in linolenic, palmitic, oleic, and linoleic acids, and in β-Sitosterol, stigmasterol and flavonoid compounds (especially rutin and apigenin) that justified their use in cosmetics and in pharmacology. The unconventional and underutilized *Z. jujuba* leaves had an interesting biochemical composition that could have a variety of uses and benefits. This work could help preserve this species by integrating it into sustainable agro-sylvo-pastoral development, especially in Tunisian arid regions.

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