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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.