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COMPREHENSIVE PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTIFUNGAL ACTIVITIES OF *INULA VISCOSA* AITON LEAVES

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ABSTRACT

In this study, a detailed phytochemical analysis of the medicinal herb *Inula viscosa* leaves was performed. Furthermore, *in vitro* antioxidant and antifungal properties of its methanolic extract were evaluated and compared with the corresponding phenolic profile obtained by high-performance liquid chromatography and mass spectrometry. Data obtained underscore the high amount of total lipids (6.14%) in leaves. Chromatographic analysis revealed its high content of unsaturated fatty acids (UFAs) with the essential ones α-linolenic and linoleic acids being the main compounds. It also showed good nutritional quality because of its high UFA/saturated fatty acid ratio and the lower values of atherogenic and thrombogenic indices. The volatile oil analyzed by gas chromatography-mass spectrometry showed the abundance of nonterpenic compounds, namely aliphatic alkanes. The total phenol content (TPC) and total flavonoid content (TFC) were higher in *I. viscosa* leaves (103 mg GAE/g dw and 99 mg CE/g dw for TPC and TFC, respectively). The use of HPLC-PDA-ESI-MS/MS allowed the identification of 17 components with hydroxycinnamic acids, namely mono- and dicaffeoylquinic acids being the most prominent components. The presence of these phenolic compound conferred strong free radical scavenging and antifungal properties to the methanol extract. Therefore, *I. viscosa* leaves could be considered as an excellent source of food functional ingredients with high nutritional value and health benefits.

PRACTICAL APPLICATIONS

The outcome of this study shows that *Inula viscosa* leaves can be considered as a consolidated source of essential fatty acids, mainly α-linolenic and linoleic acids. Moreover, they contain appreciable amounts of phenolic compounds with phenolic acids, recognized for their high biological activities, being the most abundant. In addition to its strong antioxidant activity, there is convincing evidence that *I. viscosa* extracts exhibit antifungal activity. On the light of this evidence, it is anticipated that this species may had greater potential as food additives and preservatives.

INTRODUCTION

The genus *Inula* (family Asteraceae) encompasses more than 100 species, mainly found in Africa, Asia and Europe, predominantly, in the Mediterranean area (Seca *et al*. 2014). Members of this genus have been used since ancient times as folk remedies and credited with a long list of medicinal uses, including antioxidant, anti-inflammatory, antiviral,

antibacterial, antifungal, antitumor, antidiabetic, anti-asthmatic, anti-allergic and cytotoxic, among others (Lee *et al.* 2009; Cantrell *et al.* 2010; Park *et al.* 2011; Talib *et al.* 2012). These biological activities were attributed to a plethora of bioactive components mainly sesquiterpenoids, phenolic acids, flavonoids and essential oils (Danino *et al.* 2009; Zhang *et al.* 2009; Yue *et al.* 2013; Jallali *et al.* 2014). The species *Inula viscosa* (L.) Aiton (syn. *Dittrichia viscosa* [L.] Greuter), commonly called sticky fleabane, is a well-known herb used topically in folk medicine for the treatment of skin diseases such as scabies and skin inflammations (Hernández *et al.* 2007; Danino *et al.* 2009). The antioxidant, antibacterial, antifungal, hypoglycemic, hypolipidemic, anticancer, antiparasitic and phytotoxic effects were also reported for *I. viscosa* extracts (Andolfi *et al.* 2013). Although some phytochemical investigations of this species have revealed the presence of sesquiterpenoids, triterpenoids and flavonoids, among others (Máñez *et al.* 2007), there is a dearth of information regarding the lipidic and volatile fraction, and a comprehensive analysis of such metabolites is certainly lacking. On the other hand, most of the earlier phytochemical studies have used the whole aerial parts or roots, whereas leaves have not received much attention. Bear in mind that these metabolites are particularly prone to qualitative and quantitative variations depending on genetic drift, physiological conditions, season, origin, plant part analyzed, extraction and analytical procedure; it seems reasonable to investigate the leaf chemical composition of *I. viscosa*. With regard to this topic, the present contribution was intended to characterize the fatty acid (FA), volatile oil and phenolic profiles and to evaluate the antiradical and antifungal properties of the leaf polar extract.

MATERIAL AND METHODS

Plant Material

The leaves of *I. viscosa* were collected in May 2014 from the province of Beja (north-western Tunisia), and identified by Prof. M. B. Nasri, Department of Botany, Faculty of Sciences of Tunis, where a voucher specimen was deposited. Leaves were air dried at room temperature (20 ± 2C) for 1 week, ground, sifted through 0.5 mm mesh screen and subsequently assayed for their phytochemical analyses.

Phytochemical Analyses

Determination of Total Lipids and FA Composition. The air-dried material (1 g) was extracted with chloroform : methanol (2:1 v/v) following the modified procedure of Bligh and Dyer (1959). The mixture was shaken and centrifuged at 3,000× g for 10 min to allow phase separation. The bottom (organic) layer containing total lipids was recovered and dried under a nitrogen stream.

Fatty acid methyl esters (FAMEs) were prepared using sodium methoxide (Sigma-Aldrich, Buchs, Switzerland) according to the method of Cecchi *et al.* (1985). The FAMEs were analyzed on a HP 6,890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector. Separation was performed on a RT-2560 capillary column (100 m length, 0.25 mm i.d., 0.20 mm film thickness). The oven temperature was kept at 170C for 2 min, followed by a 3C/min ramp to 240C and finally held there for an additional 15 min. Nitrogen was used as carrier gas at a flow rate of 1.2 mL/min. The injector and detector temperature was maintained at 225C. Identification of FAMEs was made by comparison of their retention time with those of reference standards purchased from Fluka (Steinheim, Germany). The FAMEs compositions (%) refer to the percentage ratio of each component to total FAs.

Indices of Lipid Quality. In addition to the unsaturated fatty acid/saturated fatty acid (UFA/SFA) ratio, the artherogenic index (AI) and thrombogenic index (TI) as well as the calculated oxidizability value (Cox) were used to evaluate the lipid quality of *I. viscosa* leaves. These indices were calculated from the data on the FA composition according to the following equations (Ulbrich and Southgate 1991):

$$\mathrm{AI} = [(4 \times \mathrm{C14:0}) + \mathrm{C16:0} + \mathrm{C18:0}] / [\Sigma\ \mathrm{MUFA} + \Sigma\ \omega 6\ \mathrm{PUFA} + \Sigma\ \omega 3\ \mathrm{PUFA}]$$

$$\mathrm{TI} = [(\mathrm{C14:0} + \mathrm{C16:0} + \mathrm{C18:0})/(0.5 \times \mathrm{MUFA} + 0.5 \times \omega 6\ \mathrm{PUFA} + 3 \times \omega 3\ \mathrm{PUFA} + \omega 3/\omega 6\ \mathrm{PUFA}]$$

where MUFA is the sum of monounsaturated FA and PUFA is the sum of polyunsaturated FAs.

The Cox value was calculated using the following equation (Fatemi and Hammond 1980):

$$\mathrm{Cox} = [1(\mathrm{C18:1\%}) + 10.3(\mathrm{C18:2\%}) + 21.6(\mathrm{C18:3\%})]/100$$

Extraction and Analysis of Volatile Oils. The air-dried leaves (100 g) were submitted to conventional hydrodistillation for 3 h. The obtained distillate was extracted twice with diethyl ether and dried over anhydrous sodium sulfate. The organic layer was concentrated at 35C using a Vigreux column and subsequently analyzed by gas chromatography-mass spectrometry (GC-MS).

The GC-MS analysis was performed on a gas chromatograph HP 6,890 (II) interfaced with an HP 5,973 mass spectrometer (Agilent Technologies) with electron impact ionization (70 eV). An HP-5MS capillary column

(60 m × 0.25 mm i.d., 0.25 mm film thickness) was used. The column temperature was programmed to rise from 40 to 280C at a rate of 5C/min. The carrier gas was helium with a flow rate of 1.2 mL/min. Scan time and mass ranges were 1 s and 50–550 m/z, respectively. Compounds were identified using Wiley 275 L, NIST and Mass Finder libraries and retention indices calculated using a series of straight chain *n*-alkanes (C_7–C_{42}) (Adams 2001).

Bioactivity and Phenolic Profile

Sample Preparation. Dried and ground leaves (1 g) were mixed with 80% methanol (1:10 w/v), stirred for 30 min and macerated for 24 h at 4C. Macerated extracts were filtered through Whatman #1 filter paper (Bärenstein, Germany) and concentrated under reduced pressure in a Heidolph rotary evaporator (Schwabach, Germany).

Determination of Total Phenol Content. Total phenol content (TPC) was determined using the Folin–Ciocalteu assay (Mau *et al.* 2001). Briefly, 125 μL of appropriately diluted extract was added to 500 μL deionized water and 125 μL Folin–Ciocalteu reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then, 1,250 μL of sodium carbonate solution (7%) was added. The volume obtained was adjusted to 3 mL using distilled water, mixed vigorously and kept in the dark for 90 min. The absorbance of the solution was then measured at 760 nm against a blank. Gallic acid was used as the standard, and the results were expressed as milligram of gallic acid equivalents (mg GAE/g dw).

Determination of Total Flavonoid Content. The total flavonoid content (TFC) was determined by the $AlCl_3$ colorimetric method (Dewanto *et al.* 2002). A 1 mL aliquot of appropriately diluted extract was mixed with 4 mL distilled water, 0.3 mL of 5% $NaNO_2$ solution, 0.6 mL of 10% $AlCl_3$ solution and 2 mL of NaOH (1 M). After 6 min of incubation, absorbance of the resulting pink-colored solution was read at 510 nm against the blank (distilled water). Catechin was used as the reference standard, and the results were expressed as milligram of catechin equivalents (mg CE/g dw).

Antioxidant Activity

2,2-Diphenyl-1-Picrylhydrazyl Assay. The radical scavenging activity of the methanolic extract against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured according to the method of Sun *et al.* (2000). An aliquot (2 mL) of methanol solution at different concentrations (10–1,000 μg/mL) was added to 0.5 mL of freshly prepared DPPH solution (0.2 mM) and left for 30 min at room temperature. Thereafter, the absorbance was measured at 517 nm. Results were expressed as radical scavenging activity percentage (%) of the DPPH according to the following formula:

$$\text{DPPH scavenging effect (\%)} = \left[\left(A_{\text{blank}} - A_{\text{sample}}\right)/A_{\text{blank}}\right] \times 100$$

where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of plant extract.

The effective concentration having 50% radical inhibition activity (EC_{50}) expressed as μg extract/mL was determined from the graph of the free radical scavenging activity (%) versus extract concentration (Yuan *et al.* 2005).

Azino-Bis(Ethylbenzothiazoline 6-Sulfonic Acid) Assay. The azino-bis(ethylbenzothiazoline 6-sulfonic acid) (ABTS) assay was based on the procedure described by Re *et al.* (1999). Briefly, the radical cation $ABTS^+$ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12 h at room temperature. The blue-green ABTS solution was diluted in ethanol to give an absorbance of 0.7 at 734 nm prior to assay. The diluted ABTS solution (2,850 μL) was mixed with 150 μL of sample extracts, and the mixture was left to stand at room temperature in the dark for 15 min, and then the absorbance was measured at 734 nm. As for DPPH assay, the antioxidant capacity of test samples was expressed as EC_{50}, the concentration necessary to 50% reduction of $ABTS^+$ (Yuan *et al.* 2005).

Antifungal Activity

All target fungal strains (*Fusarium polyphialidicum*, *F. oxysporum*, *F. equiseti*, *F. accuminatum*, *F. scirpi*, *Septoria nodorum* and *Sclerotinia sclerotiorum*) were obtained from a local collection of phytopathogenic fungi maintained at the National Agronomic Institute, Tunis, Tunisia, and preserved at 4C on potato dextrose agar (PDA).

The antifungal activity was tested by disc diffusion method (Esteban *et al.* 2005). The sterile PDA plates were inoculated with each fungal culture (7 days old) by point inoculation. The filter paper discs (5 mm in diameter) impregnated with *I. viscosa* extract (10, 20 or 40 μg/mL) were placed on test organism-seeded plates. Nystatin (10 μg per disc) was used as positive control. The activity was determined after 72 h of incubation at 28C and the diameters (mm) of the inhibition zones were measured.

Characterization of Phenolic Compounds by HPLC-PDA-ESI-MS/MS

Chromatographic separation was performed on a Waters Alliance e2695 HPLC system (Bedford, MA) equipped with a RP-xTerra MS column (150 × 4.6 mm i.d., 3.5 μm particle

size), photodiode array detector and interfaced with a triple quadruple mass spectrometer (MSD 3,100, Waters) fitted with an ESI ion source. The sample (10 μL) was eluted through the column with a gradient mobile phase consisting of A (0.1% formic acid) and B (acetonitrile acidified with formic acid 0.1%) with a flow rate of 0.5 mL/min. The following multistep linear solvent gradient was used: 0–40 min: 14–26%B; 40–60 min: 15%B; 60–75: 0%B; 75–80 min: 14%B.

PDA detection was performed in the 200–800 nm wavelength range, and the mass spectra were recorded in both negative and positive ion mode under the following operating conditions: capillary voltage: 3.5 kV; cone voltage: 25 V (ESI^-) and 15 V (ESI^+), probe temperature: 380C and ion source temperature: 120C. The spectra were acquired in the m/z range of 100–1,000 amu. The phenolic compounds present in the sample were tentatively identified based on their UV and mass spectra and retention times compared with commercial standards when available and/or literature (Hosni *et al*, 2013).

Statistical Analysis

Data were subjected to one-way analysis of variance, and the mean differences were determined by a Duncan test. Each data point was the mean of indicated replicates, and comparisons with P-values < 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Total Lipid Content and FA Composition

The average total lipid content in *I. viscosa* leaves was 6.14 ± 0.62%. This value was higher than those reported (0.2–4.5%) for the Greek specimens (Meletiou-Christou *et al*. 1998), and other edible plant species leaves from the Asteraceae family such as *Centaurea* sp. (2.5–3.16%), *Lactuca sativa* (0.1%), *Cynara cardunculus* (0.09%), *Taraxacum obovatum* (0.19–0.27%), *Chondrilla juncea* (0.09–0.76%) and *Sonchus oleraceus* (0.2–0.41%), among others (Vardavas *et al*. 2006; Aktumsek *et al*. 2013a; García-Herrera *et al*. 2014).

The proportional composition of the analyzed FAs revealed that the leaf oil of *I. viscosa* was constituted mainly by the UFAs with α-linolenic (C18:3) and linoleic (C18:2) acids being the main ones (Table 1). Palmitic acid (C16:0) was found as the most abundant FA in the saturated fraction. To the best of our knowledge, there are no data regarding the FA composition of *I. viscosa* leaves. Nevertheless, the only report on the flower FA of the closely related species *I. grantioides* points out the presence of myristic, pentadecanoic, palmitic, margaric, stearic, arachidic, behenic, tricosoic, lignoceric and heptadecanoic acids (Burdi *et al*. 1990). When compared with other edible Asteraceae species (Table 1), the same profile (C18:3 > 18:2) was also described for *Cichorium* (Blanckaert *et al*. 2000), *L. sativa* (Vardavas *et al*. 2006) and *Carthamus tinctorius* (Harrathi *et al*. 2012). Collectively, these results suggest that *I. viscosa* is a rich source of essential FAs (C18:3 and C18:2). Hence, the use of this species as a good supplement in human diet seems to be a promising alternative. To test such assertion, a set of parameters defining the quality of oil such as UFA/SFA, ω-3/ω-6, AI, TI and the calculated oxidizability value (Cox) were determined (Table 1). As shown, the UFA/SFA ratio was 1.67, which is within range observed for some edible species such as *Centaurea* (Aktumsek *et al*. 2013a), *Cichorium* (Blanckaert *et al*. 2000), *L. sativa* (Vardavas *et al*. 2006) and *Amaranthus viridis* (Guil-Guerrero and

TABLE 1. FATTY ACID COMPOSITION (% OF TOTAL FATTY ACIDS) OF *INULA VISCOSA* LEAVES AND OTHER EDIBLE ASTERACEAE SPECIES

Fatty acids	*I. viscosa* This study	*Centaurea* sp. Aktumsek *et al*. (2013a)	*Cichorium* Blanckaert *et al*. (2000)	*Carthamus tinctorius* Harrathi *et al*. (2012)	*Lactuca sativa* Vardavas *et al*. (2006)
Myristic (C14:0)	7.74 ± 1.12	0.76–0.93	–	–	–
Palmitic (C16:0)	26.63 ± 0.75	23.38–30.49	25.9–40.3	15.96	1.7
Stearic (C18:0)	3.08 ± 0.71	4.16–8.03	5.9–13.6	2.02	13
Oleic (C18:1)	8.36 ± 2.79	6.9–17.37	11.5–18	6.17	1.6
Linoleic (C18:2)	9.99 ± 1.12	20.19–29.93	10.6–26	8.86	16.6
α-linolenic (C18:3)	44.19 ± 4.64	9.96–17.27	12.9–25	21.62	59.3
Lipid quality					
SFA	37.46	33.57–43.10	31.8–53.9	62.56	20.1
UFA	62.54	56.9–66.43	46.1–68.2	37.44	79.9
UFA/SFA	1.67	1.32–1.99	0.58–2.14	0.59	3.97
n3/n6	4.42	0.29–0.56	0.8–1.21	2.44	3.58
AI	0.92	0.42–0.62	–	–	–
TI	0.26	0.42–0.68	–	–	–
Cox value	10.65	4.29–6.98	3.99–8.26	5.64	14.53

AI, artherogenic index; SFA, saturated fatty acid; TI, thrombogenic index; UFA, unsaturated fatty acid.

Rodríguez-García 1999). In contrast, the ω-3/ω-6 ratio (4.42) was much higher than those observed for the aforementioned species and underlying health benefits of the leaf oil of *I. viscosa* as it was nearly similar to the standard value of 5 as recommended by nutritionists (Garaffo *et al.* 2011). This was primarily due to high amount of C18:3 and the relatively lower amount of C18:2. It is well accepted that higher amount of these essential FAs is desirable for consumers' health as they are considered as effective agents in reducing cardiovascular and neurodevelopmental diseases as well as in preventing against some metabolic disorders (such as diabetes, hypertension and hyperlipidemia) beside their deep implication in human growth, physiological functions and maintenance (Mirmiran *et al.* 2012; Villalobos Solis *et al.* 2013; Koren *et al.* 2014).

Although the UFA/SFA ratio is an important factor from a human nutrition point of view, specific saturated and polyunsaturated FAs have different metabolic effects. FAs can either promote or prevent atherosclerosis and coronary thrombosis based on their effects on serum cholesterol and low-density lipoprotein cholesterol concentrations. For this reason, the AI and the TI have been introduced (Ulbrich and Southgate 1991). Very low values of AI and TI are recommended for a healthy diet. The C14:0 and C16:0 FAs are known to be among the most atherogenic, whereas C18:0 is thought to be neutral with respect to atherogenicity, but is instead considered to be thrombogenic (Sinanoglu *et al.* 2011). Another positive features arising from the leaf lipid of *I. viscosa* were the low AI (0.92) and TI (0.26), which were related to the higher UFA/SFA ratio. However, due to the high C18:3 amounts, the lipids from *I. viscosa* leaves were particularly susceptible to oxidation as indicated by the Cox value (10.65).

Collectively, the results of this study suggested that the lipids of *I. viscosa* leaves are of high nutritional quality and their use in human alimentation (salad or food supplement) is strongly encouraged.

Composition of Volatile Oils

The hydro-distillation of *I. viscosa* leaves offered trace amount of volatile oil as it was reported previously (Blanc *et al.* 2005). The composition of the volatile oil, along with the retention index and quantitative data, is listed in Table 2. A total of 27 compounds, covering nearly 98% of the GC profile, were identified. Nonterpenic compounds followed by sesquiterpene hydrocarbons were found as the basic group components of the oil. The former was dominated by decanoic acid, pentacosane and hexacosane, whereas α-gurjunene and α-selinene were the main components of the latter group. Oxygenated terpenes, both monoterpenes (5.24%) and sesquiterpenes (6.67%), were present in lower amounts.

TABLE 2. VOLATILE OIL CONSTITUENTS (% TOTAL PEAK AREA) OF *INULA VISCOSA* LEAVES

Peak no.	RI	Compounds	%
1	1,024	p-Cymene	6.11
2	1,041	Phenylacetaldehyde	2.92
3	1,188	α-Terpineol	1.11
4	1,377	Decanoic acid	26.39
5	1,409	α-Cedrene	0.68
6	1,416	β-Caryophyllene	1.95
7	1,419	β-Cubebene	1.47
8	1,424	α-Gurjunene	11.12
9	1,438	Hotrienol	1.21
10	1,443	Aromadendrene	3.09
11	1,459	Alloaromadendrene	1.33
12	1,479	β-Guaiene	1.52
13	1,485	α-Selinene	7.46
14	1,493	Valencene	2.37
15	1,495	Bicyclogermacrene	3.24
16	1,511	Butylated hydroxytoluene (ional)	1.81
17	1,580	Caryophyllene oxide	6.67
18	2,000	Eicosane	2.97
19	2,100	Heneicosane	0.42
20	2,200	Docosane	0.93
21	2,300	Tricosane	2.87
22	2,400	Tetracosane	0.52
23	2,500	Pentacosane	4.04
24	2,600	Hexacosane	2.73
25	2,700	Heptacosane	0.9
26	2,800	Octacosane	0.83
27	2,900	Nonacosane	1.33
		Group components	
		Monoterpene hydrocarbons	6.11
		Oxygenated monoterpenes	5.24
		Sesquiterpenes hydrocarbons	34.23
		Oxygenated sesquiterpenes	6.67
		Others	45.74
		Total identified	97.99

RI, retention indices on HP-5MS column.

Compared with earlier investigations on *I. viscosa* volatile oils, striking qualitative and quantitative differences can be outlined. The first notable difference seems to be the overall yield of essential oils and the number of identified structures. In fact, Pérez-Alonso *et al.* (1996) reported 55 components recovered in 0.2% of the essential oil of the aerial parts of *I. viscosa*. Seven years later, Marongiu and coworkers reported 48 structures identified in 0.43% of hydrodistilled leaf oil. They also found 46 structures in the essential oil (0.65%) obtained by supercritical fluid extraction (Marongiu *et al.* 2003). More recently, Haoui *et al.* (2015) underscored 30 structures recovered in 0.148% leaf oil. Regarding the main components, qualitative and quantitative differences could be observed and the presence of different chemotypes could be postulated. Although borneol, isobornyl acetate and bornyl acetate were found as the main constituents of the oil of Turkish *I. viscosa*

(Pérez-Alonso *et al.* 1996), globulol, valerianol and caryophyllene oxide were the basic constituents of the Italian specimen (Marongiu *et al.* 2003). The 12-carboxyeudesma-3,11 (13) diene, linolenic acid and palmitic acid were the main constituents of the leaf oil of Algerian *I. viscosa* (Haoui *et al.* 2015). At this point, the yield, the number and the identity of essential oil constituents seem somewhat influenced by the plant origin, plant part, extraction and analytical procedure. Another point to be considered is the noticeable amounts (17.54%) of *n*-alkanes that is congruent with literature (Marongiu *et al.* 2003; Haoui *et al.* 2015). These components are of particular interest as they are involved in different ecological and biological function such as the protection of the epidermal surface against uncontrolled water losses, the protection against UV radiations and the control of leaf temperature (van Maarseveen and Jetter 2009). They are also implicated in the defense against pathogens and insects (Guo *et al.* 2014).

TPC, TFC and Antioxidant Activities

The TPC and TFC in *I. viscosa* leaves were found to be 103 mg GAE/g dw and 99 mg CE/g dw, respectively (Table 3). These values were remarkably higher than those reported for the same species (Trimech *et al.* 2014) and the closely related species such as *I. racemosa* (Manghatayaru *et al.* 2009) and *I. crithmoides* (Jallali *et al.* 2014). In contrast, they were in the range concentration reported in other Asteraceae species where the TPC ranged from 15.98 to 227.93 mg GAE/g dw (Kenny *et al.* 2014), whereas TFC ranged from 1.13 to 182.56 mg CE/g dw (Aktumsek *et al.* 2013b; Morales *et al.* 2014).

The antioxidant activity of the methanolic extract of *I. viscosa* leaves was determined from the reduction of absorbance of the DPPH and ABTS radicals at 517 and 734 nm, respectively. The effective concentrations having 50% radical inhibition activity (EC_{50}) were found to be 23.33 and 16.75 μg/mL for DPPH and ABTS assays, respectively. These results reflect the high ability of *I. viscosa* leaf extracts to donate electrons to inactivate radical species (Yuan *et al.* 2005). Regarding the DPPH assay, the obtained value was approximately 10-fold lower than those (0.26 mg/mL) reported by Trimech *et al.* (2014). Using the same assay, the EC_{50} values were found to be in the range concentration from 12.8 to 36 μg/mL in *I. crithmoides* (Jallali *et al.* 2014). When compared with other Asteraceae species such as *Cichorium* sp. (EC_{50} of 86 μg/mL), *Sonchus* sp. (EC_{50} of 72 μg/mL) (Conforti *et al.* 2011) extract of *I. viscosa* showed the highest free (DPPH) radical scavenging activity. Consistent with the DPPH, the ABTS method also confirmed the strong radical scavenging activity of *Inula* sp. In this context, the only paper describing the antioxidant activity of *I. britannica* var. chinensis reports an EC_{50} of 39.4 μg/mL (Lee *et al.* 2009). In the case of *I. viscosa*, to the best of our knowledge, there are no data concerning its ABTS radical scavenging activity. Nevertheless, the antioxidant activity of the isolated compound 1,3-dicaffeoylquinic acid from *I. viscosa* was found to be higher than the standard trolox and BHT (Danino *et al.* 2009). The high antioxidant activity of *I. viscosa* extracts might be correlated (data not shown) with its elevated TPC, TFC and its phenolic profile particularly rich in antioxidant compounds as reported in other species (Aktumsek *et al.* 2013a). On the other hand, the putative antioxidant activity of *I. viscosa* extract could justify its use for the treatment of oxidant-related disease such as diabetes and skin inflammation.

Antifungal Activity

The antifungal activity of the leaf methanolic extract of *I. viscosa* was qualitatively assessed by the presence or absence of inhibition zones and zone diameter. The results given in Table 4 showed that the extract exhibited antifungal activity against all tested fungi strains in a concentration-dependant manner. *Sc. sclerotiorum* and *Se. nodorum* were the most sensitive strains as they were completely inhibited at a concentration of 40 μg/mL. Consequently, the concentration of 40 μg/mL was considered as the minimal inhibitory concentration for both fungi. In contrast, *Fusarium* species showed variable responses with *F. polyphialidicum* being the most resistant to the methanolic extract (46.84% inhibition at 40 μg/mL), whereas *F. accuminatum* seems to be the most sensitive (63.63% inhibition at 40 μg/mL). The results were consistent with the earlier studies in which *I. viscosa* extracts were found as fungicidal agents (Qasem

TABLE 3. TOTAL PHENOLIC AND FLAVONOID CONTENTS IN *INULA* SP. AND OTHER EDIBLE ASTERACEAE SPECIES

	Inula viscosa This study	*I. viscosa* Trimech *et al.* (2014)	*I. crithmoides* Jallali *et al.* (2014)	*Cichorium* sp. Conforti *et al.* (2011)	*Centaurea* sp. Aktumsek *et al.* (2013a)
Total phenol content (mg EGA/g dw)	103 ± 1. 1	2.57–8.48	6.7–14.1	83–139	146.06–348.56
Total flavonoid content (mg CE/g dw)	84.92 ± 1.41	29.43–55.75	5.6–6.7	–	13.12–182.56
DPPH (EC_{50} μg/mL)	23.33 ± 1.56	20–260	12.8–36	72–116	187.42–670.59
ABTS (EC_{50} μg/mL)	16.75 ± 0.26	–	–	–	–

ABTS, azino-bis(ethylbenzothiazoline 6-sulfonic acid); CE, catechine equivalent; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; EGA, equivalent gallic acid.

TABLE 4. EFFECT OF DIFFERENT EXTRACTS CONCENTRATIONS ON MYCELIAL GROWTH AND % INHIBITION OF FUNGAL STRAINS

	Concentration (µg/mL)						
	0	10		20		40	
	Mycelial growth	Mycelial growth	% inhibition	Mycelial growth	% inhibition	Mycelial growth	% inhibition
Fusarium polyphialidicum	$39.5^{a} \pm 0.71$	$39.00^{a} \pm 1.41$	1.27	$37.50^{a} \pm 3.53$	5.07	$21.00^{b} \pm 1.41$	46.84
Fusarium oxysporum	$36.00^{a} \pm 1.41$	$29.00^{b} \pm 1.41$	19.45	$22.50^{c} \pm 0.71$	37.5	$14.50^{d} \pm 0.71$	59.73
Fusarium equiseti	$40.50^{a} \pm 0.71$	$36.00^{b} \pm 1.41$	11.12	$35.50^{b} \pm 0.70$	12.35	$15.50^{c} \pm 0.70$	61.73
Fusarium accuminatum	$49.50^{a} \pm 0.71$	$47.50^{b} \pm 1.50$	4.05	$47.00^{b} \pm 1.41$	5.06	$18.50^{c} \pm 2.10$	63.63
Fusarium scirpi	$44.5^{a} \pm 8.45$	$40.50^{a} \pm 8.80$	8.99	$38.50^{a} \pm 6.00$	35.96	$18.50^{b} \pm 7.03$	58.43
Septoria nodorum	$31.00^{a} \pm 1.41$	$29.00^{a} \pm 1.41$	6.45	$26.50^{b} \pm 0.70$	14.52	$0.90^{c} \pm 0.41$	97.1
Sclerotinia sclerotiorum	$88.50^{a} \pm 0.71$	$85.50^{b} \pm 0.70$	3.39	$73.00^{c} \pm 1.40$	17.52	0	100

Values are given as mean ± SD ($n = 3$). Means for all data in each assay followed by different letters are significantly different ($P < 0.05$).

et al. 1995). Their inhibitory effects on spore germination and mycelial growth of different phytopathogenic fungi were also evidenced by Abou-Jawdah *et al.* (2004) and Wang *et al.* (2004). The efficacy of *I. viscosa* leaves against *Trichoderma harzianum*, *Tr. viride* and *F. oxysporum* has recently been reported by Omezzine *et al.* (2011). Moreover, it was reported that *I. viscosa* extract dose dependently inhibited the spore germination and the mycelial growth of the causal agent of citrus sour rot: *Geotrichum candidum* (Talibi *et al.* 2012). Because of its apparent antifungal activity, the methanolic extract of *I. viscosa* may be an alternative for the synthetic antifungal drugs. From a mechanistic standpoint, the antifungal activity of *I. viscosa* extract can be primarily attributed to some phenolic compounds endowed with fungicidal activity. The main hallmarks of such a component are the disruption of cell membrane structure, increased membrane fluidity and permeability, impairment of cellular ionic homeostasis, inhibition of respiration and alteration of ion transport processes in fungi (Lee and Sung 2008; Zabka and Pavela 2013; Tao *et al.* 2014).

In order to characterize the presumed antioxidants and/or antifungal phenolic compounds, a chromatographic analysis by HPLC-PDA-ESI-MS/MS was carried out on the methanolic extract and the results are presented in the following section.

Characterization of Phenolic Compounds

Phenolic compounds in the methanolic extract of *I. viscosa* leaves were tentatively identified based on their UV-vis, MS and MS/MS spectral data; their retention time (t_R) relative to authentic standards when available; and published information. The typical HPLC-PDA-ESI-MS chromatogram is depicted in Fig. 1. The chromatographic and MS data are listed in Table 5, including retention time, parental ion, MS/MS fragments and proposed compounds.

As can be seen, the phenolic profile was dominated by hydroxycinnamic acid, flavonols and flavanones derivatives. The former group exhibits characteristic UV absorption maxima around 325 nm with a shoulder at 290–300 nm and a common fragmentation pathway based on the loss of CO_2 group (Sánchez-Rabaneda *et al.* 2004). Consequently, peak 1 (t_R: 3.04 min) was identified as caffeic acid-*O*-glucoside. This component exhibited deprotonated molecular ion at m/z 341, and a dominant fragment ion of m/z 179 indicative to caffeic acid unit, with a neutral loss of 162 amu corresponding to hexose. Peak 2 (t_R: 4.14 min; λ_{max}: 309 nm) showed pseudomolecular ion of $[M\text{-}H]^-$ at m/z163 which produced major fragment at m/z 119. By comparison with authentic standard, this compound was identified as *p*-coumaric acid. Peak 3 (t_R: 7.25 min; λ_{max}: 325 nm) was identified as chlorogenic acid (3-*O*-caffeoylquinic acid). It exhibited deprotonated ion at m/z 353 and had main MS^2 fragments at m/z 191 and 179, indicating that the caffeoyl group is linked to the 3-OH position of quinic acid. The identity of this compound was confirmed by its UV and MS^2 spectra and HPLC retention time with the standard chlorogenic acid. Peaks 4, 8, 9 and 10 eluted at 10.51, 23.97, 25.67 and 30.07 min, respectively, with UV absorption maxima at 327 nm and exhibit the same deprotonated molecular ion at m/z 515 and MS^2 fragmentation of these ions gave a $[M\text{-}H\text{-}162]^-$ ion at m/z 353, indicating the presence of more than one caffeoyl group attached to different quinic acid OH groups. Based on the hierarchical key proposed by Clifford *et al.* (2005) and their order of elution, they were tentatively identified as 1,3-*O*-dicaffeoylquinic acid (peak 4), 3,4-dicaffeoylquinic acid (peak 8), 3,5-dicaffeoylquinic acid (peak 9) and 4,5-dicaffeoylquinic acid (peak 10). These compounds known for their biological activities have been previously reported in *I. viscosa* (Danino *et al.* 2009) and other Asteraceae species (Clifford *et al.* 2007; Gouveia and Castilho 2010; Hosni *et al.* 2013; Pandino *et al.* 2013).

Peak 11 (t_R: 30.1 min; λ_{max}: 325 nm) displayed a $[M\text{-}H]^-$ ion at m/z 499 which in MS^2 formed a fragment ion at m/z 353 (caffeoylquinic acid), representing a loss of coumaroyl group (146 amu). This compound was tentatively identified as coumaroyl caffeoylquinic acid (Clifford *et al.* 2006). Peak

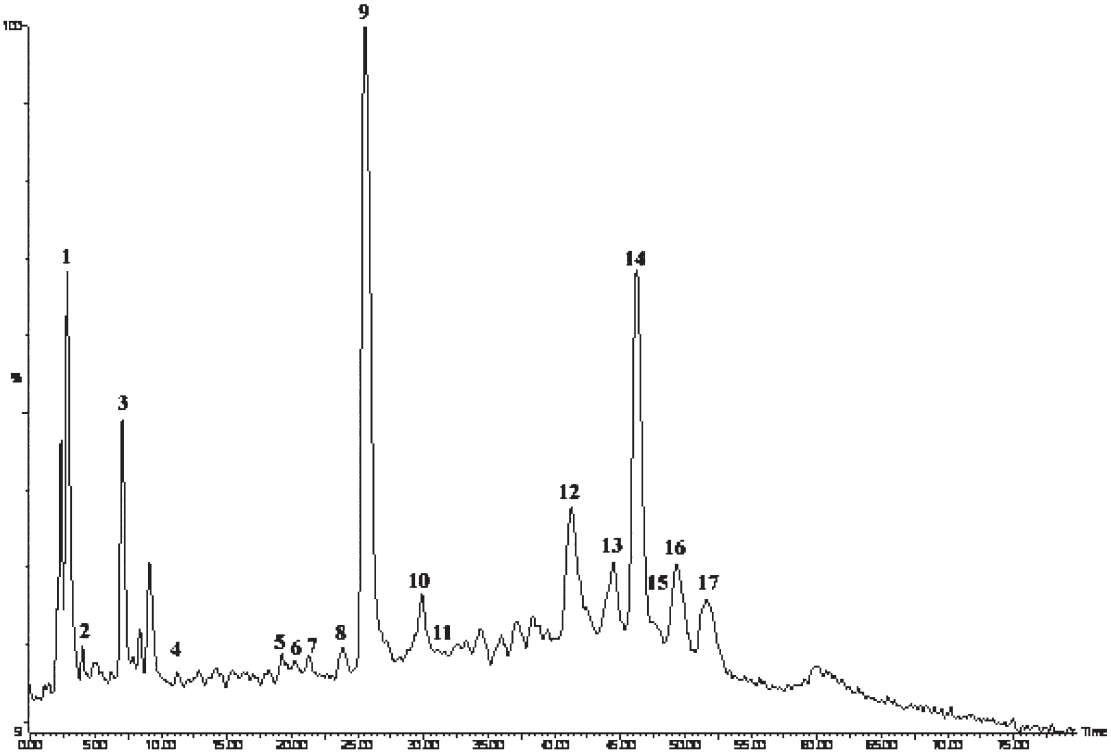

FIG. 1. HPLC-PDA-ESI-MS ANALYSIS OF THE METHANOLIC EXTRACT OF *INULA VISCOSA* LEAVES

12 (t_R: 41.41 min; λ_{max}: 286 nm) exhibited a $[M\text{-}H]^-$ ion at m/z 683 and an intense fragment ion at m/z 341 suggesting that the ion at m/z 683 is a dimer of the ion at m/z 341. The fragmentation of the latter ion gave a fragment ion at m/z 179 due to the loss of hexoside residue (162 amu), which led to the identification of caffeic acid derivative. This component was tentatively identified as a dimer of caffeic acid-*O*-hexoside (Gouveia and Castilho 2010). The only

TABLE 5. RETENTION TIME, UV AND MASS SPECTRAL DATA AND TENTATIVE IDENTIFICATION OF THE PHENOLIC COMPONENTS IN *INULA VISCOSA* LEAF EXTRACT

Peak no.	RT (min)	λ_{max}	$[M\text{-}H]^-$ (m/z)	Fragment ions (m/z)	Tentative identification
1	3.04	326	341	179; 135	Caffeic acid-*O*-hexoside
2	4.14	309	163	119	*p*-Coumaric acid
3	7.25	326	353	191; 179	Chlorogenic acid
4	10.51	326	515	353; 191; 179	1,3-*O*-dicaffeoylquinic acid
5	20.35	337	463	303; 285	Taxifolin hexoside
6	20.49	255	299	137	Hydroxybenzoic acid hexoside
7	21.44	354	477	315	Isorhamnetin-*O*-hexoside
8	23.97	326	515	353; 191; 179	3,4-Dicaffeoylquinic acid
9	25.67	326	515	353; 191; 179	3,5-Dicaffeoylquinic acid
10	30.07	326	515	353; 191; 179	4,5-Dicaffeoylquinic acid
11	30.10	325	499	353	Coumaroyl caffeoylquinic acid
12	41.41	286	683	341; 179	Dimer of caffeic acid-*O*-hexoside
13	44.21	354	285	285	Luteolin
14	44.62	343	653	315	Isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside
15	46.39	289	317	299; 289	Unknown dihydroflavonol
16	49.55	354	315		Isorhamnetin
17	51.74	291	345	303; 285	Acetyl taxifolin

hydroxybenzoic acid (peak 6) was eluted at t_R: 20.49 min with UV absorption maxima at 255 nm. It showed a pseudomolecular $[M-H]^-$ ion at m/z 299 and a strong fragment ion at m/z 137 (indicative to hydroxybenzoic acid) in MS^2, corresponding to the loss of hexoside residue. This component was tentatively identified as hydroxybenzoic acid hexoside (Hossain *et al.* 2010).

In addition to the phenolic acid described earlier, some flavonoid compounds were also identified in the methanolic extract of *I. viscosa* leaves. The flavonols luteolin (peak 13) and isorhamnetin (peak 16) were positively identified by comparison of their UV and mass spectral data with those of commercial standards. The latter component was also detected in the glucosylated forms: isorhamnetin-*O*-hexoside (peak 7) and isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside (peak 14). The former compound (t_R: 21.44 min; λ_{max}: 354 nm) presented a pseudomolecular ion at $[M-H]^-$ at m/z 477 displaying a unique MS^2 fragment ion at m/z 315 (isorhamnetin aglycone), indicating the loss of hexoside residue (162 amu) (Dias *et al.* 2013). Isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside (t_R: 44.62 min; λ_{max}: 343 nm; $[M-H]^-$ at m/z 653) showed the loss of glycosyl and feruloyl residues $[M-H-162-176]^-$ originating a base peak at m/z 315 (Barros *et al.* 2014).

Peak 5 (t_R: 20.35 min; λ_{max}: 337 nm) showed $[M-H]^-$ and $[2M-H]^-$ deprotonated molecules at m/z 463 and 925, respectively. The loss of hexoside residue in MS^2 gave a strong fragment ion $[M-H-162]^-$ at m/z 303 (indicative to taxifolin aglycone) which in turn gave another fragment ion at m/z 285 due to the loss of H_2O. This fragmentation pattern was in agreement with that of the flavanone taxifolin hexoside (Lin *et al.* 2007). Peak 15 (t_R: 46.39 min) showed UV spectrum characteristic of dihydroflavonol or flavanone structure (Sanz *et al.* 2011). It displayed a deprotonated $[M-H]^-$ ion at m/z 317 and fragment ions corresponding to losses of H_2O (commonly observed in dihydroflavonols) and CO that yielded two intense fragments $[M-H-18]^-$ at m/z 299 and $[M-H-CO]^-$ at m/z 289. The observed fragmentation pattern, however, is not informative enough to elucidate the structure of this compound.

Peak 17 (t_R: 51.74 min; λ_{max}: 291 nm) presented a deprotonated molecule at m/z 345, releasing MS^2 fragment at m/z 303 ($[M-H-42]^-$, loss of acetyl group) characteristic of the aglycone taxifolin. This component was tentatively identified as acetyl taxifolin and was already reported in *I. viscosa* (Trimech *et al.* 2014).

In general, the present phenolic profile particularly rich in mono- and dicaffeoylquinic acids as well as flavonols and flavanones could, at least in part, explain the strong antioxidant and antifungal activity of the methanolic extract of *I. viscosa*. At this point, previous studies have pinpointed the potential radical scavenging and antifungal activities of chlorogenic acid and their derivatives (Lee and Sung 2008; Suárez-Quiroz *et al.* 2013). The high ability of dicaffeoylquinic acid isomers to scavenge free radicals and inhibit lipid peroxidation was also evidenced (Danino *et al.* 2009; Yuan *et al.* 2012). The presence of functional groups (hydroxyl and caffeoyl groups) in the structure of the identified phenolics is responsible for their strong antioxidant activity. Their ability to dissolve and accumulate in the cell membrane results in cell membrane destabilization, disruption of proton transfer and blockage of ATP synthesis (Yuan *et al.* 2012; Zabka and Pavela 2013).

Collectively, the results of this study suggest that *I. viscosa* could be considered as consolidated source of bioactive components such as essential FAs, volatile oils and phenolic compounds. Its putative antioxidant and antifungal activities suggest its use as a starting bio-based material for the development of natural preservative with application in agro-food, cosmetic and pharmaceutical industries.

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