Phytochemical Investigation by Microwave-Assisted Extraction of Essential Oil of the Leaves of Walnut Cultivated in Algeria

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Walnut (*Juglans regia* L.) leaves are used traditionally as an herbal tea indicated for non-insulin-dependent diabetics. In recent years, the type-II diabetes is occurring worldwide with increasing frequency. Thus, there is an urgent need to explore the new beneficial biomolecules on the human health. Our objective was to investigate, for the first time, the volatiles profile of *Juglans regia* L. leaves from Algiers region. The extraction of essential oil of fresh plant material was performed by microwave-assisted hydrodistillation (MAHD), for the first time, a relatively recent method, then by the conventional hydrodistillation technique (HD) for comparison. The collected extracts were analyzed by GC-FID and GC/MS using two capillary columns with different polarity. Extraction time of 1 h by MAHD provided higher yields ($0.050 \pm 0.001\%$ (*w*/*w*)) than by HD ($0.030 \pm 0.006\%$ (*w*/*w*)) after 3 h. A total of 38 compounds were identified using both techniques. Essential oils had similar qualitative but different quantitative composition in terms of chemical compounds. The MAHD method improved yield while reducing the extraction time. The sesquiterpenes were the dominant family in both MAHD and HD essential oils with β-caryophyllene being the major constituent. Monoterpenes, including hydrocarbon and oxygenated, prevail in HD volatile fraction with β-pinene and eucalyptol, respectively, as major components.

Introduction. – Walnut (*Juglans regia* L.), belonging to the Juglandaceae family, is an important deciduous tree. It primarily grows in the temperate areas, but, nowadays, it is commercially cultivated and widely distributed throughout the world. In Algeria, this species called '*Al-jouz*' was once cultivated by the Berbers; there were forests only in the mountainous regions of the high plains such as the Aures. Due to its medicinal, nutritional, and economic relevance, it occures in all over the country, and walnut-tree plantations have been continously increasing. Walnuts are nutrient-rich food due to high contents of fats, proteins, vitamins, and minerals so that they are included in FAO priority-plants list. They are also a good source of flavonoids, sterols, pectic substances, phenolic acids, and related polyphenols [1]. Moreover, it was reported that phytochemicals such as phenolic compounds are considered to decrease the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation [2][3]. According to several reports, roots, bark, green walnut, shells, seeds, and extracts of the leaves have various medicinal and cosmetic properties [4–9]. In walnut leaf extracts, naphtoquinones have been the major phenolic compounds [10].

Juglone (= 5-hydroxy-1,4-naphthoquinone) is the most characteristic compound of *Juglans* spp. and is reported to occur mostly in fresh walnut leaves [11][12]. Nevertheless, because of polymerization and hydrosolubility, juglone occurs in dry leaves only in low amounts [10]. The presence of juglone in walnut leaves is considered to be the major source of their important pharmacological properties including antitumor, antitubercular, and antiviral activities [13–15]. The antimicrobial activity of *J. regia* parts, and, particularly, of the specific compound juglone have been reported [16][17]. Hypoglycemic, hypotensive, keratolytic, antifungal, antiscrofulous, and sedative activities have also been mentioned [11][18]. In Algeria, leaves have been intensively used in infusion or in decoction form for treatment of diabetes, rheumatic pain, venous insufficiency, haemorrhoidal symptomatology, against bed-bugs, as hair dyes, topical remedies for dermal inflammations and excessive perspiration of the hands and feet, and for its depurative, antidiarrheic, and antihelmintic properties. In rural areas, the bark was used as miswaks for teeth cleaning. Herbal drugs are gaining popularity in the treatment of type-II diabetes. The major advantages of herbal medications seem to be their efficiency, low incidence of side effects, and low cost. Walnut leaves are also marketed in pharmacies as an herbal tea indicated for non-insulin-dependent diabetics. Most reports on the bioactivity of *J. regia* leaves were focused on the phenolics; the volatiles profiling has been the subject of limited previous investigations, with little information on essential oil and its pharmacological effects. In 2008, *Farag* reported a dynamic headspace volatile analysis, combined with GC/MS, to profile the volatiles of *J. regia* leaves [19]. Sesquiterpenes (47%) dominated the volatile blend of the species, with germacrene D (28.6%) occurring in the highest amount. The level of monoterpenes was 24%, and the major component was (*E*)-β-ocimene (6.8%). Among the benzenoids, methyl salicylate was found as the major leaf headspace compound (16.8 %). Also in 2008, *Fojtovà et al.* [20] reported that five different extraction methods, *i.e.*, accelerated solvent extraction under elevated pressure (ASE), steam distillation, *Soxhlet* extraction, sonication, and extraction with agitation of the solvent, were tested to isolate terpenes from foliage of the walnut tree. The highest quantity of terpenes (198.7 μg/g) was obtained by ASE; the optimal conditions of pressure and temperature were 150 bars and 120°, respectively. The essential oils were analyzed by GC/MS using *HP 5 MS*™ capillary column. The main compounds in the order of elution were: α-pinene, camphene, β-pinene, myrcene, limonene, eucalyptol, linalool, bornyl acetate, *trans*-caryophyllene, and α-humulene. They have noted that the relative proportions of constituents varied depending on the extraction method.

To our knowledge, there is no previous report in the literature on the essential oil of *J. regia* leaves cultivated in Algeria. Thus, as a part of a chemical investigation program on Algerian medicinal herbs, we elucidated the volatile constituents of fresh leaves of *J. regia* from Algiers region, using two different methods. Essential-oil extractions were performed first by the relatively recent microwave-assisted hydrodistillation technique (MAHD), and then by conventional hydrodistillation (HD), recommended by the European pharmacopeia AFNOR for the extraction of edible essential oils. Several assays have been carried out in order to optimize the yield of the MAHD extraction of walnut leaves by varying *1*) the power of microwave radiations, *2*) the extraction time, *3*) the used quantity of H_2O, starting from a dried distillation, as described in our

previous works [21][22]. The extracts were analyzed by GC-FID and GC/MS. The results were compared with respect to extraction time, yield, chemical composition, and quality of the essential oil.

Results and Discussion. – *Extraction by MAHD.* Extraction time of 1 h provided yields of $0.050 \pm 0.001\%$ (*w*/*w*), higher than HD yields $0.030 \pm 0.006\%$ (*w*/*w*) obtained after 3 h. This is due to the more efficient heat flow induced by microwaves, so that the entire sample was almost simultaneously heated. It is worthy of note that, with MAHD, the time to reach the extraction temperature (100°) for the first essential-oil droplet was only 2.5 min compared to 30 min needed for HD. Both MAHD and HD essential oils were of pale yellowish color and had a characteristic walnut scent.

Qualitative and Quantitative Analyses. For the first time, the entire volatile components of the walnut leaves were investigated by the MAHD method. The chemical components were compared to those obtained by the conventional HD technique. Both essential oils were complex mixtures of mainly sesquiterpenes and monoterpenes, and the identified compounds are compiled in the *Table* in their elution order on a *HP-5MS*™ capillary column (*Table*). Thirty-eight components representing 99.47% of the MAHD essential oil have been detected (*Fig. 1*) and identified by comparing their retention indices (*RI*s) and their mass spectra with those in the literature. The major compound was β-caryophyllene (28.49%), followed by germacrene D (23.29%), α-humulene (15.64%), β-eudesmol (9.28%), β-farnesene (2.76%), γ-cadinene (2.16%), β-*trans*-ocimene (2.13%), β-pinene (1.84%), eucalyptol (1.76%), α-pinene (1.73%) α-*trans*-bergamotene (1.58%), and terpinen-4-ol (1.10%). Furthermore, 37 components have been detected (*Fig. 2*) and identified in HD extract representing 98.19% of the essential oil. The main compounds were β-pinene (20.13%), β-caryophyllene (17.55%), germacrene D (13.37%), α-pinene (9.58%), α-humulene (8.08%), eucalyptol (5.35%), β-*trans*-ocimene (4.59%), β-eudesmol (3.46%), terpinen-4-ol (2.35%), β-farnesene (2.00%), γ-cadinene (1.45%), α-*trans*-bergamotene (1.11%), and β-myrcene (1.00%). Substantially higher amounts of 18 sesquiterpene compounds (78.73% *vs.* 48.36%) and lower contents of eleven monoterpene hydrocarbons (07.18% *vs.* 37.59%) were present in the essential oil of walnut leaves extracted by MAHD compared to HD. Both extracts have shared two oxygenated monoterpenes: eucalyptol and terpinen-4-ol (2.86% for MAHD *vs.* 7.70% for HD). Five oxygenated sesquiterpenes including β-eudesmol, at a high percentage of 9.28% for MAHD *vs.* 3.46% HD, and finally two esters, *i.e.*, bornyl acetate and methyl salycilate, were obtained at 0.45% for MAHD, whereas the HD essential oil contained only bornyl acetate at 0.29%.

In this study, the extract obtained by MAHD was richer in non-oxygenated compounds (85.91% by MAHD *vs.* 85.95% by HD), unlike in several previous studies where the oxygenated fraction was higher than the non-oxygenated one [23][24]. In this context, it is worth noting that essential oils of the leaves of *J. regia*, obtained by MAHD and HD contained a relatively low amount of oxygenated compounds, and they were dominated by the sesquiterpene hydrocarbons, which were also easily extracted by techniques involving microwave heating, as it has been demonstrated in last references. Almost all of the constituents identified in our study have been already detected in volatile substances of *J. regia* leaves, using headspace analysis coupled with

Table. *Chemical Composition of Essential Oils of Walnut Leaves Obtained by MAHD and HD*

Entry	Compound[a])	*RI*[b])	*RI*[c])	MAHD [%][d])	HD [%][d])
Monoterpene hydrocarbons				7.18	37.59
1	α-Thujene	923	1028	tr	tr
2	**α-Pinene**	**929**	**1016**	**1.73 ± 0.62**	**9.58 ± 3.65**
3	Camphene	942	1054	0.37 ± 0.09	0.79 ± 0.27
4	**β-Pinene**	**975**	**1107**	**1.84 ± 0.79**	**20.13 ± 3.73**
5	β-Myrcene	994	1152	0.39 ± 0.01	1.00 ± 0.40
6	α-Phellandrene	1005	1166	0.08 ± 0.01	0.20 ± 0.00
7	α-Terpinene	1015	1168	0.10 ± 0.01	0.26 ± 0.00
8	D-Limonene	1025	1191	0.10 ± 0.01	0.18 ± 0.02
9	**β-*trans*-Ocimene**	**1047**	**1244**	**2.13 ± 0.66**	**4.59 ± 1.07**
10	γ-Terpinene	1056	1232	0.14 ± 0.02	0.40 ± 0.00
11	TerpinoIene	1090	1268	0.30 ± 0.11	0.46 ± 0.09
Oxygenated monoterpenes				**2.86**	**7.70**
12	**Eucalyptol**	**1027**	**1201**	**1.76 ± 0.46**	**5.35 ± 1.07**
13	**Terpinen-4-ol**	**1081**	**1610**	**1.10 ± 0.72**	**2.35 ± 0.66**
Sesquiterpene hydrocarbons				**78.73**	**48.36**
14	α-Cubebene	1341	1458	0.74 ± 0.08	0.71 ± 0.01
15	α-Copaene	1364	1484	0.28 ± 0.04	0.21 ± 0.07
16	β-Bourbonene	1372	1515	0.68 ± 0.23	0.48 ± 0.14
17	β-Cubebene	1380	1543	0.24 ± 0.16	0.23 ± 0.07
18	β-Elemene	1384	1540	0.44 ± 0.19	0.96 ± 0.19
19	**β-Caryophyllene**	**1408**	**1615**	**28.49 ± 0.35**	**17.55 ± 4.65**
20	**α-*trans*-Bergamotene**	**1427**	**1580**	**1.58 ± 0.71**	**1.11 ± 0.70**
21	**α-Humulene**	**1439**	**1651**	**15.64 ± 1.45**	**8.08 ± 4.11**
22	**β-Farnesene**	**1461**	**1667**	**2.76 ± 0.90**	**2.00 ± 0.76**
23	**Germacrene D**	**1472**	**1723**	**23.29 ± 2.90**	**13.37 ± 2.54**
24	γ-Curcumene	1474	1770	0.18 ± 0.08	0.17 ± 0.09
25	γ-Muurolene	1479	1646	0.94 ± 0.41	0.44 ± 0.32
26	Bicyclogermacrene	1484	1740	0,30 ± 0.40	0.43 ± 0.19
27	α-Muurolene	1492	1731	0.47 ± 0.09	0.75 ± 0.19
28	γ-Cadinene	1503	1763	2.16 ± 0.27	1.45 ± 0.28
29	α-Farnesene	1509	1751	0.16 ± 0.03	0.17 ± 0.04
30	δ-Cadinene	1515	1761	0.17 ± 0.03	0.14 ± 0.02
31	(*Z*,*E*)-α-Farnesene	1521	1725	0.06 ± 0.03	0.11 ± 0.03
Oxygenated sesquiterpenes				**10.25**	**4.25**
32	Caryophyllene oxide	1571	1992	0.13 ± 0.00	0.15 ± 0.06
33	Guaiol	1582	1983	0.70 ± 0.14	0.48 ± 0.09
34	τ-Muurolol	1635	2191	tr	tr
35	**β-Eudesmol**	**1640**	**2228**	**9.28 ± 1.49**	**3.46 ± 0.71**
36	α-Cadinol	1649	2208	0.14 ± 0.00	0.16 ± 0.04
Other compounds				**0.45**	**0.29**
37	Bornyl acetate	1286	1577	0.26 ± 0.06	0.29 ± 0.00
38	Methyl salicylate	1202	1777	0.19 ± 0.14	
Total volatile compounds [%]				99.47	98.19
Extraction time [min]				60	180
Yield [%]				0.050 ± 0.001	0.030 ± 0.006
Total oxygenated compounds [%]				13.56	12.24
Total non-oxygenated compounds [%]				85.91	85.95

[a]) Compounds in order of their elution from a *HP-5MS*™ column. [b]) Retention indices relative to C_5–C_{28} *n*-alkanes calculated on nonpolar *HP-5MS*™ capillary column. [c]) Retention indices relative to C_5–C_{28} *n*-alkanes calculated on polar *Carbowax*™ *PEG* capillary column. [d]) Percentage calculated by GC-FID on non polar *HP-5MS*™ capillary column.

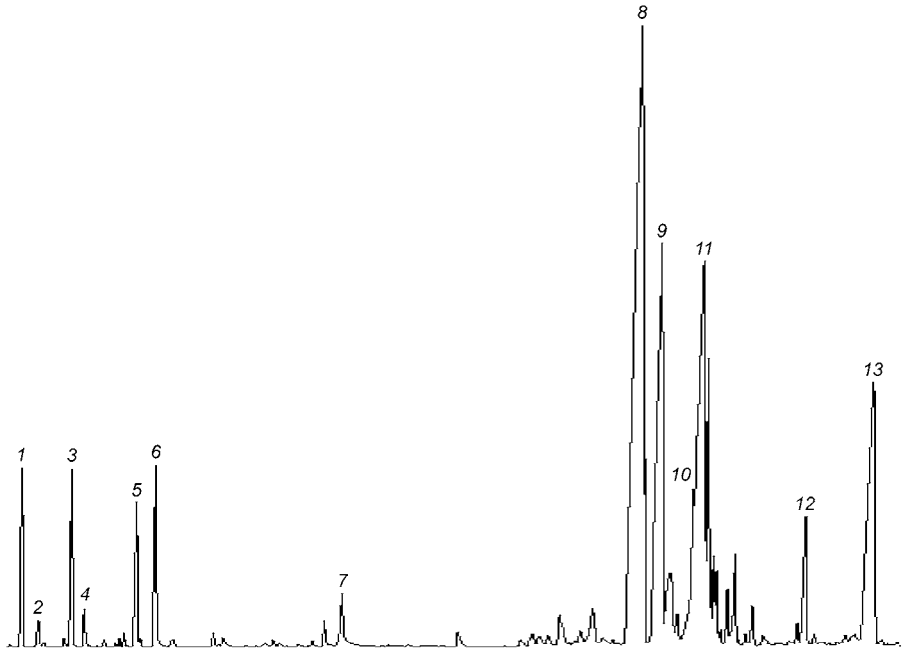

Fig. 1. *Chromatogram of the essential oil of* Juglans regia L. *leaves extracted by MAHD. 1*, α-Pinene; *2*, camphene; *3*, β-pinene; *4*, β-myrcene; *5*, eucalyptol; *6*, β-*trans*-ocimene; *7*, terpineol; *8*, caryophyllene; *9*, α-humulene; *10*, β-farnesene; *11*, germacrene D; *12*, γ-cadinene; *13*, β-eudesmol.

GC/MS and AMDIS software for data analysis; it has been reported that a high total yield of non-oxygenated compounds of 71.2% was found [19].

Conclusions. – Chemical compositions of essential oils obtained by MAHD and HD methods were qualitatively very similar. All constituents are common in both of them, except methyl salicylate which was detected only in MAHD extract in a low amount. Most of the compounds in essential oils of walnut leaves were terpenes. The sesquiterpenes were the dominant class in both MAHD and HD essential oils, with β-caryophyllene as the major constituent, followed by germacrene D and α-humulene.

Monoterpenes, including hydrocarbon and oxygenated, prevailed in HD extract with β-pinene and eucalyptol as major components, respectively. The higher proportion of sequiterpenes in the MAHD essential oils is a result of the efficiency of microwave flash heating in accelerating the flow extraction of relatively heavy volatiles.

In future, our aim is to determine which plant constituents are responsible for the hypoglycemic activity and, more precisely, are they the essential oil's or water-soluble constituents? The bioactive molecules from the total of the plant will then be considered in depth with respect to their health benefits.

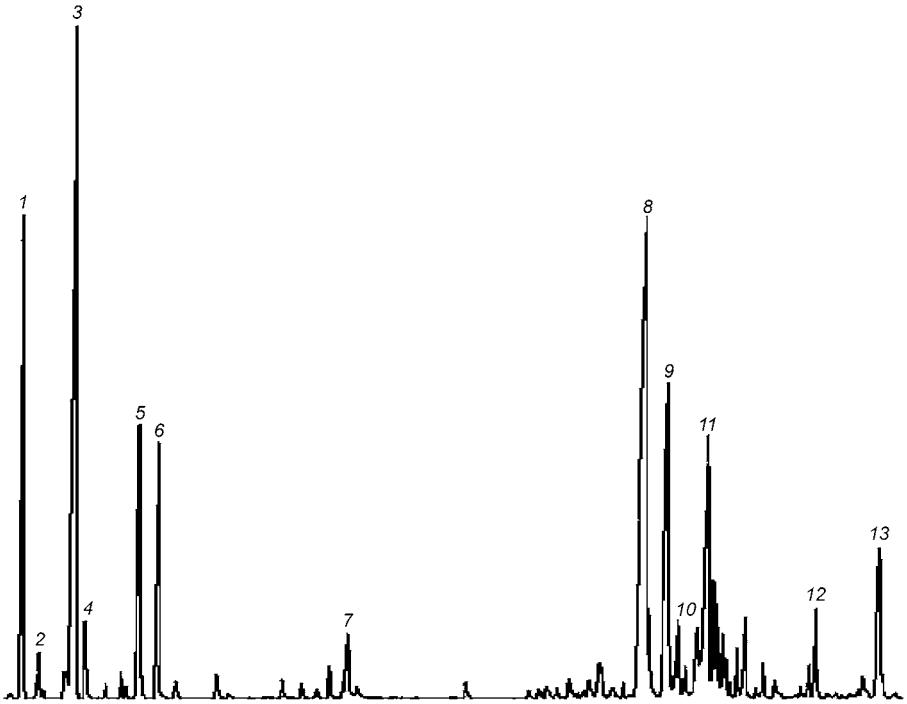

Fig. 2. *Chromatogram of the essential oil of* Juglans regia L. *leaves extracted by HD. 1*, α-Pinene; *2*, camphene; *3*, β-pinene; *4*, β-myrcene; *5*, eucalyptol; *6*, β-*trans*-ocimene; *7*, terpineol; *8*, caryophyllene; *9*, α-humulene; *10*, β-farnesene; *11*, germacrene D; *12*, γ-cadinene; *13*, β-eudesmol.

Experimental Part

Plant Material. Walnut leaves were collected in Algiers (El Achour), the north-east of Algeria on May 2011. Only fresh plant material was employed. The material was identified and authenticated by Dr. *N. Hanifi*, a plant taxonomist of the Department of Biology, University of Science and Technology Houari Boumediene, USTHB.

Hydrodistillation (HD). Fresh Walnut leaves (650 g) were submitted to hydrodistillation with a *Clevenger*-type apparatus [25], according to the European Pharmacopeia, and extracted with 2 l of H_2O for 3 h, until no more essential oil was obtained. The essential oil was collected, dried (Na_2SO_4), weighed, and stored in amber vial at 4° until subsequent analysis. The extraction was performed at least three times, and the mean value was reported.

Microwave-Assisted Hydrodistillation (MAHD). An adapted microwave oven, variable in 100-W increments (*M937, Samsung*, United Kingdom, 1000 W; 230 V/50 Hz) was used. Fresh walnut leaves (250 g) were placed in a 1-l flask containing 150 ml of H_2O. Contrary to our previous work, where the microwave extraction was performed without addition of H_2O (dry), in this work, we added H_2O to the extraction flask, because the humidity rate of fresh walnut leaves was only 25%. The flask was set up within the microwave oven cavity, and the *Clevenger* apparatus was used on the top, outside the oven, to collect the extracted essential oil. The microwave oven was operated at 800 W. The extraction time was fixed at 1 h when no more essential oil was obtained. The extracted essential oil was dried (Na_2SO_4), weighed, and stored in an amber vial at 4° until analysis. The extraction was performed at least three times, and the mean value was reported.

GC and GC/MS Analyses. A GC-FID system (*Agilent*, California, USA, 2000.) fitted with a fused-silica cap. column containing a non polar stationary phase *HP-5MS* (30 m × 0.25 mm × 0.25 μm film thickness) was used for GC analysis. The column temp. was 60° for 8 min then increased at 2°/min to 250°, and held at 250° for 15 min. Injection was performed at 250° in the splitless mode, injected volume for all samples, 0.2 μl, and flow rate, 0.3 ml/min; carrier gas N_2. The temp. of the flame ionization detector was 320°. The essential oils were analysed by GC/MS computerized system comprising a gas chromatograph coupled to a mass spectrometer (*Agilent*, Palo Alto, CA, USA, 2000.) using two fused silica capillary columns with different stationary phases. The nonpolar column was *HP-5MS*™ (30 m × 0.25 mm × 0.25-μm film thickness) and the polar one was a *Stabilwax* consisting of *Carbowax*™ *PEG* (60 m × 0.2 mm × 0.25-μm film thickness). GC/MS was performed using the following conditions: carrier gas, He; flow rate, 0.3 ml/min; splitless mode; injected volume for all samples, 0.2 μl; injection temp., 250°; the oven temp. program: 60° for 8 min, increased at a rate of 2°/min to 250°, and held at 250° for 15 min; the ionization mode, electronic impact at 70 eV. The homologous *n*-alkane series C_5 – C_{28}, injected in GC and GC/MS under the same conditions as the essential oils, were used to calculate the retention indices (*RI*s). Relatives amounts of individual components are based on peak areas obtained without FID response-factor correction. Three replicates were conducted for each sample. The average of these three values and the standard deviation were determined for each component identified. For the component identification, the MS fragmentation patterns were compared with those stored in the database *NIST 2006* and *Wiley 7*. The *RI*s of the essential-oil constituents compared with those of the published index data [26] confirmed the identification.

For both MAHD and HD techniques, experimental results on extraction yields and quant. oil compositions were subjected to statistical analysis performed using XLSTAT software on *Microsoft* Excel 2010 and represented as mean ± standard error (SE).

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