Phytochemical composition and antioxidant activity of Tunisian *Laurus nobilis*

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Abstract: We study the composition of Tunisian laurel leaves essential oil (EO), the fatty acid composition of laurel seed fixed oil and the total phenolics, flavonoids and tannins of laurel leaves methanolic extract. We also evaluated its free radical scavenging activity by the DPPH test. The predominant chemical class in Tunisian *Laurus nobilis* leaves EO was represented by oxygenated monoterpenes accounting for 64.29% of whole EO with the major compound was 1,8-cineole (46.8%). The predominant fatty acid was oleic acid (C18: 1) with an amount of 42.0%. Total polyphenols were present in the methanolic extract of Laurusnobilis leaves at an amount of 174.1 mg GAE.g-1dry matter. Total flavonoids and total tannins accounted respectively for 149.2mg CE.g-1 dry matter and 24.9mg CEg-1 dry matter. Furthermore, concerning free radical scavenging activity, *Laurus nobilis* leaves methanolic extract presented a significant IC_{50} (3mg/mL).

Keywords: *Laurus nobilis*, essential oil, seed oil, fatty acids, methanolic extract, total phenols, total flavonoids, total tannins, free radical scavenging activity.

INTRODUCTION

The Lauraceae family is composed of 32 genera with 2,000-2,500 species. *Laurus nobilis* L. Among this family, we can mention laurel (*Laurus nobilis* L.) which is originating from the southern Mediterranean region and is widely cultivated particularly in the Mediterranean area as an ornamental plant (Barla *et al*. 2007). It is an evergreen tree reputed as an aromatic plant. Bay essential oils are used in industry mainly in perfumery and cosmetic industry as perfumes and sopas ingredient. It constitutes also an ingredient in food industry since it is reputed for its antimicrobial and antioxidant properties which are interesting and useful for food preservation.

Due to its antimicrobial and insecticidal activities bay is used in the food industry as a food preservative. The essential oil (EO) is also used as a folk medicine, especially for the treatment of rheumatism and dermatitis. As a medicinal plant, Bay leaves and fruits extracts are used against rheumatism, skin rashes, and earaches and also for stimulating digestion and dieresis (Ozcan *et al*. 2010). Although several isolation and biological activity studies have been carried out to the leaves of L. nobilis, there has been very little work on its fruits.

Medicinally, Laurel leaves are renowned for their antimicrobial properties (Fiorini *et al*. 1997) and also for other several properties such as antirheumatic, antiseptic, diaphoretic, digestive and diuretic effects. Furthermore, bay laurel has been used as an antiseptic and a digestive. Laurus nobilis is also used as a fragrance component in the cosmetics and food industry (Simic *et al*. 2003).

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MATERIALS AND METHODS

Ethical approval is required from the Research Ethics Committee of Higher Institute of Biotechnology of Sidi Thabet, Universiy of Manouba, Tunisia.

Plant material

Laurus nobilis leaves were collected, in May-June 2014, in the region of Menzel-Bouzelfa, Cap-Bon (North of Tunisia) latitude 37.0726556° longitude 11.0554806°, altitude 12m. The collected material was identified by Dr. Marc El Beyrouthy from the Faculty of Agricultural and Food Sciences, University of Kaslik-Lebanon, and was air-dried at room temperature and then submitted to hydro distillation for 3h using a Clevenger apparatus. The oil was decanted and dried over anhydrous sodium sulphate.

Gas chromatography analysis (GC-FID)

EO composition was analyzed by gas chromatography (GC) using a Hewlett-Packard 6890 apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and an electronic pressure

control (EPC) injector. A HP-Innowax capillary column (polyethylene glycol: 30m x 0.25mm i.d, 0.25µm film thickness; Agilent Technologies, Hewlett-Packard, CA, USA) was used; the flow of the carrier gas (N2, U) was 1.6 mL/min and the split ratio 60: 1. Analyses were performed using the following temperature program: oven temps isotherm at 35°C for 10 min, from 35 to 205°C at the rate of 3°C/min, and isotherm at 205°C over 10 min. Injector and detector temperature were held, respectively, at 250 and 300°C.

Gas chromatography-mass spectrometry

GC-MS analysis of essential oil volatile components was carried out on a gas chromatograph HP 6890 (II) coupled to an HP 5972 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization (70 eV). A HP-5MS capillary column (30 m x 0.25mm, 0.25µm film thickness; Agilent Technologies, Hewlett- Packard, CA, USA) was used. The column temperature was programmed to rise from 35°C to 250°C at a rate of 5°C /min. The carrier gas was helium with a flow rate of 0.9mL/min; the split ratio was 60:1. Scan time and mass range were 1s and 40-300m/z, respectively. The injected volume was 1µL and the total run time was approximately 45 min. Identification of EO volatile compounds was based on the calculation of their retention indices (RI) relative to (C8-C22) n alkanes with those of authentic compounds available in our laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system and other published mass spectra 8.

Total lipid extraction

Triplicate sub-samples of 1 g were extracted using the modified method of Bligh and Dyer (1959). Thus, seed samples were fixed in boiling water for 5 min and then ground manually in a china mortar using a mixture of chloroform/methanol/hexane (3:2:1, v/v/v). After washing with water of fixation and decantation during 24h at 4°C, the organic phase containing total lipids was recovered and dried under a nitrogen stream. Finally, the residue was dissolved in a known volume of toluene–ethanol (4:1, v/v) at-20°C for further analyses.

Fatty acid transmethylation

Total fatty acids (TFA) of total lipids were transformed into their corresponding methyl esters as described by Cecchi *et al*. (1985). Transmethylation was made by the addition of 2mL of hexane, 0.5mL of 3% sodium methylate, a known amount of heptadecanoic acid methyl ester (C17:0) as the internal standard, 0.2mL of 1 N H2SO4 and 1.5mL of 10% sodium chloride. The hexanic phase that contains FA methyl esters (FAME) was recovered and its volume reduced in a stream of nitrogen, prior to analysis.

Preparation of methanolic extract

The air-dried and finely ground material (2.5g) was extracted by stirring with 25mL of absolute methanol at room temperature for 30 min. Extracts were kept for 24 h at 4°C and then filtered through Whatman filter paper before evaporation under vacuum to dryness.

Total phenolic content

Total phenolic content in the methanolic extract of *Laurus nobilis* leaves was performed according to the method of Chandler and Dodds. (1983) involving Folin-Ciocalteu reagent and gallic acid (both Sigma-Aldrich) as standard. Briefly, an aliquot of 0.1mL of extract solution containing 1mg of the extract was transferred to a volumetric flask, 46mL distilled water and 1mL Folin-Ciocalteu reagent was added and the flask was shaken thoroughly. After 3 min, 3mL of solution 2% Na_2CO_3 was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0-1000µg in 0.1mL) and a standard curve was obtained according to the equation: Absorbance = [0.0012* gallic acid (µg)] + 0.0033

Quantification of flavonoids

The total flavonoids were determined by a colorimetric method according to Dewanto *et al*. (2002). The absorbance reading was made at 510nm. The standard range is prepared with catechin at increasing concentrations ranging from 50 to 500 mg/L. The levels of flavonoids are expressed as mg catechin equivalent per gram of dry weight (mg/g DW).

Quantification of tannins

In the presence of concentrated sulfuric acid, condensed tannins depolymerize by reaction with vanillin and turn into red anthocyanidols that can be measurable by spectrophotometry (Sun *et al*. 1998). The absorbance was measured at 500nm. A calibration curve was prepared with catechin at concentrations ranging from 50 to 600 mg/l. The amount of condensed tannins was expressed as mg catechin equivalent per gram of dry weight (mg/g DW).

Antioxidant activity: DPPH radical scavenging activity

The antioxidant activity of the *Laurus nobilis* methanolic extract was evaluated by DPPH free radical-scavenging method. The DPPH free radical-scavenging activity measurements were carried out according to the procedure of Sanchez-Moreno *et al*. (1998) with some modifications. Briefly, 0.1mL of EO was added to 2.46 mL of 1,1-diphenyl-2- picrylhydrazyl radical (DPPH) (DPPH; 0.025 g/L in 50% ethanol) and mixed by vortex for 5 min. The absorbance of the samples was measured at 517nm every 1min for 60min using the spectrophotometer Genesis 2 (Milton Roy, USA). The antioxidant activity was expressed as the percentage of decline of the absorbance, relative to the control,

corresponding to the percentage of DPPH that was scavenged. The percentage of DPPH, which was scavenged (% DPPH) was calculated using:
% DPPHsc = (A_{cont} _ A_{samp}) x 100 /A_{cont}

where A_{cont} is the absorbance of the control and A_{samp} the absorbance of the sample.

Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against essential oils concentrations. Trolox was used as positive control.

RESULTS

Compositions of the EO

The qualitative and quantitative compositions of the EO of the leaves of *Laurus nobilis* L. are reported in fig. 1 and table 1. According to this table, 31 compounds accounting for 92.88% of the whole oil were identified. Among these compounds 1,8-cineole was the predominant compound with an amount of 46.8%. The major class was represented by oxygenated monoterpenes accounting for 64.29%. They were followed by hydrocarbon monoterpenes and sesquiterpenes (22.98% and 5.61% respectively).

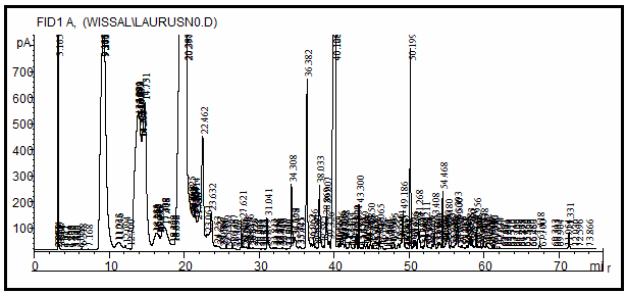

Fig. 1: Chromatogram of *Laurus nobilis* leaves essential oil

Fixed oil fatty acid composition

According to fig. 2 and table 2, *Laurus nobilis* seeds were characterized by the predominance of the oleic acid (C18: 1) with a rate of 42.0%. It was followed by linoleic acid (C18: 2) which is an essential AG. This FA accounted for 27.5%. Palmitic acid (C16: 0) was present at a considerable rate (11.9%), whereas stearic acid has a percentage of 7%. Palmitoleic (C16: 1) and linolenic acids (C18: 3) were detected at respective rates of 0.3 and 2.7% whereas lauric acid (C 12: 0) level was of 8.6%.

Methanolic extract composition and free radical scavenging activity

Table 3 illustrated the richness of the methanolic extract of *Laurus nobilis* of in total polyphenols (174.1mg/g DW). The Laurel methanolic extract was able to reduce the stable free radical DPPH to the yellow- colored 1,1-diphenyl-2-picrylhydrazyl with an IC_{50} value of 3 mg/mL.

DISCUSSION

Sangun *et al*. (2007) reported that *Laurus nobilis* L. leaves EO was characterized by high contents of 1.8-Cineole, linalool and α-terpinyl whereas fruits one was characterized by high contents of α-pinene, α-phellandrene, sabinen, 1.8-cineole and trans-β-osimen. In this contexte, Bisio *et al*. (1999) claimed that climatic and edaphic conditions have a strong influence on the EO composition.

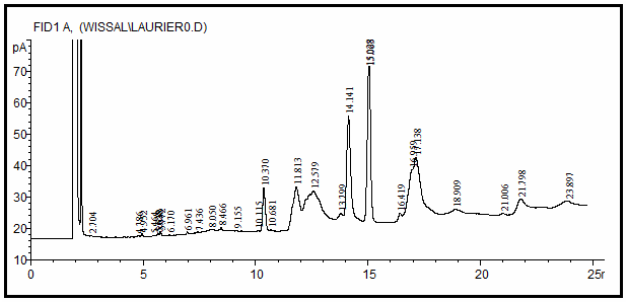

Fig. 2: Chromatogram of *laurus nobilis* seed fatty acids

According to the results of many previous studies, 1,8-cineole is the main component of *Laurus nobilis* leaves EO with percentages ranging from 31.4% to 56% (Pino *et al*. 1993). Other compounds have been reported in significant quantities, including linalool, trans- 6 sabinene hydrate, α-Terpinyl-acetate, methyl eugenol, sabinene and eugenol (Pino *et al*. 1993; Flamini *et al*. 2007). Eugenol and methyl eugenol are responsible for the spicy note of bay leaves and are very important factors in determining the sensory quality of bay leaves (Pino *et al*. 1993).

Previous studies reported differences between the essential oils of stems, leaves, buds and flowers (Bouzouita *et al*. 2001). The oil of the different laurel parts contains the same components whereas important quantitative differences were noted for the main volatiles. As example, the predominant compounds of leaves, buds and flowers such as 1,8-cineole, α-terpinyl acetate, methyl eugenol, eugenol and linalool, which are the basic components of the essential oil of, have been reported in negligible amounts in the fruits. On the contrary, (E)-β-ocimene which is the main volatile in the fruit is absent in stems, leaves, buds and flowers.

Moreover, Marzouki *et al*. (2009) reported that the yield and the composition of *Laurus nobilis* EO is influenced by climatic conditions. In addition, it is interesting to note that *laurus nobilis* EO composition depends on the extraction procedure. For example, the rate of sesquiterpenes is higher in EO extracted by supercritical

Table 1: Chemical composition of *Laurus nobilis* leaves essential oil

Volatile compound	RI[a]	RI[b]	Percentage (% of whole EO)
α-pinene	939	1030	6.1±1.3
β-pinene	980	1118	2.5±0.8
γ-terpinene	1062	1255	4.4±1.3
p-cymene	1026	1280	1.2±0.3
terpinolene	1090	1290	0.1±0.01
Δ-germacrene	1484	1726	0.1±0.02
sabinene	967	1132	7.8±1.6
α-thujene	930	1035	0.32±0.08
β-elemene	1391	1600	0.46±0.1
% hydrocarbon monoterpenes			22.98
1,8-cineole	1035	1214	46.8±3.9
cis-linalool oxide	1062	1450	0.2±0.03
camphene	954	1076	0.5±0.02
linalool	1098	1544	0.8±0.04
linalyl acetate	1257	1553	0.1±0.02
bornyl acetate	1286	1591	0.2±0.03
terpinene-4-ol	1178	1611	0.04±0.002
α-terpenyl acetate	1333	1681	0.4±0.01
myrtenyl acetatee	1239	1704	0.1±0.002
α-terpineol	1191	1706	10±2.5
borneol	1168	1719	2.4±0.9
neryl acetate	1341	1725	0.05±0.001
geranyl acetate	1359	1748	0.1±0.02
geraniol	1231	1844	0.1±0.01
eugenol	1353	2186	0.4±0.03
methyl eugenol	1404		1.8±0.8
thymol	1290	2198	0.3±0.02
% oxygenated monoterpenes			64.29
β-caryophyllene	1419	1618	2.9±0.5
α-eudesmol	1641	2216	1.62±0.2
β-eusesmol	1638	2225	1.09±0.3
% sesquiterpenes			5.61
% Total			92.88

RI[a], RI[b]: retention indices calculated using respectively an apolar column (HP-5) and polar column (HPInnowax); Means of three replicates ± S.E. Data are reported as means ± SD of three measurements. P <0.05 compared to control (C; ANOVA followed by the Duncan -test).

CO_2 than those extracted by hydro distillation (Marzouki *et al*., 2008).

Concerning FAs, laurel seed oil has a balanced composition. It is rich in C18: 1 which is renowned for its dietary and preventive role against the cardiovascular diseases. According to Marzouki *et al*. (2008), *Laurus nobilis* seed oil was characterized by the prevalence of lauric acid accounting for 27.7% of total FA (TFA). They also noted the presence of capric, oleic and linoleic acids at significant respective levels of 17.1, 27.5 and 21.5%. Furthermore, Tanrıverdi *et al*. (1989) reported that fixed oil of *Laurus nobilis* seeds contain 37.97% of SFA and 60.4% 61.37% of UFA. They reported that these percentages varied with the used solvent in oil extraction.

This value is very higher to that obtained for the ethanolic extract of Mexico leaves *Laurus nobilis L* by Muñiz-Márquez *et al*. (2014) with a mean concentration of 10.23 mg/g of plant. An important proportion of these polyphenols is represented by flavonoids (149.2mg/g DW). Several flavonoids and derivatives were detected in laurel extracts as flavonoids, O-glycosides, C-glycoside, catechin, and cinnamtannin (DallAcqua *et al*. 2009). In addition, other compounds were isolated such as lactones, sesquiterpenoids, alkaloids of isoquinolines and vitamin E (Wettasinghe and Shahidi. 2000).

Scavenging stable DPPH free radicals test is widely used for evaluating the antioxidant potential of extracts. It is a simple test since it doesn't require a long time analysis.

Thanks to the scavenging effect of the extract antioxidants, the color changes from purple to yellow thus cause a proportional decrease of absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antioxidants through a donation of hydrogen to form the stable DPPH-H molecule (Von Gadow *et al*. 1997).

Table 2: Fatty acid composition of *Laurus nobilis* fixed oil

Fatty acid	Percentage (% of TFA)
Lauric acid (C12 : 0)	8.6±1.3
Palmitic acid (C16:0)	11.9±1.8
% SFA	20.5
Palmitoleic acid (C16 : 1)	0.3±0.01
Oleic acid (C18 : 1)	42.0±4.7
Linoleic acid (C18 : 2)	27.5±2.3
Linolenic acid (C18 : 3)	2.7±0.7
% UFA	72.5

TFA: Total fatty acids; SFA: Saturated fatty acids; UFA: Unsaturated fatty acids

Table 3: Composition of *Laurus nobilis* leaves methanolic extract

Total polyphenols (mg EAG.g^{-1} DW)	174.1± 11.6
Total flavonoids (mg EC.g^{-1} DW)	149.2±8.3
Total tannins (mg EC.g^{-1} DW)	24.9±3.9

It have been reported that antioxidant capacity of plant extracts is highly correlated to the amount of 8 flavonoids. It is important to note that the extracts flavonoid content is depend on the abundance of the secretory structures in relation to plant tissues. Wang *et al*. (1998) reported that laurel leaves had an important quenching potential which explains their scavenging activity against DPPH radicals. In addition to DPPH test, other extraction methods have been used to assess the antioxidant activity of laurel phenolic (rancimat method, β-arotene/linoleic acid assay,...). According to Bozan and Karakaplan (2007), total phenol yield and activities depend on the extraction method and the solvent.

CONCLUSION

Essential oil of *Laurus nobilis* leaves from Tunisia belongs to a chemotype which is 1,8-cineole and is characterized by the predominance of oxygenated monoterpenes which have appreciable attributes highly interesting in perfumery. The FA composition of *Laurus nobilis* seeds showed that the predominant FA was oleic acid (C18: 1) with a rate of 42.0%. Total polyphenols were present in the methanolic extract of leaves and a significant free radical scavenging activity with IC_{50} (3mg/mL). As a continuation of this word and to confirm the previous findings, a further investigation of *in vivo* antioxidant activities following fractionation and purification should be explored.

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