DETERMINATION OF THE CHEMICAL AND GENETIC DIFFERENCES OF *LAURUS* COLLECTED FROM THREE DIFFERENT GEOGRAPHIC AND CLIMATIC AREAS IN LEBANON

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Abstract

Laurus (Lauraceae) is growing in many warm regions of the world, particularly in southern Europe and around the shores of the Mediterranean Sea, including Lebanon. The aim of the present study was to determine and compare leaves, fruits, essential oil and the fatty acids of *Laurus* collected from three different geographic and climatic areas (Ras-Alnaqoura, Deir-El-Qamar and Zahle), and determine the genetic identity and the Genetic distance between these samples. A phytochemical test was done on different extracts prepared from dried leaves, using solvents of different polarities to determine Alkaloids, Flavonoids, Tannins, Anthraquinones and Coumarins. Moreover additional analytical experiments were applied on the samples to determine the *Laurus* composition of trace elements and showed that it was rich in K, Ca and Si. The highest percentages have been found in Deir-El-Qamar. The determination of fatty acids showed a high concentration in stearic, oleic, vaccenic and palmitic acids. we studied the genetic differences between the three samples of *Laurus* by ISSR (Inter Simple Sequence Repeat) technique, according to this study *Laurus* was classified into two major groups and two subgroups, the genetic distance between Deir-El-Qamar sample and other samples was about 70.80%, this result indicates a significant genetic diversity between the three regions.

Keywords: *Laurus*, Volatile oil, Fatty acid, trace element, ISSR.

Introduction

Laurus is also known as sweet bay, bay laurel, Grecian laurel, true bay, and bay tree, is either an evergreen shrub or small tree, belongs to the family Lauraceae which comprises numerous aromatic and medicinal plant (Hogg et al., 1974). Laurel is usually growing to a height of from 20 to 30 feet. It is cultivated in many warm regions of the world, particularly in southern Europe and around the shores of the Mediterranean Sea (Lewis, 1984) which includes Lebanon. It has been in this country since ancient times. Lebanese people use the fruits in manufacturing soap as fruits contain fixed oils (Bozan et al., 2007). But they do not care about leaves or fruits volatile oil, which has a lot of medical effective. Bay leaves essential oil is one of main products from bay trees that are used in food, spice, flavoring and cosmetic industries (Sari et al., 2006). *Laurus* essential oil is used for the preparation of hair lotion due to its antidandruff activity and for the external treatment of psoriasis. Its fruits are generally utilized for the production of perfumed soaps and candle manufacture because of their fatty acid content (Hafizoğlu et al., 1993). It also used for relieving hemorrhoid and rheumatic pains and as a diuretic (Kivçak et al., 2002), It has antibacterial and antifungal properties (Ertürk, 2006). Different studies made on the essential oil show influence of the

area of culture, of variety and harvest season on the chemical composition (Rohloff et al., 2005; Flamini et al., 2007). The volatile oil content of bay leaves ranges from 1 to 3% on fresh weight basis. The main constituent of the essential oil includes 1-8 cineole (45-50%), α-pinene, sabinene, linalool, eugenol, eugenol acetate, methyleugenol, ternineol acetate, and phellandrene, also Fruits contain many fatty acids as lauric 7.5%, palmitic 25%, oleic 39.6%, and linoleic 22% (NURBAŞ et al., 2005). Also *Laurus* contains a very important sesquiterpene lactones called Costunolide (Ferrari et al., 2005), which is anticancer compounds (Rasul et al., 2012). Given the scarcity of local studies on Laurel widespread in Lebanon, has not been reported to date. The aim of the current study was to determine and compare the essential oil and the fatty acids of *Laurus* leaves and fruits which collected from three different geographic and climatic situations, in the end the genetic analysis were done to show the relationship between *Laurus* samples.

Materials and methods

Plant material

The samples of *Laurus* leaves and fruits were collected from three Areas in Lebanon, the first from the coast (Ras-Alnaqoura), the second from the mountain (Deir-El-Qamar), and last one from the Plains (Zahle) in year 2013. The Samples were air-dried at room temperature in the shade for some weeks. They had a final moisture content of 10.0 %. Before using them, the dried samples were grinded in a blender. At the end of the milling process, the particle sizes were in the range of 0.8–0.9 mm.

Phytochemical Tests

Phytochemical tests are performed on different extracts prepared from the dried leaves and ground, using many solvents of different polarities. They are generally simple, quick to implement, performed mostly in test tubes. The detection method of the different families of chemical compounds co-existing is a precipitation reaction or staining reagents. These reactions result in the appearance of turbidity, flocculation or a colour change which may, depending on the intensity of the result, the concentration of certain constituents.

Supplementary analysis (Extraction and Determination of trace elements from the leafs of Laurus)

The supplementary analysis of *Laurus* leaf is done in Monocrystal Laboratory of Ukraine. 2g of Laurus leaf powder was placed in a capillary tube of fluorized polymers, in order to disperse it under pressure and microwaves. Then 5ml of HNO3 (70%), the capillary was then closed firmly and put in a steam room for 20 min, under a pressure that do not exceeds 120 psi (pounds per square inch). After cooling and filtering, the substance was put in a 50ml tube. Water was added afterwards to complete the volume (50ml) (solution 1). From this solution 1ml was take and put into a 100ml tube and completed with water up to 100ml, obtaining the solution 2.In order to determine macro elements percentages a spectrometer od the type of Thermo Jarred Ash atomic absorption spectrometer was used:

Solution 1 was used to determine the percentage of Zn, Fe, Cu, Ni, Mn, Al and P. Solution 2 was used to determine the percentage of Ca, Mg, K and Na.Conditions: liquid flow speed: 1,58ml/1min 2 sec.

The flow speed of added Argon (Ar): 1L/min.

The flow speed of initial Argon (Ar): 14L/min.

Volatile oil extraction

The volatile oils of *Laurus* leaves or fruits were obtained by the process of hydro distillation in the Clevenger apparatus. (100g) of *Laurus* (leafs or fruits), were placed in a

flask (2.5L) and hydro distilled for 2.5h. The oil samples were dried over anhydrous sodium sulphate and stored at 4°C in the dark.

Fatty oil extraction

Fruits oil is extracted by using petroleum ether in ultrasonic bath 15 min up for twice. Then it filtered and evaporated by using a rotary evaporator at 50°C. The fatty oil Dried and stored in the dark at room temperature until use.

Volatile oil analysis

50 microns of volatile oil sample was Taken and extended to 250 microns by hexane, it mixed then 1 micron from it was injected in Gas chromatography (GC) for analyse. Gas chromatography (GC) analysis was carried out on a Shimadzu GC 2010 with FID detector and a DB23 capillary column (60 m×0.25 mm; film thickness 0.25 μm). The carrier gas was helium with a flow rate of 0.72 ml/min., the oven temperature for first 4 min. was kept at 60 °C and then increased at a rate of 4 °C/min. until reached to the temperature of 250 °C and keep on for 5 mints, injector and detector temperature were set at 250 °C.

Fatty oil analysis

The fatty acid components of lipids are converted to the simplest convenient volatile derivative, usually methyl esters. So 50 microns of fatty oil sample was Taken and extended to 2 microns by hexane, it mixed then the free fatty acids was esterification by using methanol sodium hydrate solution (29g NaOH in 250 ml methanol). The solution was centrifuged for 2mins at 4000 rpm. The upper layer hexane was taken and washed with water, and then the upper layer (hexane) was taken again and dried on anhydrous sodium sulphate. 1 microns of hexane containing fatty acids was injected in GC. Gas chromatography (GC) analysis was carried out on a Shimadzu GC 17 AFW with FID detector and a DB23 capillary column (60 m×0.25 mm; film thickness 0.25 μm). The carrier gas was helium with a flow rate of 0.81 ml/min., the oven temperature for first 4 min. was kept at 75 °C and then increased at a rate of 3 °C/min. until reached to the temperature of 180 °C and keep on for 10 mints, injector and detector temperature were set at 260 °C.

Determination of fatty acids from *Laurus*

The following test was performed in the institute of Monocrystals, Kharkov, Ukraine. Approximately 1.2 g of dry the leaves previously grounded into particles of 0.5 mm was extracted with methanol-chloroform in portions of 10 ml three times for 3 hours. The mix was filtered through the paper filter into a 10 ml flask. 1g of anhydrous sodium sulphate was added to the extract obtained, which was evaporated at 60°C in the nitrogen stream until dryness (a residue of 40mg). 1 ml of diethyl ester, 5 ml of methanol and 0.2 ml of acetyl chloride were added to the residue and the flask was filled with nitrogen, and then it was boiled with the reflux condenser on the glycerine bath for 45 min at 70°C. The solution obtained was evaporated in the nitrogen stream to a volume of 0.3 ml. Then 2 ml of cyclohexane were added and stirred for 1 min. After the complete stratification of the layers, the upper cyclohexane layer was used as a test sample. It was filtered through a filter with 0.2 g of sodium sulphate. The resulting solution was subjected to analysis by gas chromatography Shimadzu GC-14B, FID chromatography under the following conditions: capillary column (60m x 0.32mm HP-23; 0.25μm), the column temperature was held at 175°C for 2 min, and then raised to 225°C with a rate of 3°C / min, injector and detector temperatures were 240°C and 250°C respectively, the carrier gas flow rate (nitrogen) was 1.0 ml/min, split ratio was 1:60. The content of each fatty acid was calculated by the internal regulation method (Kanaan et al., 2005).

The genetic study

This study is done to identify differences among Laurus collected from Lebanon (Ras-Alnaqoura, Deir-El-Qamar and Zahle) by ISSR technique (inter simple sequence repeat) which considered as a powerful technique to determine genetic relations among individuals. A total of 12 ISSR primers were used and analysed according to the polymorphism. Genomic DNA was extracted from leaves (Figure 3, A) by using the sodium dodecyl sulphate protein precipitation method described by (Milligan, 1998). The purified DNA was stored at 4°C. The DNA with the primers was generated thousands to millions of copies of a particular DNA sequence by using PCR (Polymerase chain reaction) then the samples were separated on 6% polyacrylamide gels. The DNA bands were photographed by UV source (Figure 3, B). The presence or absence of polymorphic ISSR marker bands was manually scored (1 present, 0 absent), and the statistic program PopGen 32was using for droning the genetic tree.

Results and discussion

Phytochemical Tests (table1)

The phytochemical tests showed the chemical groups that exist in Laurus leaves (Table 1), as shown in the table, *Laurus* leaves are rich in Alkaloids, Flavonoids, Tannins, Anthraquinones, Saponins, Sterols steroids and Reducing carbohydrates, while *Laurus* has no Coumarins or starch.

Table 1: The Phytochemical groups in the leaves of *Laurus*

Region Chemical group	The Sea Coast (Ras-Alnaqoura)	The Mountain (Deir-El-Qamar)	The Plains (Zahle)
Alkaloids	+	++	++
Flavonoids	+	++	+
Tannins	+	++	+
Anthraquinones	+	+	+
Coumarins	-	-	-
Saponins	++	+	+
Sterols steroids	+	+	+
Starch	-	-	-
Reducing carbohydrates	++	++	+

Supplementary analysis (Extraction and Determination of trace elements from the leafs of Laurus)

The trace elements were detected in Laurus samples and their concentration were determined and shown in (figure1). Fifteen trace elements were found in *Laurus* analysed. *Laurus* appeared rich in K, Ca, Mg, Si, and Na.

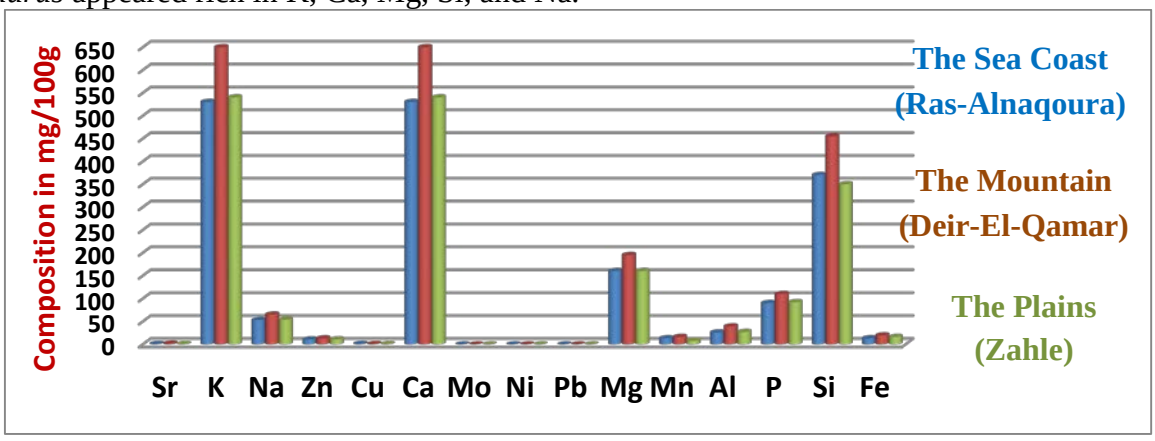

Figure 1 Comparison of trace elements in Laurus leaves

Volatile oil extraction

As seen in the (table 2), *Laurus* which grow in the Mountain gives the high rate of leaves volatile oil while, *Laurus* which grow in the plains gave the high rate of fruit oil:

Table 2: The Volatile Oil amount of Laurus leaves and fruits

Region	The Sea Coast (Ras-Alnaqoura)		The Mountain (Deir-El-Qamar)		The Plains (Zahle)	
	Leaves	Fruits	Leaves	Fruits	Leaves	Fruits
V.O amount mL/100g	1.43	0.63	2.51	0.75	0.77	0.95

Fatty oil extraction

As seen in the (table 3), *Laurus* which grow in plains gives the high rate of fruits volatile oil:

Table 3: The Fatty Oils amount of Laurus fruits

Region	The Sea Coast (Ras-Alnaqoura)	The Mountain (Deir-El-Qamar)	The Plains (Zahle)
F.O mL/100g	22.53	17.52	28.14

Volatile oil analysis

As seen in (Table 4), 18 different compounds were determined. Although there was no marked difference in the composition of leaves or fruits oils:

Table 4: the essential oil composition of Laurus leaves and fruits

Region Volatile oil	The Sea Coast (Ras-Alnaqoura)		The Mountain (Deir-El-Qamar)		The Plains (Zahle)	
	Leaves	Fruits	Leaves	Fruits	Leaves	Fruits
α-Pinene	3.00	7.69	6.03	11.69	2.14	17.96
Camphene	0.17	1.08	0.14	1.31	0.16	2.61
β-pinene	2.88	3.91	4.17	6.91	2.51	9.51
Sabinen	9.74	2.93	6.10	4.49	4.06	3.47
Myrcene	0.43	0.90	0.20	1.01	0.12	1.50
α-Phellandrene	0.12	8.29	0.14	14.55	0.06	17.07
Limonen	2.33	1.43	2.50	2.42	1.18	2.89
γ-terpinene	0.82	2.42	0.78	3.41	0.44	4.53
Trans β ocymen	0.73	11.82	1.08	0.57	0.51	3.92
1.8 Cineol	57.05	48.01	58.45	31.78	65.99	17.64
P-Cymen	1.06	0.48	2.18	1.57	1.46	0.98
Linalyl acetate	0	0.24	0	0.45	0	0
Linalool	0.75	0.40	0.59	0.24	0.51	0.24
Bornyl acetate	0	2.07	0	2.87	0.02	3.40
Terpinen-4-Ol	2.48	0.35	3.63	1.26	3.77	0.77
β-Caryophllen	0.41	1.56	0.17	2.36	0.53	2.35
α-Terpineol	2.90	1.29	3.64	2.07	3.26	0.87
α-humulene	11.02	2.39	6.80	4.48	7.92	4.38

Fatty oil analysis

As seen in the (Table 5), many different compounds were determined.

Table 5: the fatty acids composition of Laurus leaves and fruits

Region Fatty acid		The Sea Coast (Ras-Alnaqoura)		The Mountain (Deir-El-Qamar)		The Plains (Zahle)	
		Leaves	Fruits	Leaves	Fruits	Leaves	Fruits
C8:0	Caprylic acid	-----	-----	2.284	-----	-----	-----
C10:0	Capric acid	3.190	0.146	2.333	0.321	-----	0.175
C10:1n3	----------	1.997	-----	2.030	-----	1.731	-----
C10:1n6	----------	-----	-----	1.599	-----	1.533	-----
C10:2n	----------	-----	-----	2.149	-----	-----	-----
C11:0	Heptacosanoic acid	-----	-----	1.250	-----	-----	-----
C12:0	Lauric acid	-----	9.109	10.905	21.432	2.124	15.617
C12:1n6	----------	-----	-----	2.346	-----	-----	-----
C13:0	Nonacosanoic acid	-----	-----	0.880	-----	1.710	-----
C13:1n	----------	2.514	-----	1.175	-----	-----	-----
C14:0	Myristic acid	2.983	0.391	0.796	0.876	1.693	0.813

C14:1n6	----------	-----	-----	0.849	-----	3.599	-----
C14:1n9	Myristoleic acid	-----	-----	1.993	-----	-----	-----
C15:0	Pentadecanoic acid	-----	-----	0.884	-----	-----	-----
C15:1	Pentadecenoic acid	3.021	-----	1.651	-----	1.865	-----
C16:0	Palmitic acid	24.588	19.479	14.097	15.483	15.01	20.512
C16:1n6	----------	-----	-----	1.042	-----	-----	-----
C16:1n9	Palmitoleic acid	-----	0.877	1.019	0.402	-----	0.548
C16:2	Hexadecadienoic	-----	-----	0.819	-----	-----	-----
C16:3	----------	2.114	-----	1.824	-----	2.252	-----
C17:0	Margaric acid	-----	-----	1.772	-----	-----	-----
C18:0	Stearic acid	1.288	0.905	1.092	0.422	4.640	0.873
C18:1n9	Oleic acid	7.123	43.098	4.786	34.373	4.157	36.388
C18:2n9,12	Linoleic acid	17.746	24.492	7.790	25.062	9.786	20.141
C18:3n9,12,15	Linolenic acid	30.716	1.094	23.522	0.908	21.362	1.081
C20:0	Arachidic acid	0.322	0.114	0.747	0.327	2.380	0.461
C20:1	Arachidonic acid	1.402	0.295	2.242	0.394	24.426	3.391
C20:2	----------	0.996	-----	6.124	-----	1.732	-----

The genetic study

In this study genetic similarities and differences in Laurus were determined for the first time in Lebanon. According to this study *Laurus* was classified into two major groups and tow subgroups ;(figure 2)

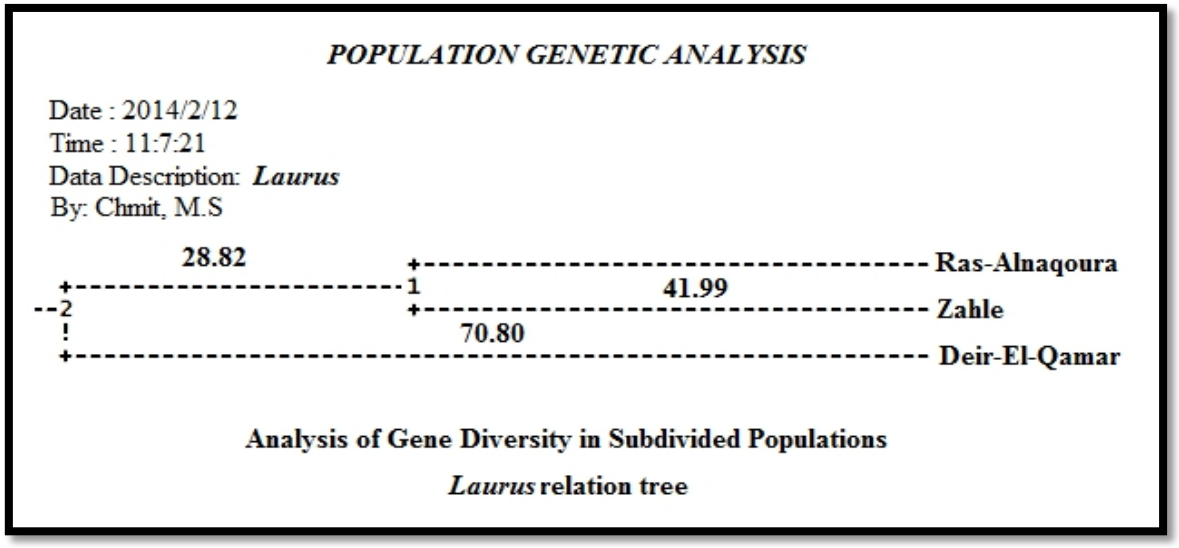

Figure 2 ISSR Dendogram of genetic distance showing grouping of the three populations of *Laurus*.

The statistic study by PopGen 32 program shown the genetic identity and distance between Laurus samples, it is clear in the table below:

Table 6: Comparison of Genetic identity (above diagonal) and Genetic distance (below diagonal) for the three populations

POP ID	Ras-Alnaqoura	Zahle	Deir-El-Qamar
Ras-Alnaqoura	****	0.4318	0.1364
Zahle	0.8398	****	0.4318
Deir-El-Qamar	1.9924	0.8398	****

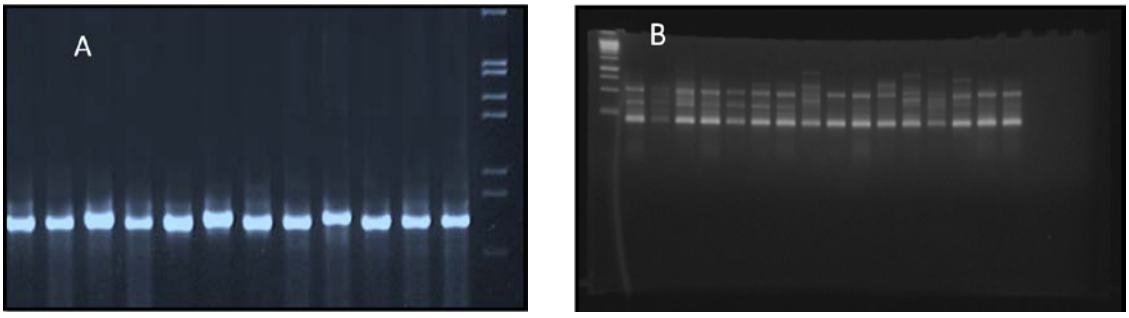

Figure 3 A: DNA extraction B: DNA bands

Conclusion

The plant Laurus has been selected from Mount Lebanon (Deir-El-Qamar) due to its high content in essential elements when compared to other regions. Our study has shown that the plant Laurus contains high amounts of trace elements, such as K, Ca, Si, and fatty acids. The same thing applies to tannins flavonoids. In conclusion and at large, the study has shown that the active substances present in this plant such as essential oil and fatty acids may be play a non-negligible role in medicine, pharmacy, food and industry.

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