IJPSR (2019), Volume 10, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 14 November 2018; received in revised form, 20 February 2019; accepted, 08 March 2019; published 01 August 2019

PHYTOCHEMICAL SCREENING, POLYPHENOLS CONTENT AND A NOVEL SOURCE OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF ESSENTIAL OIL OF *LAURUS NOBILIS* FROM MOROCCO

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Keywords:

Laurus nobilis, Antibacterial, Nosocomial infections, DPPH, Antioxidant

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ABSTRACT: Bacterial resistance and oxidative stress are an important etiology of chronic diseases. This is the reason why new alternatives were established to overcome the incidence of some pathologies. Thus, the present study was aimed to evaluate the antibacterial activity of the extracted oil against bacteria that causes nosocomial infections in neonatal intensive care by using disc diffusion method. The antioxidant activity was evaluated using various methods (1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, reducing power assay, total antioxidant activity) and the content of total phenolic and flavonoids were determined. In the essential oil, the twenty-eight compound was identified by GC/MS analysis. The major components were: 1,8-cineol (26.76%), 1-α-pinene (8.63%), linalool (7.78%). The oil strongly reduced the DPPH radical (IC_{50} = 4. 4 µg/ml) and reducing of molybdate (0.53 ± 0.0027 mg Eq ascorbic acid/µg EO. The oil showed strong antibacterial activity against nosocomial bacteria tested. This study confirms the essential oil of *laurus nobilis*. L processes antibacterial and antioxidant properties *in-vitro*.

INTRODUCTION: Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH °) and singlet oxygen (O_2) are highly toxic molecules. The excess of these molecules causes cellular damage, including the denaturation of DNA and proteins, lipid peroxidation, inducing chronic diseases such as cancer, diabetes, cardiovascular, inflammatory and neurodegenerative diseases [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(8).3770-76

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(8).3770-76

Currently, there is global interest in finding new antioxidants from natural sources to minimize oxidation of living cells and prevent oxidative deterioration of foods. Antioxidants are classified as primary or secondary antioxidants depending on their site of action. Primary antioxidants can give a hydrogen atom to a lipid radical thus giving a more stable radical. Secondary antioxidants react with the initiating radicals or reduce the oxygen level without formation of reactive species [2]. Antioxidants can act by several mechanisms; however, their activity must be detected and evaluated using various assays. *In-vitro*, antioxidant assays in biological systems and foods can be divided into two categories: those that measure the free radical scavenging capacity and those that evaluate lipid peroxidation [3].

Another problem affecting public health is the emergence of antibiotic resistance, following their massive use [4]. This leads to the large demand of consumer for new antibiotics against pathogens and has prompted scientists to use herbal medicine with antibacterial properties.

Plants are very rich in secondary metabolites including phenols and flavonoids that have beneficial effects on human health [5, 6]. These natural secondary metabolites can reduce free radicals and prevent chronic diseases [7].

Essential oils contain volatile aroma compound from aromatic plants. They are complex mixtures of compounds belonging to diverse chemical families (esters, phenolic compounds, alcohols, terpenes aldehydes). The antioxidant ability of several essential oils extracted from aromatic plants, as well as some factors that can influence the chemical composition of the oils and consequently the biological activity [3].

The genus Laurus is a shrub or tree of the family *Lauraceae* evergreen of Mediterranean origin, but widely grown in Europe and USA, the noble laurel enjoys an important place both in the medication and culinary field as a condiment these leaves are commonly used as an aromatic spice for soups, fish and meats [8]. The biological activities and phytochemistry of *Laurus nobilis* have previously been extensively investigated. Several studies have been done on the genus *Laurus nobilis*, which have shown its richness in different secondary metabolites [8, 9, 10]. Several types of research have been made on the chemical composition of the essential oil of laurel. In all cases, 1-8-cineole is the majority compound with percentages varying between 31% and 55%. Linalool, α-terpinyl acetate, methyl eugenol, sabinene, and eugenol were present in appreciable quantities [10, 11]. Many studies have been conducted on the essential oil of laurus nobilis; these works have confirmed a huge potential antibacterial, antioxidant and antifungal [12, 13, 14, 9, 15] and also it is used for food preservation [16] and as a bioinsecticide [17]. Traditionally, the leaves of *laurus nobilis* are used as the aroma, and they are widely used in traditional medicine to treat earache, rheumatism, and to promote perspiration, the infusion of its fruit has diuretic and carminative effects.

Pharmacological research has shown that bay leaf can be used in the treatment of diabetes, cardiovascular diseases and improves the lipid profile in type 2 diabetes [18]. The aqueous extract of the laurel is a higher inhibitor of tumor cell lines (HeLa, MCF7, NCI-H460, and HCT15) [19], and a gastroprotective effect [20].

This work is part of research on natural antibiotics and antioxidants. To our knowledge, this is the first report on their antibacterial effect against nosocomial bacteria. Thus, the objectives of this study are to evaluate for the first time the antibacterial effect against resistant bacteria. Furthermore, total antioxidant activity was quantified by the ascorbic acid equivalents; free radical scavenging activity was also determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Spectroscopic analysis was determined of the total phenol, and total flavonoid content from essential oils of *Laurus nobilis* leaves. Since this plant is used for treat various therapeutic purposes by indigenous people, we have chosen this plant for our investigation.

MATERIALS AND METHODS:

Plant Material and Isolation of the Essential Oil: Leaves of *Laurus nobilis* L. is collected during April 2015 in Larach region West of Morocco. Identification was confirmed by Professor Amina Bari Botanist (Department of Biological Sciences, Faculty of Sciences, Sidi Mohammed Ben Abdellah University Fez, Morocco). Plant material was dried for 7 to 10 day in the shade at temperature. Then stored in cloth bags at 5 °C and transferred later to the laboratory for preparation of the plant extracts.

Isolation of the Essential Oils: A total (100 g) of the *Laurus nobilis* leaves was submitted for 3 h to water distillation, using a Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia [21]. Oils obtained were collected and dried over anhydrous sodium sulphate and stored in a refrigerator at 4–5 °C before analysis. Yields based on dried weight of samples were calculated.

Identification of Essential Oil Constituents by Gas Chromatography-Mass Spectrometry (GC-MS): The analysis of the volatile constituents was

run on a Thermo Fischer capillary gas chromatograph directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 210729), using an HP-5MS non polar fused silica capillary column (60 m × 0.32 mm, 0.25 mm film thickness). The operating condition of GC was: oven temperature was maintained as initial temperature 40 °C for 2 min, programmed rate 2 °C/min up to a final temperature of 260 °C with isotherm for 10 min; injector temperature 250 °C. The carrier gas was helium, flow rate 1 ml/ min. Samples were run in hexane with a dilution ratio of 10:100. 1µl of the diluted oil specimen was injected using the splitless injection technique with ionization energy of 70 eV in the electronic ionization mode. The ion source temperature was 200 °C; the scan mass range was 40-650 m/z and the interface line temperature was 300 °C. Components identification was made by determination of their retention indices (KI) relative to those of a homologous series of n-alkanes (C_8–C_{20}) (Fluka, Buchs/sg, Switzerland) and by matching their recorded mass spectra with those stored in the spectrometer database (NIST MS Library v. 2.0) and the bibliography [22].

Antimicrobial Activity Assessment: All strains tested were isolated from the newborn in the neonatal and intensive care rooms in the University Hospital Centre of Fez, Morocco. Gram-negative bacteria included: *Escherichia coli* (*E. coli*), *Klebsiella pneumonia* (*K. pneumonia*), *Pseudomonas aeruginosa* (*P.aeruginosa*) and *Proteus mirabilis* (*P. mirabilis*). While Gram-positive bacteria included only *Staphylococcus aureus* (*S. aureus*). For the bacterial susceptibility screening test, we used the agar-disc diffusion method as mentioned earlier [23, 24]. Each microorganism stock was suspended in Mueller-Hinton (MH) broth and then incubated at 37 °C for 18-24 h. The overnight cultures were diluted and adjusted to get a density of 100 CFU/ml (0.5 McFarland turbidity standards). They were flood-inoculated on to the surface of MH agar. Sterile 6 mm diameter filter discs (Whatman paper no. 3) were impregnated with 10 µg/disc of the essential oil and were put onto the surface of the inoculated agar (MH). The plates were incubated for 18 h at 37 °C. Antimicrobial activity was evaluated by measuring the inhibition zones on the tested bacterial strains. The antibiogram discs of Imipenem (IMP), Cefotaxime (CTX), Kanamycine (K), Pristinamycine (PT) were used as standard drugs for comparison. The tests were carried out in triplicates. Results were interpreted in terms of a diameter of inhibition zone: resistant (D < 6 mm), intermediaries (6 mm < D < 13 mm) and sensitives (D > 13 mm). An average zone of inhibition was calculated for three replicates. ANOVA test was used to determine whether there are any significant statistical differences between all inhibition tests.

DPPH Scavenging Activity: The ability of extracts to scavenge the DPPH radical was measured using the method described by [25] 0.1 ml of various concentrations of essential oil or standard was added with 1.5 ml of an ethanol solution containing 0.1 mmol of DPPH (2, 2-diphenyl-1-picrylhydrazyl). The absorbance of the mixture was measured at 517 nm with a spectrophotometer (Jasco V-530) after 30 min of incubation time at room temperature in the dark. The following equation calculated the percentage of inhibition:

$$I\ (\%) = (A_b - A_s / A_b) \times 100$$

Where A_s is the absorbance of the control and A_b is the absorbance of the sample. Butylated hydroxytoluene (BHT) served as positive control. The IC_{50} values were calculated as the concentration providing a 50% inhibition of DPPH radical.

Reducing Power Capacity: The reducing capacity of the tested essential oils was determined by the procedure of Oyaizu [26]. 100 µl of the extract was mixed with 500 µl of phosphate buffer (0.2M, pH 6.6) and 500 µl of potassium ferricyanide [K_3 F_e $(CN)_6$]1%. The obtained solution was incubated at 50 °C for 20 min. The mixture was acidified with 500 µl of trichloroacetic (TCA) 10% which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 500µl of distilled water and 100 µl of F_eCl_3 (0, 1%), and the absorbance was measured at 700nm (Jasco v-530). BHT was used as the standard. The results were expressed as EC_{50} (mg/ml). EC_{50} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration. All samples were analyzed in triplicate.

Total Antioxidant Capacity (TAC): The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex in acid pH [27]. A total volume of 25µl of essential oils dissolved in ethanol was added to 1 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The mixtures were incubated at 95 °C for 90 min then cooled to room temperature. The absorbance was measured at 695 nm (Jasco v-530). The total antioxidant activity was expressed as the number of equivalence of ascorbic acid.

Determination of Total Phenolic Compounds Content: Total phenolic content of the essential oils was determined by Folin-Ciocalteu method [28]. The 0.5 ml of a known dilution of the oils and 2 ml of 7% sodium carbonate solution were added to 2.5 ml of 10% (v/v) Folin-Ciocalteau reagent. The absorbance was read at 760 nm (Jasco v-530) after 2h of reaction at room temperature in the dark. Gallic acid was used as the standard for the construction of a calibration curve. Total phenols contents were expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/µg EO).

Total Flavonoids Contents: Total flavonoids contents of essential oils were measured by the aluminum chloride colorimetric assay [29]. 1 ml of sample or rutin standard solution was added into a 10 ml volumetric flask containing 4 ml of distilled water. To the flask 0.30 ml 5% $NaNO_2$ was added, after five minutes 0.3 ml $AlCl_3$ 10 % was added to react for six min. After that, 2 ml of 1M NaOH was added and the total was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm (Jasco v-530). Rutin was used as the standard for the construction of the calibration curve. Total flavonoids contents were expressed as mg Rutin equivalents per gram dry weight of each extract (mg RE/µg EO). All samples were analyzed in triplicate.

RESULTS AND DISCUSSION:

Phytochemicals: Essential oils obtained from the leaves of *Laurus nobilis* from Larache region, Morocco were yellow with yields of 1.5%. The obtained yield from *Laurus nobilis* L. are higher than the leaves yielded from Algeria and Tunisia which are respectively 0.46% and 0.58% [17]. The chemical composition of leaves *Laurus nobilis* L. essential oils was determined by GC-MS analysis. Twenty-eight compounds accounting for more than 99.98% of the total essential oil were identified in **Table 1**. Six predominant components followed in the essential oils from laurel Larach were: 1, 8-cineol (26.76%) followed to 1-α-pinene (8.63%), Linalool (7.78%), 2-carene (7.18%), α- terpineol (6.94%) Eugenyl methyl ether (6.63%), representing 63.92% of the total oil. The high relative amount of 1, 8-cineole in the essential oil of *laurus nobilis* L. confirms the previous results for the same species collected in Turkey, Tunisia and Algeria [17, 30] with values between 24 % and 60 %. A previous study is made on the chemical composition of *Laurus azorica* from four different regions of the Macaronesian Island shows that the major compound is α-pinene with values up to 36.4% followed by 1,8-cineole.

TABLE 1: CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM LEAVES OF MOROCCAN *LAURUS NOBILIS* L.

Compounds	Retention Index (RI)	Air (%)
α –Thujen	921	0.87
1- α-pinene	930	8.63
2- α-pinene	932	1.27
Camphene	945	1.11
α –terpinene	1014	1.27
1,8-cineole	1029	26.76
γ-terpinene	1045	2.09
α – terpinolene	1063	0.9
Linalool	1080	7.78
4-terpienol	1125	1.16
Terpinen-1-ol	1126	2.99
α –terpineol	1189	6.94
Endobornylacetate	1292	1.32
1-bornylacetate	1293	1.18
Phenol	1312	0.43
2-Carene	1358	7.18
Eugenol	1367	3.98
Belemene	1511	1.2
Eugenylmethylether	1415	6.63
βCaryophyllene	1426	1.13
Sabinene	975	1.05
3-Carene	1010	1.42
Iso-eugenol	1455	1.98
Isobornyl acetate	1291	0.59
𝛘-Cadinene	1526	0.61
Isopspthulenol	1644	1.01
α –Spathulenol	1625	5.7
α -Cadinol	1660	3.18
Total		99.98

Antibacterial activity : The antibacterial activity of essential oils from the leaves of laurel was tested by the agar disc diffusion method against five bacteria strains: *E. coli*, *P. aeruginosa*, *S. aureus*, *K. pneumonia* and *P. mirabilis*, responsible for nosocomial infections, isolated from newborn in neonatal unit in the university center hospital of Fez Morocco. Laurel essential oil demonstrated significant antibacterial activity against all bacteria strains tested to a varying degree with significant diameters of the inhibition zones. The results revealed that the laurel essential oil could inhibit the growth of the following bacteria such as *P. aeruginosa* (13.3 mm), *S. aureus* (25.6 mm), *P. mirabilis* (20 mm), *E. coli* (26.6 mm), *K. pneumonia* (18 mm), compared to the standard antibiotics used as positive controls (IMP, K, PT, CTX) **Table 2**.

However, we showed strong activity against Gram-negative bacteria, moderate activity against Gram-positive. These results are in agreement with previous studies [10]. The essential oil extracted from different aromatic plants has an antibacterial power [23, 24]. The essential oil of the Lebanese laurel shows antibacterial activity against the tested bacteria [9]. These results suggest that the antibacterial activity of the essential oil of laurus nobilis is mainly due to its wealth in molecules, this activity is explicable by the presence of the high contents of the components: 1,8-cineole, α-pinene and linalool or by a synergy between these major components and other minors in this essential oil. It is clear that there is a relationship between the presence of phenolic compounds and strong antibacterial activity [31].

TABLE 2: ANTIBACTERIAL ACTIVITY OF *LAURUS NOBILIS* L. ESSENTIAL OIL

Bacterial species	Inhibition zone (mm)	
	Essential oil (15 µl/disc)	Antibiotics
E. coli	26.6 ± 0.4	15(K), 18(PT), 20(CTX), 37(IMP)
S. aureus	25.6 ± 0.4	17(K), 23(PT), 24(CTX), 39(IMP)
P. aeruginosa	13.3 ± 0.4	0(AMP), 0(NOR), 4(CTX), 12(IMP)
K. pneumonia	18 ± 0.8	0(PT), 17(K), 12(CTX), 24(IMP)
P. mirabilis	20 ± 0.8	0(PT), 18(K), 14(CTX), 22(IMP)

Data are expressed as mean ± SD

Antioxidant Activities: The antioxidant activity of *Laurus nobilis* L. essential oils was evaluated by three *in-vitro* antioxidant methods: DPHH free radical scavenging, reducing power assay and total antioxidant capacity. The results were shown in **Table 3** and **Table 4.**

TABLE 3: DPPH RADICAL SCAVENGING AND REDUCING POWER ACTIVITIES OF *LAURUS NOBILIS* ESSENTIAL OILS

Samples	DPPH (µg/ml)	FRAP (µg/ml)
Laurus nobilis essential oil (µg/ml)	4.44 ± 0.05	2.87 ± 0.07
BHT	10	12
Ascorbic acid	25.20	40.62

The antioxidant activity of the essential oil of *laurus nobilis* vis-a-vis the free radical DPPH was evaluated by colorimetric method following the reduction, which is accompanied by its passage from the violet color (DPPH) to the yellow color (DPPH-H) inactive form. IC_{50} with values lowest reflect a good protective action. The antioxidant activity (IC_{50}) of Laurus nobilis L. oils is 4.44 ± 0.05 µg/ml and 2.87 ± 0.07 µg/ml for DPPH scavenging and ferric-reducing power assays respectively. The antioxidant activity of laurel essential oil is higher compared to BHT and ascorbic acid as standard antioxidants (IC_{50} values were 10 µg/ml and 25, 20 µg/ml for DPPH, 12 µg/ml and 40.62 µg/ml for ferric-reducing power) **Table 3**. The results show us that our essential oil has a strong ability to free radical scavenging [32, 33]. The total antioxidant capacity (TAC) of the laurel essential oil is corresponding to the equivalent of ascorbic acid is high 0.53Eq ascorbic acid mg/µg EO **Table 4**.

Total Phenols and Flavonoids Contents: The total phenolic contents (TPC) of *Laurus nobilis* L. oil was determined using the Folin-Ciocalteu assay, while the total flavonoid contents were determined using the chelating power of flavonoid with aluminum III. The result is shown in **Table 4**, the values were 19 mg Eq Gallic acid/mg EO and 36 mg Eq Rutin/mg EO for TPC and TFC respectively. The essential oils of laurel were very rich on the phenol and flavonoid total. Several studies have confirmed the positive relationship between phenol and flavonoid content and strong total antioxidant capacity, and the power to reduce free radicals [34, 35]. Plants rich in flavonoid and

phenolic a are a good source of natural antioxidant and antibacterial activities [36, 37].

TABLE 4: PHENOLIC, FLAVONOINDS AND TOTAL ANTIOXIDANT CAPACITY (TAC) CONTENT

Sample	Phenolic content[1]	Flavonoids content[2]	TAC[3]
Laurus nobilis essential oil (mg Eq/µg)	19 ± 0.14	36 ± 0.1	0.53 ± 0.027

[1]Equivalent of gallic acid, [2] Equivalent of rutin, [3]Equivalent of ascorbic acid. Data are the mean of three replicates (n=3) and presented as mean ± SD.

CONCLUSION: The essential oil of *Laurus nobilis* L. presented good antibacterial and antioxidant activities *in-vitro*, and it could be exploited and used as a natural antibacterial drug against bacteria causing nosocomial infections in neonatal service, and as a natural antioxidant to prevent food oxidation. Further studies are required to investigate the *in-vivo* efficacy of this essential oil.

ACKNOWLEDGEMENT: The authors are thankful to University Sidi Mohamed Ben Abdellah, Fez, Morocco for supporting this study.

AUTHORS CONTRIBUTIONS: All the authors have contributed equally.

CONFLICT OF INTEREST: The authors declare that there are no conflict of interest.

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How to cite this article:
Taroq A, El-Kamari F, Aouam I, El-Atki Y, Lyoussi B and Abdellaoui A: Phytochemical screening, polyphenols content and a novel source of antibacterial and antioxidant activities of essential oil of *Laurus nobilis* from Morocco. Int J Pharm Sci & Res 2019; 10(8): 3770-76. doi: 10.13040/IJPSR.0975-8232.10(8).3770-76.

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