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Evaluation of the antibacterial activity of essential oil of *Laurus nobilis* against *Pseudomonas syringae* pv. *phaseolicola* and potential biocidal action

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Summary With a continuously growing human population on our planet, the chemical burden of our environment is also growing. In addition, the resistance of microorganisms, like bacteria, to widely used chemicals is evident. Therefore, the application of bactericidal products that reduce the risks for development of resistance as well as the environment and human safety is of great benefit. In this work, we have screened the essential oil extracted from plants of *Laurus nobilis* L. (laurel) grown at the base of the Greek mountain Olympus for its antimicrobial activity against two strains of the *Phaseolus vulgaris* pathogen, *Pseudomonas syringae* pv. *phaseolicola* as well as human pathogenic bacteria (biocidal use). Our results, obtained with established methods, like Well diffusion and Disc diffusion assay, reveal that laurel essential oil is a very effective bacteriostatic and bactericidal agent. Importantly, the activity of laurel essential oil as growth inhibitor of the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* is reported for the first time. This opens the field for more extended investigations regarding its use in crop protection. Additionally, the laurel essential oil tested showed significant antibacterial properties against several human pathogenic bacteria, namely *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Additional Keywords: phytopathogenic bacteria, *Pseudomonas syringae* pv. *phaseolicola*, Well- Disc- diffusion assay

Introduction

In modern times there is a continuously growing interest of researchers to explore the bioactive and bioeffective properties of plants for therapeutic or food preservative purposes, as a green alternative or in complement to established synthetic, chemistry-based agents (Elshafie and Camele, 2017). The plant extracts with such biological activity belong to the secondary metabolites (SMs) of plants. Plants which accumulate bioactive SMs are known as "Medicinal plants". The plants that produce also aromatic SMs (volatiles) are known as "Aromatic and Medicinal plants" (AMPs) (Hussein and El-Anssary, 2018).

The Mediterranean Basin is one among the first twenty-five Global Biodiversity Hotspots on our planet with 25.000 native and endemic plant species (Myers *et al*., 2000). Greece, which is located in the Eastern Mediterranean, hosts the largest plant biodiversity of the Basin per unit of area with up to 1520 taxa. This large plant diversity is attributed to the climate, the geographical position and geomorphology of Greece. Interestingly, many of the endemic species are characterized as AMPs (Georghiou and Delipetrou, 2010).

The plant family Lauraceae comprises over 2.500 species. *Laurus nobilis* L. (Lauraceae) is a broadly and wildly growing evergreen AMP which is endemic in the Mediterranean Basin where it grows spontaneously or it is cultivated (Greece, Turkey, Spain, Portugal and Morocco) (Stace, 2010). It is locally known as bay laurel, sweet bay, bay, true laurel, Grecian laurel or laurel tree. Laurel is known from the ancient times for its beneficial medicinal properties (Stefanova *et al*., 2020). Pedanius Dioscurides, the an-

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cient Greek botanist and pharmacologist, mentions the therapeutic values of this AMP in his book "De Materia Medica". According to this work, crushed leaves can "heal bee and wasp stings and any kind of inflammation" (Dioscurides, 77AC, 2001). As to the Greek mythology, the plant was dedicated to the God Apollo, whose priestess, Pythia, used to chew *L. nobilis* leaves and uttered her famous prophecies (Giesecke, 2014).

The essential oil (EO) of *L. nobilis* has been recognized to possess many interesting medicinal properties such as antimicrobial, antioxidant (Ramos *et al*., 2012), anti-inflammatory (Kupeli *et al*., 2007) and anticarcinogenic (Saab *et al*., 2012) ones. EO extracted from all the parts of this plant is reported to exhibit antimicrobial activity (Bennadja *et al*, 2013). Since laurel is well acknowledged in the field of traditional medicine, the chemical composition of its EO has been studied extensively. The main chemical classes of volatiles in *L. nobilis* EO are oxygenated monoterpenes, monoterpene hydrocarbons, phenylpropanoids, sesquiterpene hydrocarbons and oxygenated sesquiterpenes (Pino *et al*., 1993). The metabolite 1.8-cineole was reported as the major component in the EO of laurel plants from Argentina (Huergo *et al*., 1978), Tunisia (Bouzouita *et al*., 2001), Turkey (Kilic *et al.,* 2005), Croatia–Serbia (Politeo *et al*., 2007), Italy (Flamini *et al*., 2007) and Iran (Moghtader and Farahmand, 2013). The spicy aroma of leaves is attributed to benzene compounds (eugenol, methyl eugenol, and elemicin) present in percentages ranging between 1% and 12% (Pino *et al*., 1993). Additionally, the stressful Mediterranean climate (heat and drought stress) is thought to be one reason for the biosynthesis of a broad spectrum of active metabolites (Mamoucha *et al*., 2018).

The chemical components of EO from *L. nobilis* growing in Greece (mountain Athos) and Georgia, and their antimicrobial activity against 20 human pathogenic microorganisms i.e., *Enterococcus faecalis*, *Staphylococcus aureus* and *Candida albicans* have been formerly studied by the authors (Stefanova *et al*., 2020). Previous work by Bozkurt *et al*. (2020) has provided evidence of inhibitory activity of EO of *L. nobilis* against gall-forming phytopathogenic bacteria (*Rhizobium radiobacter*, *Pseudomonas savastanoi* pv. *savastanoi* and *P. savastanoi* pv. *nerii*). Here, the antibacterial activity of the Greek *L. nobilis* EO is tested on the growth of *Pseudomonas syringae* pv. *phaseolicola*, an important plant pathogen which causes the disease 'halo blight' in *Phaseolus vulgaris*. Halo blight is ascribed globally yield losses of up to 45%, but the phytoprotection against this disease is mainly based on preventive measures. For chemical control, mainly copper preparations are used, which are known to be particularly burdensome for the environment and for the human health (Arnold *et al*., 2011). Moreover, the Greek laurel EO was tested against several human pathogenic bacteria for its potential biocidal activity, namely *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. Our work contributes to the research on development of alternative to chemical compounds for use in crop protection and as biocides.

Material and methods

Plant material

The upper part (young leaves) of *L. nobilis* plants was collected during the period of full flowering, from a cultivated area at Litochoro (300 m altitude), at the base of the eastern side of mountain Olympus (Greece). The plant tissue was collected on sunny days, under an atmospheric temperature of approximately 20°C and was kept under shade, in open wooden basket, for 1 hour until used.

Extraction of essential oils and preparation

The EO extraction process was conducted using the hydrodistillation method, in a modified Clevenger-type apparatus for 3 h with 3 l of H_2O. One thousand grams of leaves were extracted and yielded 9 ml of EO. Fresh leaves were chopped, placed in a

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volumetric flask and water was added. The temperature was maintained at approximately 100°C. EO was removed with a Pasteur pipette and stored at refrigeration temperature (4°C) in glass flasks wrapped in aluminum foil.

For the antibacterial screening, pure and diluted EO was used. The dilution was performed in different emulsifying agents (ethanol 70%, 80%, 90% (v/v), DMSO 0,2%, 0,5%, 5%, 20% (v/v) and Tween20 0,5%, 5%, 20% (v/v)). Additionally, a combination of 0,02% Tween and LB broth (PanReac, U.S.A.) was tested.

Preparation of bacterial cultures

Human pathogenic bacteria, commonly used to evaluate antimicrobial activity of EOs, were obtained from the American Type Culture Collection ATCC; the Gram-positive *Bacillus subtilis* (ATCC9372), *Staphylococcus aureus* (ATCC29213), *Micrococcus luteus* (ATCC9341) and the Gram-negative *Escherichia coli* (ATCC25922). The plant pathogenic bacteria, *Pseudomonas syringae* pv. *phaseolicola* strains, were kindly provided by Dr. Jesus Murillo (strain 1448A, race 6) and Dr Maria Holeva (strain BPIC593, Benaki Phytopathological Institute Collection, Dr A. Alivizatos, 1976). All strains were stored in glycerol stock solution, at -80° C. To ensure optimal growth conditions and purity, all the ATCC strains were sub-cultured in Blood and Mac Conkey Agar plates (Oxoid, UK) before each test. Accordingly, *P. syringae* pv. *phaseolicola* was cultured in Nutrient Broth Agar (PanReac, U.S.A.). The bacterial strains, the incubation conditions and the culture media used are given in Table 1.

Antimicrobial screening

For the antimicrobial assays, both in liquid and on solid bacterial cultures, the applied techniques assure repeatable results due to the continuously direct contact of EO with the bacteria**.** Laurel EO extracted from *L. nobilis* leaves was tested in the screening using two standard methods with agar plates: the Disc diffusion assay (DDA) and the Well diffusion assay (WDA). Moreover, the minimal inhibitory and the bactericidal concentration (MIC and MBC) of the EO in liquid medium were determined.

Inoculum suspension

The bacterial inoculum was prepared according to Clinical and Laboratory Standards Institute (2015) direct colony suspension method. Briefly, 24-hour bacterial colonies grown on solid media were suspended in sterile saline solution (0.9% NaCl) to achieve a turbidity of 0.5 McFarland (OD_{630}= 0,05) standard, corresponding to approximately 1 to 2×10^8 colony-forming units (CFU)/ml. The turbidity was measured using a spectrophotometer. For the broth dilution method, the inoculum (0.5 McFarland) was diluted 1:1000 in fresh LB broth for ATTC strains or NB for *P. syringae* pv. *phaseolicola* strains to obtain a concentration of $1\text{-}5\times10^5$ CFU/ml (National Committee for Clinical Laboratory Standards, 2015). The inoculum was used within 15 minutes from its preparation.

Table 1. The bacterial strains, culture media and growth conditions used in this study for the evaluation of antibacterial activity of the essential oil of *Laurus nobilis*.

Microorganisms	Culture media	Incubation conditions (time/temperature)
Pseudomonas syringae pv. *phaseolicola* BPIC593	Nutrient Broth Agar	24-48 hours/22 ˚C
Pseudomonas syringae pv. *phaseolicola* 1448A	Nutrient Broth Agar	24-48 hours/22 ˚C
Bacillus subtilis ATCC 9372	Blood Agar	24 hours/ 37˚C
Micrococcus luteus ATCC9341	Blood Agar	24 hours/ 37˚C
Staphylococcus aureus ATCC29213	Blood Agar	24 hours/ 37˚C
Escherichia coli ATCC25922	Mac Conkey Agar	24 hours/ 37˚C

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Disc diffusion assay

Five hundred µl of the inoculum in saline solution (1-5×10^5 CFU/ml) was spread over the plates of pre-dried Mueller–Hinton agar (MHA, Oxoid) using a sterile cotton swab (National Committee for Clinical Laboratory Standards, 2015). It was allowed to dry for 1 min. Then, sterile filter paper discs of 6 mm diameter (Schleicher and Schuell, Dassel, Germany) were placed on the plates and immediately 8 µl of the *L. nobilis* EO extracted in this work were spotted. The negative and positive controls were sterile saline solution and antibiotics containing discs (gentamicin, 10 µg/ml for the ATCC strains and kanamycin, 50 µg/ml for the *P. syringae* pv. *phaseolicola* strains), respectively. The plates were incubated at 37°C for 24 h for the ATCC strains or at 22°C for 48 hours for the *P. syringae* pv. *phaseolicola* strains. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the paper disks. Each bioassay was carried out three times.

Well diffusion assay

Similarly to the procedure used in the Disc diffusion method, the Mueller–Hinton agar plate surface was inoculated by spreading 500 µl of the bacterial inoculum in saline solution (1-5×10^5 CFU/ml) over the entire agar surface. Then, a hole with a diameter of 6 mm was punched aseptically with a sterile tip and 8 µL of the *L. nobilis* EO extracted in this work was added into the well. Then, agar plates were incubated at 37°C for 20 hours for the ATCC strains or at 22°C for 40 hours for the *P. syringa*e pv. *phaseolicola* strains and the inhibition zones around the wells were measured.

Minimum Inhibitory Concentration (MIC)

The Tube Dilution Assay was performed as following: The MIC values of the EO were determined using a two-fold broth dilution procedure to get a concentration of the EO in the medium ranging from 5.0 to 0.156%, according to the Clinical and Laboratory Standards Institute M27-A3 guidelines (Clinical and Laboratory Standards Institute, 2008). Briefly, 20 µl of the EO were added to the first test tube containing 380 µl LB for the ATCC strains or Nutrient broth for the *P. syringae* pv. *phaseolicola* strains. Then, 200 µl from the first tube were transferred and serially diluted into the next tubes, which contained 200 µl of the same medium. This two-fold dilution was continued until the 10^{th} tube. The 200 µl from the 10^{th} tube were discarded. The 11th tube was used as the sterile control (negative control: the medium without bacterial inoculum) and the 12^{th} tube was reserved for the growth control (positive control: the medium with bacterial inoculum). Then, 200 µl of the bacterial inoculum containing 10^5 CFU/ml were added in tubes 1 to 10 and 12. All the tubes contained finally the same volume (400 µl) of medium. Ampicillin for the ATCC strains (100 µg/µl) or kanamycin (50 µg/µl) for *P. syringae* pv. *phaseolicola* strains were also used as positive antibacterial controls in an extra tube containing 200 µl of the antibiotic containing medium and 200 µl of the bacterial inoculum. Finally, tubes were incubated in the dark on an orbital shaker (160 rpm) at 37°C for 20 hours (Kavanaugh *et al*., 2012) for the ATCC strains or at 22°C for 40 hours for the *P. syringae* pv. *phaseolicola* strains. The lowest concentration of EO preventing visible growth (no turbidity) of a strain was identified as the MIC and expressed as % (v/v) of EO in culture medium. For *S. aureus*, 100 µl instead of just 20 µl of EO were also tested for antibacterial activity.

Minimum Bactericidal Concentration (MBC)

The complete absence of growth was considered as the MBC. To confirm the results of the MBC, 10 µL of the bacterial suspensions of the tube dilution assay (see 2.4.4), withdrawn from the tubes with little or no visible growth, were streaked on 1,5 % w/v LB-agar plates for the ATCC stains or 1.5 % w/v NB-agar plates for *P. syringae* pv. *phaseolicola* strains. Then the plates were incubated for 24 h at 37°C for checking of viable cells (National Committee for Clinical Laboratory Standards, 2015) for the ATCC strains or at 22°C for 48 hours for the *P. syrin-*

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gae pv. *phaseolicola* strains. Total absence of bacterial colonies on the agar plate was determined as the MBC. Both the growth control (containing inoculum but no EO) and negative control (containing EO but not inoculum) were also plated on agar plates. Values were recorded as % (v/v) of EO in culture medium.

Statistical analysis

The mean values of the diameter (mm) of the inhibition zones ± standard deviations were calculated from three independent experiments.

Results

All the emulsifying agents used for the dilution of the laurel EO did not permit the production of a homogenous solution. Therefore, pure laurel EO, instead of diluted, was used for all the antibacterial assays.

Zones of inhibition

The zones of inhibition that were produced by the *L. nobilis* EO extracted from plants grown in the Greek area of the mountain Olympus are given in Table 2 and Figure 1. In the Disc diffusion assay, the EO inhibited the growth of all applied bacterial strains. Depending on the strain, the diameters of inhibition zones ranged from 7.5 to 20.8 mm. The EO exhibited the greatest antibacterial effect against the *M. luteus* with a diameter of inhibition zone 20.8±3 mm, followed by the *P. syringae* pv. *phaseolicola* that exhibited an inhibition zone of 15.0±3 mm (strain 1448A) and 14.0±3 mm (BPIC593 strain). A smaller inhibition zone was observed against *E. coli* (10.0±2 mm) and *S. aureus* (8.0±2 mm). The weaker antibacterial activity was noted against *B. subtilis* (7.5±2 mm). Negative control discs (saline solution) did not produce any zones of inhibition. Positive inhibition controls included: a) kanamycin disks (50μg/ml) that caused an inhibition zone of 19 mm for *P. syringae* pv. *phaseolicola* strain 1448A, and b) gentamicin disks (10μg/ml) that caused an inhibition zone of 23 mm for all other bacteria.

In the Well diffusion assay, the diameter of the inhibition zone for *M. luteus* was 18.0±3 mm and it was the biggest one. On the other hand, the smallest zone was noted for *E. coli* (9.0±3 mm), *B. subtilis* (8.0±3 mm) and *S. aureus* (8.1±2 mm). *P. syringae* pv. *phaseolicola* displayed a zone of 14.5±3 mm for strain 1448A and of 18.0±2 mm for the BPIC593 strain.

The MIC and MBC of the EO

The EO was tested by the broth dilution method to determine the MIC visually and the MBC by growth on agar medium, expressed as a percentage of % (v/v) of EO in the medi-

Table 2. Zones of bacterial growth inhibition of six bacterial strains by essential oil of *Laurus nobilis* using two agar diffusion methods.

Method	Diameter* of the zone of growth inhibition of six bacterial strains (mean of three replicates ± standard deviation in mm)					
	Pseudomonas syringae pv. *phaseolicola* (1448A)	*Pseudomonas syringae* pv. *phaseolicola* (BPIC593)	*Bacillus subtilis* ATCC 9372	*Micrococcus luteus* ATCC9341	*Staphylococcus aureus* ATCC29213	*Escherichia coli* ATCC25922
Disc diffusion assay	15.0±3	14.0±3	7.5±2	20.8±3	8.0±2	10.0±2
Well diffusion assay	14.5±3	18.0±2	8.0±3	18.0±3	8.1±2	9.0±3

*The diameter of the paper discs was 6 mm.

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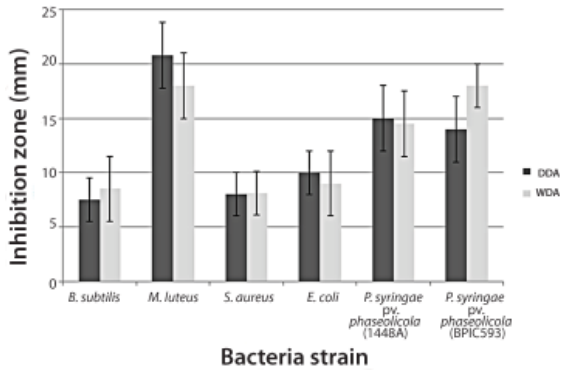


Figure 1. Histogram of inhibition zones (mm) measured in Disc diffusion assay (DDA) and Well diffusion assay (WDA) for the evaluation of the antibacterial activity of the essential oil of *Laurus nobilis* on six bacterial strains. Details are given in Table 2. Error bars represent the mean ± standard error from three independent experiments.

um. Since the addition of solvents and detergents in EO did not result in a homogenous solution, pure EO was used. As the antibacterial activity of the EO might be affected by the shaking rate and air exchange during culture (Wang *et al*., 2016), all liquid antibacterial assays were performed under constant agitation conditions on an orbital shaker (160 rpm) in tubes of 5 ml volume. By doing so, a direct-contact of bacteria and EO was achieved as well as the aeration of the media. Each experiment was performed three times. The results are summarized in Table 3. The EO was more potent against *M. luteus* (MIC and MBC were 1.25% and 2.5% respectively). The EO was also very effective against *P. syringae* pv. *phaseolicola* displaying a MIC of 1.25% for both strains and an MBC of 5% for the 1448A strain and 2.5% for the BPIC593 strain. The MIC for *B. subtilis* and *E. coli* were 2.5% and the MBC were 5% and 2.5%, respectively. On the other hand, the lowest antibacterial activity was displayed against *S. aureus,* since starting with 20 µl of EO in the first of the ten tubes for the antibacterial screening did not affect its growth. Therefore, 100 µl of EO were also used in the first tube instead of 20 µl. The MIC recorded was 1.563% and the MBC was 6.25%.

Table 3. Growth inhibitory effect of serially diluted essential oil of *Laurus nobilis* against six bacterial strains.

Bacterial strain		EO (v/v%)								
		5	2.5	1.25	0.625	0.3126	0.156	GrC[a]	StC[b]	SoC[c]
Pseudomonas syringae pv. *phaseolicola** (1448A)	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	+	+	+	+	+	+	-	-
Pseudomonas syringae pv. *phaseolicola** (BPIC593)	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
*Bacillus subtilis** ATCC 9372	MIC	-	-	+	+	+	+	+	-	-
	MBC	-	+	+	+	+	+	+	-	-
*Micrococcus luteus** ATCC9341	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
*Escherichia coli** ATCC25922	MIC	-	-	+	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
		12.5	6.25	3.125	1.563	0.781	0.391	GrC[a]	StC[b]	SoC[c]
*Staphylococcus aureus*** ATCC29213	MIC	-	-	-	-	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-

a: Growth control (tube containing bacteria), b: Sterile control (tube without bacteria), c: Solvent control,
-: no growh, + : growth
* *B. subtilis*, *M. luteus*, *E. coli* and *P. syringae* pv. *phaseolicola* were incubated with 20 µL EO in the 1st tube
** *S. aureus* was incubated with 100 µL (20 µL EO did not inhibit its growth).

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Discussion

Many EOs extracted from AMPs have been reported to have antimicrobial properties (Horváth and Ács, 2015). Therefore, *in vitro* studies including EOs which have been used in traditional medicines from the medieval times are important to improve antimicrobial treatments. EOs have unique metabolic profile (co-existence of phenols, flavonoids, alkaloids, terpenes, tannins etc) and exhibit their activity by many different inhibition mechanisms. Consequently, they can affect a variety of pathogens by changing membrane permeability, denaturing proteins and inhibiting enzymes (Nazzaro *et al*., 2013).

Standardizing the experimental methods for the bioassays

Testing plant EOs in liquid antimicrobial bioassays encounters problems mainly due to the EOs' water insolubility and complexity, i.e., a non-homogenous distribution of the oil through the medium appears and gives bacteria a greater chance of survival. To avoid these difficulties, many researchers propose the use of different solvents (methanol, ethanol, DMSO, Tween) (Wang *et al*., 2016). Unfortunately, the chemical properties of EOs do not permit a standardized methodology to dissolve them and the appropriate method depends on the EOs' nature. Laurel EO in this work was one of these cases, i.e., the low dispersion of water insoluble compounds of EO in the liquid growth medium (Tan and Lim, 2015). Different solvents and detergents (DMSO and Tween20) were tested. The dilution of laurel EO in all emulsifying agents resulted in a white 'color' suspension when the culture medium was added. The presence of the suspension did not permit the determination of MIC. The suspension might be caused by the lipophilic molecules of the laurel EO which form micelles, thereby suppressing EO's attachment to the microorganisms. These hydrophobic molecules interact with the lipids of the bacterial cell membrane, disturb cell structures and render them more permeable (Chouhan *et al*., 2017).

Only by using pure laurel EO with continuous shaking during incubation could we observe the antibacterial activity. Thus, for the *in vitro* investigation of the laurel EO antimicrobial activities in liquid culture, shaking could prevent the adherence clumping and phase separation between aqueous phase and EO. Additionally, Juergensmeyer *et al.* (2007) reported that shaking causes a decreased lag phase and a higher final yield. Our method is based on the National Committee for Clinical Laboratory Standards recommended standards for broth dilution assays. Moreover, the use of pure EO (without solvent) enhances the antibacterial activity. Undoubtedly, modifications should be made on proposed techniques since not all the EOs have the same diffusibility and solubility in liquid cultures (Rios *et al*., 1988). In field experiments, a totally different approach has to be applied. In this case, the EO/water dilution mixture would be directly sprayed on leaves.

In order to better evaluate the antimicrobial effectiveness of laurel EO, two different methods were applied, the Disc diffusion and the Well diffusion assay. The Disk diffusion assay is accepted by the FDA (Food and Drug Administration of the USA) and is established as a standard assay for the analysis of antimicrobial activity by the National Committee for Clinical Laboratory Standards (2015). The Well diffusion assay offers a higher sensitivity, faster and better diffusion of the EO into the agar (Valgas *et al*., 2007). Both antimicrobial assays resulted in similar results. We concluded that the Disc diffusion assay is an easier and less demanding technique than the Well diffusion assay. In the Well diffusion assay the main disadvantage is the difficulty in making the well.

In addition to the method of agar plates, a broth dilution method was used to determine the MIC of EO against the tested bacteria. The main advantage of MIC is the small quantity of reagents and samples required, which enables a greater number of repetitions and, thus, increases the reliability of the results. MBC was also evaluated, which is reported to be helpful in severe infectious

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diseases (Nemeth *et al*., 2015).

Antibacterial properties of laurel EO

This work aims to perform an initial screening of the antibacterial properties of the specific EO, with emphasis on its properties over a plant pathogen. Data from our study has confirmed the antimicrobial potential of EO of *L. nobilis*. Furthermore, this is the first report regarding the antibacterial effect of laurel EO on the growth of two stains of *P. syringae* pv. *phaseolicola*, the well-studied 14484A and the strain BPIC593 isolated from plants grown in the Regional Unit of Florina, Greece.

P. syringae pv. *phaseolicola* is an important plant pathogen that mainly affects *P. vulgaris* crop production (Arnold *et al*., 2011). From our results it is well documented that the EO has a significant effect on the *P. syringae* pv. *phaseolicola* growth. Therefore, this study, together with previous research work by Bozkurt *et al.* (2020) on EO of *L. nobilis* against gall-forming phytopathogenic bacteria, establishes the grounds for the potential use of laurel EO as natural compounds alternative to chemically synthesized plant bactericides, whose use has already been deemed problematic (Lamichhane *et al*., 2015). Moreover, other EOs have been shown to exhibit *in vitro* antibacterial activity against *P. syringae* pv. *phaseolicola*, e.g. EO of *Origanum* species (Della Pepa *et al*., 2019). As there are increasing efforts in research to combat phytopathogenic bacteria with natural agents, we should note that most relevant studies employ culturing methods to assess the antibacterial effect of EOs on phytopathogenic bacteria and do not consider the viable but non culturable condition (VBNC) that may occur with bacterial cells. Therefore, in order to assess such phenomena, future studies should also incorporate viability testing using additional methods e.g., RT-PCR.

The EOs of *L. nobilis* growing in different countries have been reported to display antibacterial activity. Moghtader and Farahmand (2013) and Ouibrahim *et al.* (2013) noted antibacterial activity of laurel EO (by the Disc diffusion assay) against *E. coli*. In our screening, we noticed a rather small inhibitory activity of laurel EO towards *S. aureus*. This finding is in agreement with the results of Bennadja *et al.* (2013). Interestingly, even though *M. luteus* is a notoriously pathogenic microorganism in skin acne (Chiller *et al*., 2001) and *L. nobilis* EOs are used in cosmetics (Sahin Basak and Candan 2013), we found only few papers investigating *L. nobilis* EOs' antibacterial activity against *M. luteus*. In our work, we noticed the largest zone of inhibition for *M. luteus*.

Being an AMP, *L. nobilis* biosynthesizes a variety of active secondary metabolites. There are numerous phytochemical investigations on Mediterranean *L. nobilis* EOs. According to their results, the major constituents in laurel EOs are 1.8-cineole (eucalyptol), linalool and a-terpinyl acetate (Saab *et al*., 2012; Sahin Basak and Candan 2013). They are terpenoids and belong to the chemical subclass of oxygenated monoterpenes. 1,8-cineole is the major chemical component in leaf-derived EOs for the majority of medicinal species of *Eucalyptus* sp. followed by a-terpinyl acetate (Silva *et al*., 2011). Along the same line, Stefanova *et al.* (2020) have found in EO from Greek *L. nobilis* leaves the highest content in 1,8-cineole (30.8% of total ion current), but also a high content of α-terpineol (8%) and sabinene (7.9%) which belong to the oxygenated monoterpenes and monoterpene hydrocarbons, respectively. Other studies report the antimicrobial activity of these metabolites, such as linalool and 1,8-cineole. Sato *et al.* (2007) noted the highest antimicrobial activity for 1,8-cineole. According to the results by Hendry *et al.* (2009), a synergistic antimicrobial activity was noted for 1,8-cineole and chlorhexidine digluconate. The antimicrobial activity of 1,8-cineole might be attributed to the reduction of cellular activity by interacting with membrane enzymes and proteins (Stojković *et al*., 2011). Thus, the high content of oxygenated monoterpenes may, in a synergistic effect with minor compounds, provide the high antimicrobial activity of the Greek *L. nobilis* EO.

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Conclusions

The current study reports, for the first time, the antibacterial activity of the Greek medicinal plant *L. nobilis* assessed by different *in vitro* assays against the plant pathogen *P. syringae* pv. *phaseolicola* and four human pathogenic bacterial species. Its EO is a valuable source of bioactive compounds that apparently have antimicrobial properties that inhibit the growth of different strains of the Gram-positive and Gram-negative bacteria tested. The chemical characterization of individual compounds could further shed light on the most effective combination that can be used as an antimicrobial agent. In conclusion, in a world of increasing antibiotic resistance of pathogenic bacteria, where there is an urgent need for new bioactive compounds, this initial step sets the basis for future use of natural agents of low cost and low environmental imprint to control bacterial plant and human pathogens.

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Declarations

Authors' contributions

S.M., A.G. and A.P. conceived and designed the experiments. S.M. and A.G. executed the experiments. S.M., A.G. and A.P. discussed the results and wrote the paper.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Αξιολόγηση της αντιβακτηριακής δράσης αιθέριου ελαίου δάφνης, *Laurus nobilis*, έναντι του βακτηρίου *Pseudomonas syringae* pv. *phaseolicola,* και πιθανής βιοκτόνου δράσης

Σ. Μαμούχα, Α. Προμπονά και Α. Γαλέου

Περίληψη Με τη συνεχή αύξηση του ανθρώπινου πληθυσμού στον πλανήτη μας, η επιβάρυνση του περιβάλλοντος από χημικά επιδεινώνεται συνεχώς. Επιπλέον, μεγάλο πρόβλημα αποτελεί η εμφάνιση ανθεκτικότητας των μικροοργανισμών, όπως τα βακτήρια, σε ευρέως χρησιμοποιούμενες χημικές ουσίες. Ως εκ τούτου, η εφαρμογή βακτηριοκτόνων προϊόντων που μειώνουν τους κινδύνους ανάπτυξης ανθεκτικότητας, για το περιβάλλον και την ανθρώπινη υγεία είναι εξαιρετικά ωφέλιμη. Στην παρούσα εργασία εξετάσαμε το αιθέριο έλαιο που εξάγεται από φυτά του είδους *Laurus nobilis* L. (δάφνη), τα οποία καλλιεργούνται στις παρυφές του όρους Όλυμπος, για την αντιμικροβιακή του δράση έναντι δύο στελεχών του παθογόνου *Pseudomonas syringae* pv. *phaseolicola* που προσβάλλει το φυτό *Phaseolus vulgaris*, και παθογόνων βακτηρίων για τον άνθρωπο (βιοκτόνος δράση). Τα αποτελέσματά μας, που ελήφθησαν με καθιερωμένες μεθόδους, όπως η Well diffusion assay και η Disc diffusion assay, αποκαλύπτουν ότι το αιθέριο έλαιο δάφνης είναι πολύ αποτελεσματικός βακτηριοστατικός και βακτηριοκτόνος παράγοντας. Για πρώτη φορά αναφέρεται η δράση του αιθέριου ελαίου δάφνης ως αναστολέα της ανάπτυξης του φυτοπαθογόνου βακτηρίου *P. syringae* pv. *phaseolicola*. Έτσι, η παρούσα εργασία ανοίγει το πεδίο για πιο εκτεταμένες έρευνες σχετικά με τη χρησιμοποίησή του στη φυτοπροστασία. Επιπλέον, το αιθέριο έλαιο δάφνης που δοκιμάστηκε έδειξε σημαντικές αντιβακτηριακές ιδιότητες έναντι πολλών παθογόνων βακτηρίων για τον άνθρωπο, όπως τα *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* και *Bacillus subtilis*.

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