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ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OILS ISOLATED FROM TWO SPECIES *CUPRESSUS ARIZONICA* GREENE AND *CUPRESSUS SEMPERVIRENS* L. (VAR. *HORIZONTALIS* AND *PYRAMIDALIS*)

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ABSTRACT
Cupressus sempervirens L. and *Cupressus arizonica* Greene are two cone-bearing coniferous woody plant species of the *Cupressaceae* family found in Lebanon. The essential oils of the introduced species *Cupressus arizonica* Greene and those of each of the two native varieties of the species *C. sempervirens* L. (var. pyramidalis and horizontalis), both collected in Lebanon, were isolated from their cones by hydrodistillation. By using the agar disc diffusion method, we aim at studying the antibacterial activity of each of these oils against two bacterial strains, namely the Gram-positive *Staphylococcus epidermidis* and the Gram-negative *Escherichia coli*. A significant antibacterial activity was obtained by all plant extracts tested. However, this study revealed that both varieties of the species *C. sempervirens* exhibited an inhibitory activity slightly greater than that of the *C. arizonica* against the Gram-positive bacteria, whereas *C. arizonica* showed a slightly greater inhibitory activity against the Gram-negative bacteria used in this study.

KEYWORDS: *Cupressus sempervirens*, *Cupressus arizonica*, Antibacterial activity, Essential oils, *Staphylococcus epidermidis*, *Escherichia coli*.

INTRODUCTION
The genus *Cupressus* of the two species in this study is commonly known as “Cypress”. Members of this genus are evergreen trees or tall shrubs with single or multiple trunks.[1] These tall evergreen trees are characterized by slender branches and a statuesque conical shape. The flowers of cypress are small and the cones are brownish grey in color.[2] The first cypress species in this study namely *C. sempervirens* L. is an evergreen, branched quadrangular and horizontal or erect tree.[3] Its crown varies from extremely spire-shaped to about as broad as tall and is usually accompanied with upright or spreading branches that are uniformly dense. Hence, it appears to show a symmetrical canopy with regular outlines. The two studied varieties (var. *horizontalis* and *pyramidalis*) are basically distinguished by the size of the angle between the branches and the stem; the angles of the *horizontalis* variety are wide while those of the *pyramidalis* variety are small.[1-4] Though these varieties exhibit different morphologies, hybridization can naturally take place between these varieties and the produced progenies may be characterized by a form varying between the two forms.[4-5] On the other hand, the other studied species *Cupressus arizonica* Greene var. *arizonica* (CA) is a medium-sized evergreen tree with a conic to ovoid-conic crown.[6-7] This species is mostly known by its blue-green, grayish-green, or silvery foliage arranged oppositely in pairs and tightly clasping the cord-like or four-sided twigs. Unlike *C. sempervirens*, *C. arizonica* is not endemic to the Lebanese region. Indeed, *C. arizonica* is originally native to some regions in southern and western United States[8-9] and has been abundantly introduced in numerous areas of Middle East, including Lebanon, during the past two decades.[7-10] Throughout history, essential oils (EOs) and medicinal plant extracts have evoked interest as sources of natural products, as well as they attracted the attention in the discovery of biologically active compounds.[11-12-13] Essential oils (also called volatile oils) are aromatic oily liquids obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots). The aerial parts of members of the family *Cupressaceae*, especially the two species of study, have been widely used in traditional medicine to treat various infections such as colds (where it combats coughing), parasitic infections, inflammation, hemorrhoids, and others where

the fruits of the plant were traditionally used as antiseptic compounds and to cure diabetes.[14-15-16] Nowadays they are sometimes used for suffumigations, in solution for washing, and bandages for the treatment of circulatory diseases.[14] Though few studies have investigated the antimicrobial potentials of Eos[17-18-19-20] most of their medicinal and pharmacological advantages originate from the potential of these oils as sources of new antimicrobial compounds especially against bacterial pathogens.[12-21] According to several reports, all of the EOs extracted from the two studied medicinal cypresses proved to be effective against at least two bacteria, one of which was the Gram-positive *Staphylococcus aureus* and the other was the Gram-negative bacteria *Escherichia coli*.[2-3-17-22-23] Because the Antimicrobial Resistance (AMR) has increasingly become a serious threat to global public health,[24] and due to the perception that herbal products may be safe and have been used for many years as traditional medicines, studies are currently focussing on the therapeutic and pharmacological effects of natural products of specifically plant origin.[25] Therefore, the main objective of this study was the assessment of antibacterial efficiencies of the oil extracted from the cones of the two above mentioned species of Lebanese cypress; *Cupressus sempervirens* L., with its two varieties (*var. horizontalis* and *pyramidalis*) and *Cupressus arizonica* Greene. These efficiencies were justified by relevance to the active phytochemicals comprised within the EOs of these plants which are in turn based on previous reports.

MATERIALS AND METHODS

Plant material

The plant cones were first collected in winter (beginning of March) and then in spring (April 2015) in the second time. The cones of the species *C. sempervirens* L. were collected from different regions of Lebanon; the horizontalis form (CSH) was collected from Kfarjarra-Saida, at an altitude of 210 m from sea level, South Lebanon, whereas the pyramidalis form (CSP) was collected from Ain el Mir-Jezzine, at an altitude of 400 m from sea level, South Lebanon. On the other hand, the cones of the species *C. arizonica* were collected from Roum- Jezzine, at an altitude of 880 m, South Lebanon. The collected plant materials are aromatic and the derived essential oils will be prepared by hydro-distillation. Both the identification and classification of the studied plant species were done by Dr. Katia Saade, the botanist of the Department of Life and Earth Sciences of the faculty of Sciences - Lebanese University.

Extract preparation

The fresh cones of each plant sample (CSH, CSP, and CA) were separately chopped into small pieces and then crushed into powder. Because extraction of essential oils requires a large amount of plant material,[24] about 700 g of each sample powder were individually placed in a 2000 ml round-bottomed flask in which 700 ml of distilled water were added. Later, each of the oils that will be produced each by distillation would consequently have a fixed concentration of 10^3 µg/ml. The essential oil extraction was done by hydro-distillation of each plant sample using a "Clevenger-type" apparatus for 4 hours according to the European Pharmacopoeia (1975).[26-27] After 4 hrs, the accumulated oil was stored in sealed glass vials at 4 ˚C prior to analysis.

Bacterial strains

In this study, we tested our oils against two facultative anaerobic standard reference bacteria: a Gram-negative strain represented by *Escherichia coli* (*E. coli*) [K12 E.coli strain carrying plasmid, DH5-alpha/pTY250 (PTA-4079)] and a Gram-positive one represented by *Staphylococcus epidermidis* (*S. epidermidis*) (ATCC® 14990™). The two non-pathogenic microorganisms were obtained from the Department of Microbiology, Lebanese University (Fanar), Lebanon. The cultures of bacteria were maintained in their appropriate agar slants at 4 ˚C throughout the study and used as stock cultures.

Antibacterial screening

Media

Mueller Hinton agar was used as base medium for the screening of antibacterial activity and Nutrient broth in order to prepare the inocula in this experiment.

McFarland 0.5 $BaSO_4$ turbidity standard

The standard was prepared by adding sulfuric acid to an aqueous solution of barium chloride, which resulted in the formation of a suspended barium sulfate precipitate of 0.85 % salinity and reproducible turbidity.[28] Barium sulphate turbidity standard (4-6 ml) was taken in screwed cap test tube and used to compare the turbidity. The prepared bacterial suspension, once adjusted to the same turbidity of the McFarland Standard (0.5), produces the intended bacterial plate counts (1.5×10^8 CFU/ml).

Preparation of Inocula

A 24 hours old colony of bacteria was taken out of its culture and mixed with a nutrient broth and was later placed in incubator for 18-24 hours. After 24 hours, each of the selected bacterial stains was mixed with an autoclaved saline solution until the turbidity in each test tube of inocula reached the same turbidity as that of the previously prepared McFarland 0.5 $BaSO_4$ turbidity standard. Each inoculum was used for culturing the nutrient agar plates.

Disc-Diffusion Method

The agar disc-diffusion method has been employed for the determination of antibacterial activities of the essential oils since 1997.[23] The freshly prepared and cooled Mueller Hinton medium was poured into 18 plastic and flat- bottomed Petri dishes on a horizontal surface to give a uniform depth of approximately 4 mm (25 to 30 ml for plates with a diameter of 100 mm to provide the best nutrient medium distribution). The Petri dishes were capable of gelatinizing for 30 min at room temperature before their incubation in an incubator at 37

°C for 24 hours. After 24 hours, an inoculating swab was dipped in broth of the prepared inoculums of each bacterium and then separately swabbed on the agar plates in different directions. The spreading technique that was adapted was the "Roll Plate swabbing Method", described in NCCLS Document M40.[28] A quantity of 30 µl of each sample of the essential oils (CSH, CSP, and CA) was poured each on a separate filter paper disc (5 mm in diameter) where six discs were prepared (two discs soaked with the same oil sample for each of the two bacteria). After which, the discs were dried for 30 min at room temperature before being later placed in the inoculated plates of each bacteria. Tetracycline (30 µg) was used as a positive control in each Petri-dish. All tests were performed in triplicates. The plates were incubated at 37 °C for 24 hours. After the incubation, the diameter of clear zones around each disc was measured and compared against the zone of inhibition produced by the standard antibiotic.

RESULTS AND DISCUSSION

Isolation of the essential oils

The "Percentage Yield" (w/w) of essential oils of each of the two varieties of the species C. *sempervirens* (CSP and CSH) and the species *C. arizonica* (CA) ranging between 0.8-1.5 w/w are provided By Table (1).

Table 1: List of essential oils studied with their respective sites, locations and % Yield.

Latin name	Family name	Location	Altitude	Material Studied	Yield %
C. sempervirens (horizontalis)	*Cupressaceae*	Kfarjarra	210 m	Cones	**0.8%**
C. sempervirens (pyramidalis)	*Cupressaceae*	Jezzine	800 m	Cones	**1%**
C. arizonica	*Cupressaceae*	Jezzine	800 m	Cones	**1.5%**

Table 2: Antibacterial test results for the three oils.

Bacterial strain	Essential oils						Negative Control (mm)	Positive Control* (mm)
	CA		CSH		CSP			
	IZ	MIC	IZ	MIC	IZ	MIC		
S. epidermidis	8	200	9	100	9	50	n.a	22
E. coli	10	200	8	100	8	50	n.a	18

IZ: Diameter of inhibition zone (mm) including disc diameter of 5 mm
MIC: Minimum inhibitory concentration; values given as µg/ml (values specific to each bacteria were obtained by our own lab calculations)
n.a: No activity
*: Tetracycline (30 µg/disc) (values specific to each bacteria were obtained from reference)

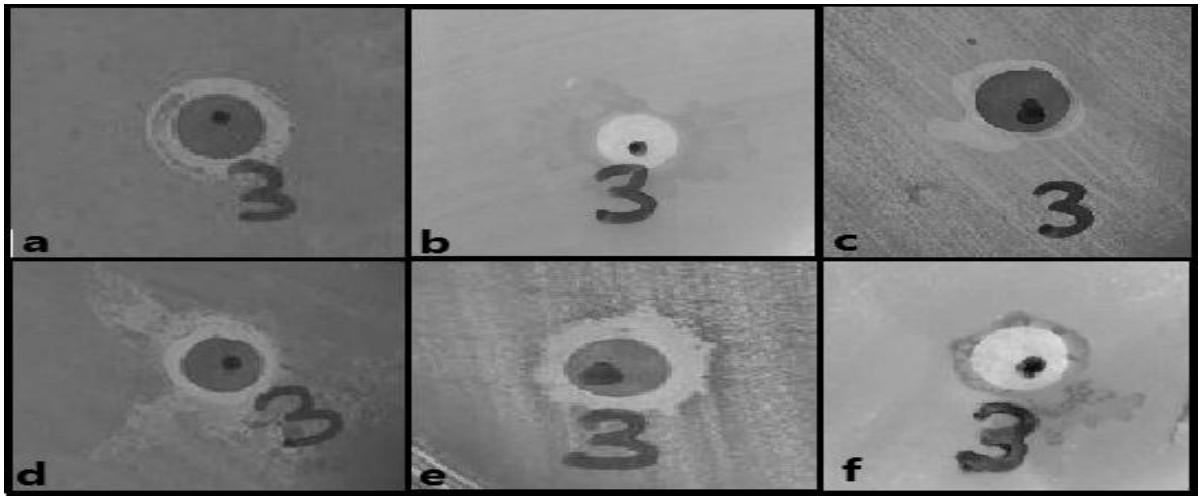

Figure 1: bacterial activity on culture; (a) *E. coli* + CA, (b) *E. coli* + CSH, (c) *E. coli* + CSP, (d) *S. epidermidis* + CA, (e) *S. epidermidis* + CSH, (f) *S. epidermidis* + CSP.

Anti-bacterial activity test results

The anti-bacterial activity of each of the three studied essential oils against the two selected bacterial species is summarized in Table (2). The best results out of the three repeated cultures were exhibited in this table as final results. These results revealed that the selected essential oils showed an antibacterial activity with varying magnitudes. The zone of inhibition above 8 mm in diameter was considered as a positive result. The results shown in Table (2) are exhibited in Figure (1) exactly as

they appeared in cultures. The degree of the essential oil activity is revealed by the size of the inhibition zone that is expressed by the diameter of the referred inhibition zone (in mm or cm) and usually the diameter of the disc is included.[29] However, this technique is less suitable for quantification purposes, such as the determination of the MIC. Moreover, the highest MIC values of *C. sempervirens* essential oils are usually recorded at a higher extraction concentration of (4x103 µg/ml) [23-30] and are not usually observed at a small one as in this experiment (103 µg/ml). Hence, according to the facts discussed above, the MICs registered in table (2) and which are calculated according to our own database wouldn't be considered as true MICs. However, minimal inhibitions were actually observed and therefore these concentrations deserve to be partially considered as MICs.

In reference to Table (2), each of the three oils alone was able, to some extent, to destroy the cultured bacterial strains in different levels of effectiveness. However, their effectiveness (diameter of IZ) slightly varied at the level of the bacteria (Gram-positive or Gram-negative) utilized. Indeed, both studied varieties of *C. sempervirens* (CSH and CSP) gave exactly same results (same IZ) with both bacterial strains (9 mm IZ with *S. epidermidis* and 8 mm IZ with *E. coli*). However, slight but detectable differences in inhibition zones (IZs) were noticed between the two *Cupressus* species: *C. sempervirens* L., and *C. arizonica*. Indeed, oils of the two varieties of *C. sempervirens* were more effective in inhibition of the Gram-positive bacterium *S. epidermidis* than *C. arizonica* oil (8 mm IZ with CA oil < 9 mm with CSH and CSP oils). On the contrary, *C. arizonica* oil was more effective in inhibition of the Gram-negative *E. coli* bacterium than the oils of both *C. sempervirens* L. varieties (10 mm with CA > 8 mm with CSH and CSP). In other words, under application of *C. sempervirens* oils, larger IZs (9 mm) were reported for the Gram-positive bacterium, and lower ones (8 mm) were reported for the Gram-negative bacterium. On the contrary, *C. arizonica* showed a higher activity against the Gram-negative bacterium rather than against the Gram-positive one (10 mm IZ with *E. coli*>8 mm with *S. epidermidis*). Still, the IZ diameters obtained are too small, due to the low concentrations obtained for the oils, to show the full antibacterial activity of these EOs. In addition to this, the documented differences were too slight to be considered as a major difference in activity. Hence, it was only scientifically mandatory to ignore this difference according to standards.[31] This documented anti-bacterial activity can be explained in reference to the GC/MS qualitative analysis in previous studies. According to previous GC/MS qualitative analysis conducted on the exact same oils tested in this study, the existence of variable numbers of different constituent compounds which belonged to the "Terpene" or "Terpenoid" (terpenes with added oxygen molecules or that have had their methyl groups moved or removed by specific enzymes) universal chemical groups was revealed. These latter complex chemical compounds are normally synthesized within the cytoplasm of the vegetal cell.[32] Among the terpenes, mainly δ-3-carene, α-humulene, α-pinene, β-phellandrene and α-cedrol are usually attributed to the antimicrobial activities of EOs.[3-23] These terpenes range between monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$).[11-32-33] According to many researchers, it was demonstrated that all the above mentioned terpenes possessed an antimicrobial activity especially against Gram-positive and Gram-negative bacteria.[3-17-34] Moreover, the antimicrobial activity of EOs might also be attributed to the presence of phenolics, alkaloids, flavonoids and polyacetylenes.[35] The antimicrobial action of the essential oils (EOs) was mainly limited to the toxic effects of the above mentioned terpenes on the structure and function of the bacterial cell membranes.[32-36] In fact, as a result of their lipophilic character or hydrophobicity, these oil components will preferentially dismantle the lipids of the bacterial cell membrane and those of the mitochondria.[12-29-33] This results in the disturbance of cell structure (membrane expansion, increased membrane fluidity and permeability and disturbance of membrane-embedded proteins) affecting other cellular structures in a cascade type of action.[29] This cascade involves extensive leakage of critical molecules and ions leading to an alteration in ion transport processes and reducing the intracellular ATP pool via decreased ATP synthesis resulting in inhibition of respiration, loss of the control on turgor pressure,[32] and consequently the absolute death of the bacterial pathogen.[12-33] However, each of these actions cannot be considered a separate event; but instead, it may be a consequence of the other not well- characterized activities.

CONCLUSIONS

The EO was principally acting on the cell membrane of each of the two bacterial strains used. Indeed, being the key element for the fundamental biological activities taking place within the cells, the integrity of the cell membrane is essential for the survival of bacteria. Hence, the ability of these EOs to disturb this integrity has somehow made them capable of eradicating a bacterial growth. Thereafter and based on the findings of this study, the use of essential oils from *C. arizonica* and *C. sempervirens* as antibacterial material might be of great value for pharmaceutical industry. Still, the limitations in this study were due, first to the absence of adequate GCMS machinery, which in turn imposed the urge to use GCMS results of the exact same plant species done by previous studies; second to the small amounts of EOs obtained after collection and extraction. Therefore, further work should be done to better investigate the documented antibacterial activities, with the availability of the appropriate equipment for the required chemical analysis and with greater concentrations of EOs allowing a better determination not only of the IZ but also of MIC for each of the used oils. In conclusion, the chemical characterization and identification of the biological activities of the volatile oils of the two species *C.*

arizonica Greene and *C. sempervirens* L. var. horizontalis and var. pyramidalis will fill a gap in the understanding of *Cupressus* genus.

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