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# Analysis of the chemical compositions of six essential oils and evaluation of their antioxidant and antibacterial activities against some drug-resistant bacteria in aquaculture

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**ABSTRACT**

**Introduction:** The extensive use of chemicals and antimicrobial agents in aquaculture has decreased the immune mechanisms of cultivated species and promoted the emergence of drug-resistant microorganisms leading to diseases among cultivated fish, affecting consumers' health. Thus, the investigation of natural antibacterial and anti-stress agents is crucial. In the current study, we focused on the evaluation of the potential use of essential oils (EOs) as antioxidant and antimicrobial agents in aquaculture.

**Methods:** The EOs, obtained by hydrodistillation from clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum verum*), rosemary (*Rosmarinus officinalis*), artemisia (*Artemisia herba-alba*), cedarwood (*Cedrus atlantica*) and oregano (*Origanum compactum*) were analyzed by gas chromatography/mass spectrometry (GC/MS). Their antibacterial activities were carried out against five bacteria, pathogenic to fish in aquaculture, using the well diffusion and microatmosphere methods. The pathogens used were *Vibrio anguillarum*, *Photobacterium damselae* subsp *damselae*, *Aeromonas salmonicida*, *Edwardsiella tarda,* and *Lactococcus garvieae*. Then, the minimum inhibitory and bactericidal concentrations of each EO were determined. Furthermore, the antioxidant activity was performed *in vitro*.

**Results:** The investigated EOs were effective against the pathogenic strains. They showed variable constituents such as phenols, sesquiterpenes, and monoterpenes. Regarding the antioxidant activity, cinnamon, clove, and oregano EOs showed their abilities to donate hydrogen to 2,2-diphenyl-1-picrylhydrazy (DPPH) radical and scavenge free radicals produced by 2,2-azino-bis-3-ethylbenzothiazoline6-sulfonic acid (ABTS), respectively.

**Conclusion:** These results gave insight into the potential use of phytobiotics in aquaculture as a safe strategy to substitute antibiotics to protect fish from oxidative stress and inhibit the emergence of drug-resistant bacteria for safer consumption of cultivated fish.

*Implication for health policy/practice/research/medical education:*
The essential oils (EOs) of clove, cinnamon, rosemary, and oregano showed good antibacterial and antioxidant activities. This research can be continued to the *in vivo* assay to obtain more evidence for their efficacy. EOs could be used as an alternative to chemicals in order to overcome infectious diseases and oxidative stress occurring in fish to limit related health problems affecting fish consumers.

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## Introduction

Aquaculture is a fast-growing sector increasing seafood production. It is under intense pressure to satisfy the growing demand for fish products of the increasing world population (1). The global production of finfish and shellfish reached 172.6 million tons in 2017, approximately half of which is currently derived from aquaculture, which demonstrates the crucial role of this sector in economic

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stability worldwide (2).

The expansion of aquaculture is hampered by several constraints such as the emergence of marine infectious agents that can be transferred through feed, water intake, or infected broodstock. Additionally, fish species are exposed to oxidative stress due to internal or environmental factors such as temperature, salinity, feed, and deprivation (3). This can lead to an increase in the mortality of marine species, a decrease in their growth performance, and a decrease in the response of their immune system, which may threaten the biological diversity of marine organisms (4,5). Thus, the plethora of chemical substances and antibiotics are applied in fish farming through food, baths, or injections in aquatic organisms in order to control and limit aquaculture outbreaks impacting productivity, economy, environment, and health (6). However, drug intensive use creates reservoirs of antibiotic-resistant microorganisms and transferable resistance genes in bacteria residing in the aquatic environment. Hence, resistance genes may disseminate by horizontal gene transfer and reach human pathogens in the food chain or may reach the human directly (7). Consequently, the resistance phenomenon made antibiotics become no longer effective (2); the reason why many countries have banned their use in aquaculture as it damages not only public health but also the environment (8). Hence, we focused on the use of phytobiotics in aquaculture, especially essential oils (EOs) as eco-friendly alternative agents to antibiotics and chemical compounds for healthier cultivated fish. Their active molecules such as flavonoids, alkaloids, glycosides, organic acids, and tannins enhance many functions in aquatic animals comprising digestibility, metabolism, and growth performance with no environmental pollution and no drug-induced diseases. They may also improve fish immune defense, reduce oxidative stress, control microbial pathogens, and improve water quality (9). This study aims to analyze the chemical compositions of six EOs using gas chromatography/mass spectrometry and evaluate their antibacterial and antioxidant activities against five marine pathogenic strains responsible for serious infections in aquaculture species, affecting consumer's health besides analyzing their antibiotic resistance profile. Moreover, we aim to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of each EO.

## Materials and methods

### Preparation of phytobiotics

The phytobiotics used in this work were EOs, obtained from the dried raw material of clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum verum*), rosemary (*Rosmarinus officinalis*), artemisia (*Artemisia herba-alba*), cedarwood (*Cedrus atlantica*), and oregano (*Origanum compactum*) (Table 1). The botanical identification was carried out in Laboratory of plant, animal and agro-industry productions in Faculty of Sciences of Kenitra, University of Ibn Tofail (Morocco), where a collection of voucher specimens was deposited. EOs were extracted by 3.5 hours of hydrodistillation using the Clevenger apparatus and were obtained from the distillate with hexane and dehydrated through anhydrous sodium sulfate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35°C and the pure EOs were stored in sealed vials protected from light at 4°C until their use (10).

### Chemical analysis of essential oils

The GC/MS unit consisted of a Shimadzu GC-2010 gas chromatograph, equipped with DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; SGE, Ltd.), and interfaced with a Shimadzu QP2010 Plus mass spectrometer (software version 2.50 SU1). The oven temperature was programmed as described for GC analysis; transfer line temperature, 300°C; ion source temperature, 200°C; carrier gas, helium, adjusted to a linear velocity of 36.5 $cm.s^{-1}$; split ratio, 1:40; ionization energy, 70 eV; scan range, 45-400 u; scan time, 1 second.

Component identification was carried out by comparison of their retention indices relative to $C_9$-$C_{20}n$ alkanes on the DB-5 column (11), confirmed by the comparison of recorded mass spectra with those of a computer library (Shimadzu corporation library and NIST05 database/ ChemStation data system) and from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesized components, commercially available standards, and other literature data (12).

### Antibacterial activity tests

#### *Bacterial strains*

Bacterial strains used in this study belonged to the

**Table 1.** Plant species used for the extraction of essential oils

| Plant species | Family | Harvest site | Part used |
|---|---|---|---|
| *Syzygium aromaticum* | Myrtaceae | Fes | Buds |
| *Cinnamomum verum* | Lauraceae | Fes | Buds |
| *Rosmarinus officinalis* | Lamiaceae | Rabat | Aerial parts |
| *Artemisia herba-alba* | Asteraceae | Ouarzazate | Aerial parts |
| *Cedrus atlantica* | Pinaceae | Rabat | Leaves |
| *Origanum compactum* | Lamiaceae | Fes | Aerial parts |

collection of the Microbiology group of the University of Santiago de Compostela. They were isolated over years from rainbow trout (freshwater) and from marine farms, especially from turbot (*Scophthalmus maximus*), seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) during different episodes of mortalities in the Atlantic coast as well as the Mediterranean coast of Spain. *Vibrio anguillarum* AQV60.1; *Photobacterium damselae* subsp *damselae* AQP16.1; *Aeromonas salmonicida* RM305.1, and *Edwardsiella tarda* ACR333.1 were isolated in Trypticasein Soy Agar (Condalab, Spain) with 1% of Sodium Chloride (NaCl) added (TSA-1). *Lactococcus garvieae* CC30.1 was isolated from rainbow trout using TSA. All the strains were identified and kept frozen at -65°C in Trypticasein Soy Broth (Condalab, Spain) supplemented with 1% of NaCl and 15% of glycerol (TSB-1).

### *Antibiotic susceptibility test of the five isolated marine bacteria*

The susceptibility test was carried out using the agar diffusion assay. Susceptibility to the following antibiotics was tested: Amoxicillin (A; 25 µg), amoxicillin + clavulanate (AUG; 20 µg + 10 µg), penicillin (G; 10 units), cefixime (CFM; 5 µg), ceftazidime (CAZ; 30 µg), cefuroxime (CRX; 30 µg), cephalothin (KF; 30 µg), cephalexin (CFX; 30 µg), ciprofloxacin (CPR; 30 µg), norfloxacin (NOR; 2 µg), azithromycin (AZM; 15 µg), gentamycin (GM; 10 µg), piperacillin (PRL; 75 µg), oxacillin (OX; 1 µg), colistin (CT; 25 µg), tetracycline (T; 30 µg), vancomycin (VAN; 30 µg), kanamycin (KAN; 30 µg), imipenem (IMP), sulfamethoxazole + Trimethoprim (TS; 1.25 µg + 23.75 µg), and Amikacin (AK; 30 µg) (13).

### *Well diffusion assay*

A well diffusion assay was used to determine the growth inhibition of bacteria by EOs according to Klūga et al (14) method with some modifications. *V. anguillarum*, *P. damselae* subsp *damselae*, *A. salmonicida*, *E. tarda,* and *L. garvieae* were used as indicator strains in assays of antibacterial activity. They were cultured in TSB-1 at 22°C for 24 hours. Then, 20 mL of TSA-1 was inoculated with $10^6$ CFU of indicator strains fresh culture and was poured into a petri dish. Wells of 5 mm diameter were performed into the inoculated medium of each petri dish and filled with 10 µL of EOs. The plates were then incubated for approximately 24 hours at 22°C and the diameter of the inhibition zones was measured in mm.

### *Microatmosphere test*

The microatmosphere technique was used to assess the antibacterial activity of volatile compounds of EOs. Briefly, 20 mL of TSA-1 was inoculated with $10^6$ CFU of indicator strains fresh culture and was poured into a petri dish. Then, a sterilized filter paper disc was placed in the middle of the petri dish lid and soaked with 10 µL of EO. The plates were sealed with parafilm and incubated at 22°C for 24 hours. The absence of bacterial growth resulted in a translucent zone on the agar and its diameter was measured (15).

### *Microdilution assay*

A resazurin microtiter plate assay was used for the determination of the MIC of the EOs. The resazurin reagent was prepared at a concentration of 0.01% (w/v) and sterilized by filtration through a 0.22 µm membrane. 50 µL of TSB-1 was distributed in all wells of the microtiter plates; then 10 µL of EOs dissolved in 10% dimethylsulfoxide (DMSO) was added and serially diluted. 50 µL of $10^6$ CFU of indicator strains were added to all the wells and then incubated at 22°C for 24 hours. A 30 µL of 0.01% (w/v) resazurin solution was then added to each well and the plates were incubated for 4 h at 22°C. The controls used in each plate were: a column with all solutions except the bacterial culture, a column with DMSO solution as negative control, and a column with all solutions except the EOs (16). The MIC of each EO was determined; it represented the lowest concentration inhibiting visible bacterial growth, which corresponded to the absence of change in resazurin color.

### *Determination of the minimum bactericidal concentration*

The MBC of each EO represents the lowest concentration at which inoculated bacterial strains are completely killed. It was determined by inoculating 10 µL of each culture medium that presented no visible growth in the microtiter plates on TSA-1 and incubated at 22°C for 24 hours (17).

## Antioxidant activity

### *DPPH assay*

The DPPH free radical scavenging activity of EOs was carried out following the method presented by Bounatirou et al (18). 50 µL of each EO with different concentrations (0.25-4 mg/mL) was added to 1950 µL of DPPH ethanol solution and shaken. Then, the mixture was kept in the dark for 30 minutes at room temperature and the absorbance was detected with a spectrophotometer at 517 nm. Ascorbic acid was used as a positive control. The percentage of DPPH scavenging activity was calculated according to the following formula:

DPPH scavenging activity (%) = [(A0-At)/A0] x100

Where A0 = absorbance of the control sample and At = absorbance of each sample.

## ABTS radical scavenging assay

The ABTS free radical scavenging activity of EOs was carried out according to El Ghallab et al (19) procedure with some modifications. 20 µL of each EO with different concentrations (0.25-4 mg/mL) was added to 1980 µL of ABTS. After incubation in the dark at room temperature for 6 minutes, the absorbance was measured with a spectrophotometer at 734 nm. Ascorbic acid was used as a positive control. Then, the ABTS scavenging activity was

calculated according to the following formula:
ABTS scavenging activity (%) = [(A0-At)/A0] x100

Where A0 = absorbance of the control sample and At = absorbance of each sample.

### Statistical analysis

The experiments carried out in this study were conducted on triplicate and the results were given as means ± standard deviation (SD). Then, the data were analyzed using Prism 8.0 software using two-way ANOVA followed by Tukey's multiple comparisons test where a statistical significant difference was shown when $P < 0.05$.

## Results

### The chemical composition of essential oils

The percentage of compounds constituting each EO chemical GC/MS analysis of EOs is shown in Table 2. The results of the analysis showed the presence of diverse

**Table 2.** The main chemical constituents of essential oils identified by Gas Chromatography/Mass Spectrometry (GC/MS)

| Compounds | RI[a] | LRI[b] | *S. aromaticum* (Area%)[c] | *C. verum* (Area%) | *R. officinalis* (Area%) | *A. herba-alba* (Area%) | *C. atlantica* (Area%) | *O. compactum* (Area%) |
|---|---|---|---|---|---|---|---|---|
| Bicyclo[3.1.0]hexan-3-one | 793 | 790 | | | | 47.95 | | |
| β-Myrcene | 988 | 990 | | | | | | 2.86 |
| α-Pinene. | 932 | 930 | | | 10.66 | | | 0.83 |
| Camphene | 946 | 947 | | | 4.29 | 1.88 | | |
| β-Pinene | 974 | 975 | | | 4.23 | | | |
| (+)-4-Carene | 1003 | 1003 | | | | | | 2.83 |
| ortho-Cymene | 1022 | 1023 | | | 1.92 | 0.31 | | 14.59 |
| D-limonene | 1024 | 1026 | | 21.49 | 1.57 | | | |
| β-Phellandrene | 1025 | 1027 | | | | 3.34 | | |
| 1,8-Cineole | 1026 | 1028 | | | 46.27 | | | |
| γ-Terpinene | 1054 | 1056 | | | | | | 20.89 |
| β-Linalool | 1095 | 1094 | 0.29 | | 1.39 | | | 2.37 |
| α-Thujone | 1101 | 1104 | | | | 8.37 | | |
| Camphor | 1141 | 1142 | | | 14.76 | 14.75 | | |
| Borneol | 1165 | 1167 | | | 3.47 | | | 0.31 |
| Terpinen-4-ol | 1174 | 1176 | | | | 0.75 | | |
| α-Terpineol | 1186 | 1188 | | | 2.42 | | | 0.69 |
| D-(+)-carvone | 1256 | 1254 | | 71.78 | | | | |
| Thymol | 1289 | 1288 | 16.27 | | | | 1.10 | 5.29 |
| Carvacrol | 1298 | 1287 | | | | 0.86 | | 35.27 |
| Eugenol | 1356 | 1354 | 43.04 | 0.28 | | 1.04 | | |
| 1H-Indene, 2,3,3a,4,7,7a-hexahydro-2,2,4,4,7,7-hexamethyl- | 1367 | 1369 | | | | | 3.04 | |
| Longifolene | 1407 | 1409 | | | | | 18.35 | |
| Caryophyllene | 1408 | 1405 | 28.44 | 0.16 | 1.86 | | | 3.84 |
| α-Caryophyllene | 1417 | 1415 | 3.32 | | 0.21 | | | 0.19 |
| cis-(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9-tetramethyl(1H)benzocycloheptene | 1478 | 1480 | | | | | 10.64 | |
| (-)-β-himachalene | 1505 | 1506 | | | | | 47.00 | |
| δ-Cadinene | 1522 | 1524 | 0.53 | | | | 1.84 | |
| Eugenyl acetate | 1526 | 1527 | 4.25 | | | | | |
| Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- | 1528 | 1530 | | | | | 2.27 | |
| α-Calacorene | 1544 | 1546 | | | | | 1.16 | |
| Caryophyllene oxide | 1582 | 1584 | 0.29 | | 2.48 | | 0.38 | 0.99 |
| β-Himachaleneoxide | 1615 | 1618 | | | | | 1.08 | |
| α-Bisabolol | 1685 | 1687 | | | | | 1.05 | |
| Heptadecane | 1700 | 1702 | | | | 1.01 | | |
| 1-Tetracosanol | 2456 | 2455 | | | | | | 1.03 |

[a] Experimental linear retention index.
[b] Relative linear retention index to $C_9$–$C_{22}$ *n*-alkanes on the DB-5 column taken from Adams (11) for DB-5 capillary column in literature.
[c] Percentage values.

**Table 3.** Resistance of the studied strains to antibiotics

| Bacterial strain | Resistance profile |
|---|---|
| *V. anguillarum* | Trimethoprim + sulfamethoxazole (TS), cefixime (CFM), amikacin (AK), colistin (CT) |
| *P. damselae* subsp *damselae* | Trimethoprim + sulfamethoxazole (TS), cefixime (CFM), amikacin (AK), ceftazidime (CAZ), colistin (CT), cefuroxime (CRX), cephalothin (KF), cephalexin (CFX) |
| *A. salmonicida* | Cefixime (CFM), amikacin (AK), ceftazidime (CAZ), colistin (CT), cefuroxime (CRX), cephalothin (KF), cephalexin (CFX) |
| *E. tarda* | Cefixime (CFM), amikacin (AK), ceftazidime (CAZ), colistin (CT), cefuroxime (CRX), cephalexin (CFX) |
| *L. garvieae* | Kanamycin (KAN), oxacillin (OX), Trimethoprim + sulfamethoxazole (TS), gentamycin (GM), norfloxacin (NOR) |

**Table 4**. Antibacterial activity of essential oils against marine pathogenic strains

| Bacterial strains | *S. aromaticum* | *C. verum* | *R. officinalis* | *A. herba-alba* | *C. atlantica* | *O. compactum* |
|---|---|---|---|---|---|---|
| *V. anguillarum* | 12.66 ±0.44$^{a}$ | 31 ±0.0 | 12.66±0.44$^{a}$ | 9 ±0.0$^{a}$ | 8 ±0.0$^{b}$ | 40.33±0.44$^{a}$ |
| *P. damselae* subsp *damselae* | 22 ±0.66 | 28 ±0.0 | 13 ±0.0$^{a}$ | 8.66±0.44$^{ab}$ | 8.66±0.44$^{ab}$ | 26±0.0 |
| *A. salmonicida* | 11.33 ±0.44 | 27 ±0.66$^{a}$ | 14 ±0.0 | 9 ±0.0$^{a}$ | 9 ±0.0$^{a}$ | 27±0.0 |
| *E. tarda* | 12.33 ±0.44$^{a}$ | 26.33 ±1.55$^{a}$ | 12.66±0.44$^{a}$ | 9 ±0.0$^{a}$ | 9 ±0.0$^{a}$ | 40 ±0.0$^{a}$ |
| *L. garvieae* | 8±0.0 | 8±0.0 | 8±0.0 | 8±0.0$^{b}$ | 8±0.0$^{b}$ | 8±0.0 |

The inhibition diameter zones were measured in mm. Column with different superscript letters differs significantly ($P$ <0.05).

chemical constituents in the investigated EOs including phenolic compounds, monoterpenes and sesquiterpenes.

## Antibacterial activity of essential oils

### *Antibiotic sensitivity test*

First, the gram staining was carried out; the results revealed one gram-positive bacteria over five corresponding to *L. garvieae*. All these bacteria are pathogenic to fish in aquaculture and cause different infectious diseases such as furunculosis, pasteurellosis, vibriosis, lactococcosis and edwardsiellosis. Consequently, they lead to high mortality rates of fish. Then, the antibiotics profile resistance of each strain was conducted using several drugs, and the results were represented in Table 3.

The antibacterial activity of EOs was investigated *in vitro* against five pathogenic strains using the well diffusion technique, and then the inhibition zone diameters were measured as shown in Table 4. The results obtained by the well diffusion method showed that four EOs had the highest antibacterial activities against four strains. Oregano and cinnamon EOs were extremely effective, with inhibition zones ranging from 26 to 40 mm. The greatest inhibition diameter was obtained by oregano EO, which revealed the extreme sensitivity of *V. anguillarum, E. tarda, P. damselae* subsp *damselae,* and *A. salmonicida.* The less active EOs were cedarwood and artemisia, which showed inhibition zones of 8-9 mm against all the tested strains.

Moreover, the antibacterial activities against the five pathogenic strains were also assessed by gaseous contact of the EOs in order to evaluate the activities of their volatile compounds. Results showed that cinnamon and oregano out of six EOs possessed volatile compounds, which exhibited antibacterial activities against the tested strains, with inhibition zones ranging from10 to 25 mm, as shown in Table 5. They inhibited all strains by the microatmosphere method except *L. garvieae*, which correlates with the previous results of the well diffusion method, where *L. garvieae* was weakly inhibited.

Due to good inhibition of strains, the MIC and MBC of each EO was determined by the microdilution technique (Table 6). Oregano EO revealed a strong inhibition of bacterial growth, with MIC values ranging from 0.136 to 17.5 mg/mL and MBC values ranging from 0.273 to 35 mg/mL, while cedarwood EO was the less effective and showed the lowest antibacterial activity.

## Antioxidant activity

The antioxidant activity of EOs was carried out *in vitro* using the DPPH radical scavenging method. They exhibited variable degrees of scavenging activities. Clove EO showed the strongest scavenging capacity with a half maximal inhibitory concentration ($IC_{50}$) value of 0.413 mg/mL (0.015 mg/mL for ascorbic acid), followed

**Table 5.** Antibacterial activity of essential oils by microatmosphere method

| Essential oil | Bacteria | Diameter of inhibition (mm) |
|---|---|---|
| *O. compactum* | *V. anguillarum* | 14.33±1.11 |
| | *E. tarda* | 23±0.00$^{a}$ |
| | *P. damselae* subsp *damselae* | 23±0.66$^{a}$ |
| | *A. salmonicida* | 20.66±0.44 |
| *C. verum* | *V. anguillarum* | 9.33±0.44 |
| | *E. tarda* | 20±0.00$^{a}$ |
| | *P. damselae* subsp *damselae* | 21.33±0.44$^{a}$ |
| | *A. salmonicida* | 21±1.33$^{a}$ |

Column with different superscript letters differs significantly ($P$ <0.05).

**Table 6.** Minimum inhibitory concentration (MIC; mg/mL) and minimum bactericidal concentration (MBC; mg/mL) of essential oils against five marine pathogens

| Bacterial strains | *S. aromaticum* | | *C. verum* | | *R. officinalis* | | *A. herba-alba* | | *C. atlantica* | | *O. compactum* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *V. anguillarum* | 19.035[a] | - | 38.46[a] | 19.23[a] | 33.46 | - | 36.92[b] | - | 110.76[a] | - | 0.136 | 0.273 |
| *P. damselae* subsp *damsela*e | 38.07[b] | 38.07[a] | 38.46[a] | 38.46[a] | 33.46[a] | - | 92.30[a] | - | 110.76[a] | - | 17.5[a] | 4.375[a] |
| *A. salmonicid*a | 4.758 | 38.07[a] | 19.23 | - | 33.46[a] | - | 92.30[a] | - | 110.76[a] | - | 17.5[a] | 35[b] |
| *E. tard*a | 19.035[a] | 19.035 | 38.46[a] | 38.46[a] | 33.46[a] | - | 36.92[b] | - | 92.30 | - | 17.5[a] | 35[b] |
| *L. garvieae* | 38.07[b] | 38.07[a] | 115.38 | - | 50.19 | - | 110.76 | - | 110.76[a] | - | 4.375 | 4.375[a] |

Column with different superscript letters differs significantly ($P$ <0.05).

by cinnamon EO with an $IC_{50}$ value of 3.223 mg/mL. On the other hand, clove, cinnamon and oregano EOs exhibited good scavenging activities against free radicals produced by ABTS (Figure 1). Using this assay, the highest antioxidant capacity was obtained with clove, with an $IC_{50}$ value of 0.457 mg/mL.

## Discussion

EOs represent a source of natural substances with diverse chemical structures, which can provide a broad spectrum of biological activities. In this study, six EOs were investigated, their chemical composition was carried out by GC/MS, which showed variability within the identified compounds. The constituents present at the highest concentrations at clove EO were Eugenol (43.04%), Caryophyllene (28.44%), and Thymol (16.27%). Cinnamon had D-(+)-carvone (71.78%) as the major chemical component with D-limonene 21.49%. Oregano EO was mainly composed of carvacrol (35.27%), γ-terpinene (20.89%) and ortho-cymene (14.59%), while cineole (46.27%), camphor (14.76), and a-pinene (10.66%) were present at high concentrations in rosemary. Cedarwood had (-)-β-himachalene (47%) and Longifolene (18.35%) as the major constituents, while artemisia had Bicyclo[3.1.0] hexan-3-one at a high concentration (47.95%) in addition to camphor (14.75%). In general, the various biological activities characterizing the EOs are related to their high yield chemical constituents (20,21).

The emergence of antibiotic resistant bacteria has been dramatically increasing in the aquaculture sector because of the extensive use of drugs. Consequently, it has resulted in the increase of antibiotic resistance in fish pathogens, the transfer of the resistance determinants, and finally, the development of serious health problems not only in cultivated fish but also in human (22). In this study, the resistance profile to antibiotics determined for each pathogenic strain clearly showed their multiresistance. Thus, we studied the antibacterial and antioxidant activities of six EOs to evaluate their potential use as phytobiotics in aquaculture.

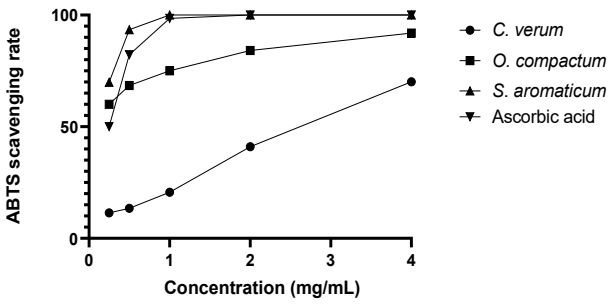


**Figure 1.** Antioxidant activity of essential oils with ABTS assay. ABTS = 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

The antibacterial activities of clove, cinnamon, rosemary, artemisia, cedarwood, and oregano were performed and the inhibition diameters were measured. EOs exhibited a broad spectrum inhibitory effect. Oregano EO was the most effective against four strains with inhibition diameters ranging from 27 to 40 mm. It showed a strong bactericidal activity against *V. anguillarum* with MIC value of 0.136 mg/mL and MBC value of 0.273.

EOs possess various antimicrobial potentials and are known to act differently. This may be by disrupting the cell wall, membrane permeabilization, targeting drug efflux pumps, targeting quorum sensing, biofilms, and R-plasmids (23). Many studies have reported that EOs are more effective against gram-positive than gram-negative bacteria, possibly due to the different compositions in the cell walls, as gram-negative bacteria possess a rigid outer membrane rich in lipopolysaccharides limiting the diffusion of hydrophobic compounds through it, making these bacteria less susceptible (24,25). In our study, the EOs were effective on both gram-positive and gram-negative bacterial pathogens, as shown clearly in the results of the microdilution assay. Eugenol, linalool, α-terpineol, thymol, 1,8-cineole, camphor, and carvacrol are among the bioactive components, which have prominent inhibitory effect against bacteria (26). This broad range of compounds in EOs make their antibacterial activity not reliant on one specific mode of action; they exhibit various mechanisms and can attack several targets in a cell to inactivate the bacterium (27). Additionally, the potential inhibitory mechanisms regarding microbial growth may be due to the synergistic effect of a complex mixture of terpenoids and phenolic acids in EOs (28). For example, the presence of phenolic content in EOs displayed more specificity for the inhibition of microbial growth based

on their effective hydroxyl group in chemical structures, which help in disruption of plasma membrane structure and thus disorganize the membrane permeability, while terpenoids could affect the plasma membrane fatty acids leading to alteration in membrane dynamicity, permeability, and leakage of cytoplasmic constituents (29).

Moreover, some EOs are known to possess a bioactive vapor phase, which does not require direct contact to exhibit antimicrobial activity. Thus, the microatmosphere assay was performed to evaluate this hypothesis for our EOs. In this study, oregano and cinnamon EOs possessed volatile compounds that inhibited the bacterial growth of the tested strains with inhibition zones ranging from 10 to 23 mm.

Some fish species are exposed to the imbalance between the production of reactive oxygen species and the antioxidant defense system. This can lead to cell damage to the structure of nucleic acids, lipids, and proteins, causing cell death (3). Thus, in this study, we evaluated the antioxidant activity of EOs using two assays: DPPH and ABTS. The DPPH assay measures the capacity of the extracted EO to donate hydrogen to the DPPH radical, resulting in color depletion of the solution. The higher antioxidant activity reflects the lowest $IC_{50}$. EO showed the strongest radical scavenging effect (73.58%) at 4 mg/mL followed by cinnamon EO (55.14 mg/mL). Moreover, clove EO also exhibited the highest scavenging activity against free radicals produced by ABTS, and revealed the lowest $IC_{50}$ value of 0.457 mg/mL. These results correlate with previous studies confirming that antioxidant activities follow a pattern of dose dependent type (30). Hence, the potent antioxidant activities of EOs may strongly be due to their composition from terpenes and terpenoids, such as monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes (31). Finally, previous studies have reported that EOs are able to provide other biological functions (32). They can provide immunostimulating and anti-stress effects, so they can be used as phytobiotics in aquaculture to improve the immune and physical status of fish through appetite stimulation and growth promotion (13).

## Conclusion

The antibacterial and antioxidant activities of the investigated EOs against marine pathogens showed a good potential to substitute drugs in order to limit the emergence of antibiotic resistant microorganisms and solve the oxidative stress outbreaks occurring in aquaculture field. Therefore, further *in vivo* studies are needed to study the possibility of applying these phytobiotics in aquaculture for a better quality of fish products.

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## Authors' contributions

AC performed the work, analyzed the results, and drafted the manuscript. AE analyzed the chemical composition of EOs by GC/MS. BEK contributed in conception and review. MRM, JLB, and AS conducted critical reviews to the manuscript prior to submit to the journal.

## Conflict of interests

Authors have no conflicts of interest to declare.

## Ethical considerations

All authors have inspected the ethical issues of plagiarism, misconduct, data fabrication, falsification, double publication or redundancy related to the manuscript.

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