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Antibacterial activities and Chemical Composition of Essential Oils of *Cupressus sempervirence* L. and *C. funebris* Endl. in Khuzestan, Iran

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Abstract

Essential oils of different plants are widely used in the pharmaceutical and food fields. The chemical composition of essential oils of *Cupressus sempervirence* L. and *C. funebris* Endl. Were obtained by a Clevenger apparatus and analyzed by chromatography–mass spectrometry (GC/MS) to assess the composition of the essential oils, study antimicrobial properties, and compare the effect of the essential oils of two *Cupressus* species with imipenem as a carbapenem antibiotic on wound infections. The essential oil efficiency was estimated at a rate of 0.3%. In total, ten compounds were identified from the essential oils of each species. The results showed that *C. sempervirens* mainly consisted of 21.5% totarol, 15.54% delta-3-carene, 14.37% α-pinene, and 11.78% phenanthrene; *C. funebris* mainly contained 24% α-cedrol, 18.11% naphthalene, 12.96% α-pinene, 10.05% delta-3-carene, and 9.3% α-cedrene. The results of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) showed that the essential oils of two species could inhibit the growth of most strains of *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 (DSM 50071^T). Time-killing assay revealed that essential oils further reduced bacterial colony growth after 24 hours' incubation compared to imipenem. However, the essential oils of two Cupressus species showed more efficient bactericidal effects versus imipenem.

1. Introduction

Aromatic plants with medicinal properties are widely used in traditional therapy, so many researchers have focused on plant compounds with natural antimicrobial activity. The Cupressaceae family, known as Cypress trees, are usually cultivated as ornamental trees, but their aerial parts have been widely used in folk medicine. These species have important biological and medicinal properties and are used to treat many diseases, such as colds, flu, rheumatism, suffumigation, antitussives, toothache, and gum ulcers. The essential oil of *Cupressus* species has antimicrobial, antiviral, and antifungal activity (Selim et al., 2014; Nouri

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et al., 2015; Saad et al., 2017) and is consumed as a natural food and medicine preservative. These species are also widely used in the cosmetics industry, such as skin tightening, skin inflammation, anti-dandruff, and anti-aging (Larousse, 2001; Emami et al., 2004; Selim et al., 2014). They can also be used as a natural pesticide (Kabiri et al., 2014), to control plant pathogens, and to manage human diseases as an astringent, anti-inflammatory, anti-flatulence, and dyspepsia (Milos et al., 2002; Boukhris et al., 2012; Salem et al., 2018; Nozohour et al., 2019).

The major components of different aerial parts of *C. sempervirens* and *C. atlantica* are α-pinene, δ-3-carene, limonene, α-terpinolene, germacrene, delta-cadinene, α-phellandrene, δ-cadinene, β-caryophyllene, and α-humulene (Ariouni et al., 2011; Fadel et al., 2021). *C. funebris* is a main source of cedar wood oil in China and rich in α-cedran, β-cedran, thujopsene and cedrol (Adams & Li, 2008).

The composition of essential oils is affected by the type of organ, various environmental factors, and some agricultural factors (Asgary et al., 2013; Venditti et al., 2018; Fadel et al., 2021).

The best-known and most widespread species in Khuzestan Province (Iran) are *C. sempervirens* L. and *C. funebris* Endl. They are medium-sized coniferous, evergreen trees up to 35 m tall and are slightly aromatic. This study aimed to identify the chemical composition of the essential oils of these species and the estimation of their antibacterial activity against pathogenic microbial strains.

1. Material and Methods

1.1. Plant materials

The studied plant species were collected from the Agricultural Sciences and Natural Resources University of Khuzestan in Mollasani, located 35 km from Ahvaz, Khuzestan province. Fresh leaves and stems were air-dried and powdered by an electrical herb grinder.

1.2. Extraction of essential oil

Essential oils of leaves and stems were isolated by the Clevenger apparatus (Egharevba et al., 2015). For this purpose, 50 g of dried powder and 1500 mL of distilled water were added to balloons. After a 5 h extraction, essential oils were collected in a container. Essential oils obtained after dehydration with anhydrous sodium sulfate were injected into the GC/MS system. Extraction was repeated three times (Elyemni et al., 2019).

1.3. Gas chromatography/mass spectrometry (GC/MS) analyses

Essential oils were analyzed using the GC/MS system (Agilent, Palo Alto, CA, USA), with a Capillary HP – 5MS column (30 m length, 0.25 mm diameter), with helium (0.8 ml/min) as the carrier gas. The oven temperature program was as follows: 5 min at 50 ˚C ramped from 50 to 240 at 3 ˚C min^{-1} and 240 ˚C to 300 ˚C at 15 ˚C min^{-1}. The temperature of the carrier gas was regulated at 290 ˚C. A comparison of the mass spectra and retention index based on relative retention times of saturated hydrocarbons with standard references was used to identify the essential components (Carroll et al., 2011; Elyemni et al., 2019).

1.4. Bacterial strains and culture media

The clinical isolates of *Pseudomonas aeruginosa* were obtained from specimens from patients at the Baqiyatallah Hospital of the Baqiyatallah University of Medical Science. Five clinical isolates of *P. aeruginosa* (Schröter) Migula and a standard strain of *P. aeruginosa* (ATCC 27853) were used for the antibacterial experiments. The standard strain was obtained from the Iranian Research Organization for Science and Technology (IROST). Antibacterial activities of *C. sempervirens* and *C. funebris* were examined against clinical bacterial isolates and a standard strain. Mueller Hinton agar (MHA, Merk Germany) and Mueller Hinton broth

(MHB, Merk Germany) were used for relevant antibacterial experiments (Hall, 2015).

1.5. MIC and MBC evaluations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *C. sempervirens* and *C. funebris* and imipenem were compared against the clinical isolates and the standard strain using broth microdilution following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Initial bacterial inoculum and evaluation of MIC and MBC were performed according to Boukhris et al. (2012).

1.6. Time-killing assay

The bactericidal activity of *C. sempervirens* and *C. funebris* against clinical isolates of P. aeruginosa was also evaluated using the time-killing assay method. The isolates were cultured in Muller-Hinton broth (MHB) to obtain the exponential phase through cultivation. Preparation of bacterial suspension, determination of MICs concentration of *C. sempervirens*, *C. funebris* and imipenem, and obtaining the bacterial cell density were accomplished according to Balouiri et al. (2016) and Shahbandeh et al. (2021).

2. Results and Discussion

2.1. Chemical composition

The essential oil content was 0.3% of dry weight (in three replicates). In total, ten compounds were isolated from *C. sempervirens* and *C. funebris* essential oils. Results showed that *C. sempervirens* mainly contained about 21.5% Totarol (a natural meroterpene) as a major constituent and 15.54% delta-3-carene (a natural bicyclic monoterpene), 14.37% α-pinene (an organic compound of the terpene), and 11.78% phenanthrene (a polycyclic aromatic hydrocarbon). The other compounds were found in smaller amounts (ranging from 4-6.4%) (Table 1). The GC Chromatogram of *C. sempervirens* is shown in (Fig 1). The most important compounds in *C. funebris* were 24% α-Cedrol (sesquiterpene alcohol), 18.11% naphthalene, 12.96% α-Pinene, 10.05% delta-3-carene, and 9.3% α-Cedrene. All other compounds were found in smaller amounts (ranging from 2.5-6.1%) (Table 2).

These compounds are often terpenoid, which plays an important role in the interaction between plants and pathogens (Filho et al., 2011). Terpenoids comprise the main part of plant essential oils and have antioxidant, anti-cancer, and anti-diabetic properties. Active oxygen includes a set of chemical substances derived from molecular oxygen with high reactivity, which are involved in the pathological improvement of many diseases, such as neurological diseases, cardiovascular problems, diabetes, and other diseases. The best strategy to prevent oxidative damage is to use compounds with antioxidant properties (González-Burgos & Gómez-Serranillos, 2012; Cox-Georgian, 2019). These results are highly consistent with other research results (Boukhris et al., 2012; Riahi et al., 2012; Nouri et al., 2015; Fadel et al., 2021).

No	RT (min)	RI	Compound %	Compounds
1	10.06	902	14.37	α-Pinene
2	13.78	975	15.54	delta-3-carene
3	14.74	994	6.37	Limonene
4	17.74	1052	4.66	α-Terpinolene
5	25.35	1203	4.00	Carvacrol methyl ether
6	30.13	1305	5.86	α-Terpinyl acetate
7	52.91	1891	5.48	Propanamide
8	53.92	1921	10.45	Data MS
9	55.88	1982	11.78	Phenanthrene
10	63.19	2251	21.50	Totarol

Table 1. Chemical composition of essential oil of *C. sempervirens* L. obtained by GC-MS System

RI = Retention index, RT=Retention time

Table 2. Chemical composition of essential oil of *C. funebris* Endl. obtained by GC-MS System

No	RT (min)	RI	Compound %	Compounds
1	5.854	800	7.30	Data MS
2	10.08	902	12.96	α-Pinene
3	13.8	976	10.05	delta-3-carene
4	14.76	994	5.90	Limonene
5	17.76	1053	3.78	α-Terpinolene
6	30.13	1305	2.56	3,4-Methylenedioxyamphetamine
7	32.79	1365	9.30	α-Cedrene
8	33.13	1373	18.11	Naphthalene
9	34.51	1404	6.04	α-Humulene
10	40.45	1548	24.00	α-Cedrol

RI = Retention index, RT=Retention time

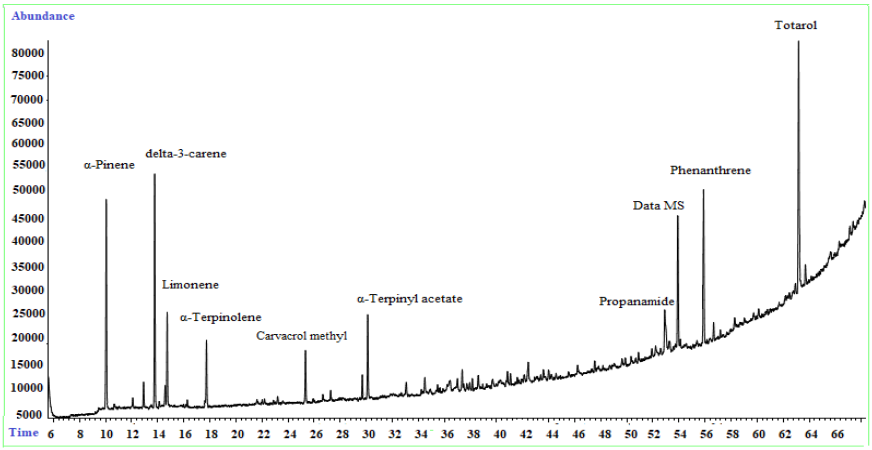

Figure 1. GC Chromatogram of *C. sempervirens* L. The horizontal axis shows the retention time (min), and the vertical axis shows the intensity (abundance) of the signal.

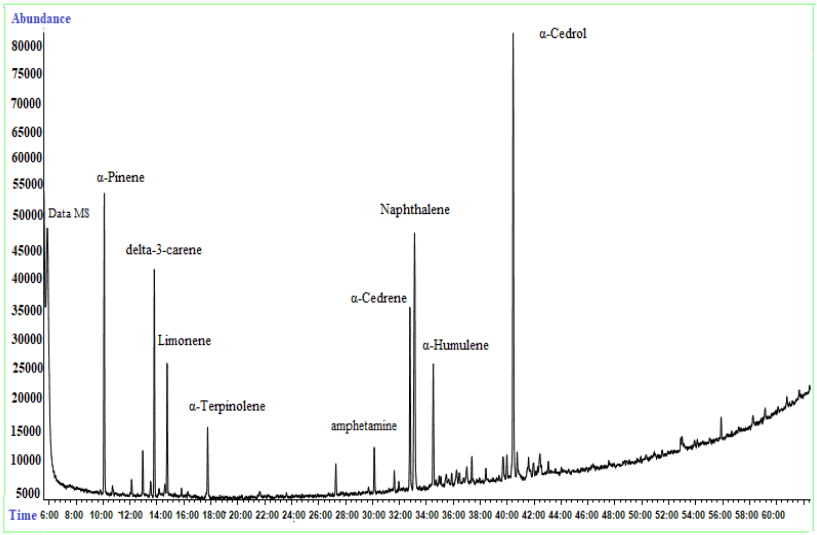

Figure 2. GC Chromatogram of *C. funebris* Endl. The horizontal axis shows the time of retention (min), and the vertical axis shows the intensity (abundance) of the signal.

2.2. Antibacterial activity

The MIC and the MBC determination and the time-killing assay of *C. funebris*, *C. sempervirens*, and imipenem against *P. aeruginosa* are shown in (Fig 3) and 4, respectively. Based on the obtained results, the MICs of *C. funebris* and *C. sempervirens* essential oils on standard *P. aeruginosa* bacteria were 64 and 32 µg/ml, respectively. Also, MIC results indicated that the essential oil of *C. sempervirens* could inhibit the growth of strains 2, 3, and 4 with concentrations of 64, 64, and 32 µg/ml, respectively. These results revealed that *C. sempervirens* is more effective and has more inhibitory effects compared to *C. funebris*.

The MBC values of *C. funebris* and *C. sempervirens* essential oils against the ATCC strain were 128 and 64 µg/ml, respectively. Also, they have a bactericidal effect against all clinical strains of *P. aeruginosa*, which was more effective than imipenem due to lower MBC value. The essential oils of the two species destroyed strain 2 with a concentration of 128 µg/ml. Also, the essential oil of *C. sempervirens* was able to destroy strains 3 and 4 with a lower concentration than *C. funebris.* The results of MICs and MBCs showed that the two mentioned Cypress extracts have better antibacterial activity than imipenem.

The time-killing assay is important for post-treatment analysis to determine the viability time of bacteria and to determine the minimum time to achieve an inhibitory effect on their growth. In this study, the counting of *P. aeruginosa* colonies indicated that the essential oils of two species of Cypress and imipenem were able to reduce the number of colonies to zero after 24 hours. Also, these results demonstrated that the essential oil of the two species was more effective and able to reduce the speed of bacterial colony growth faster than imipenem after the first hour.

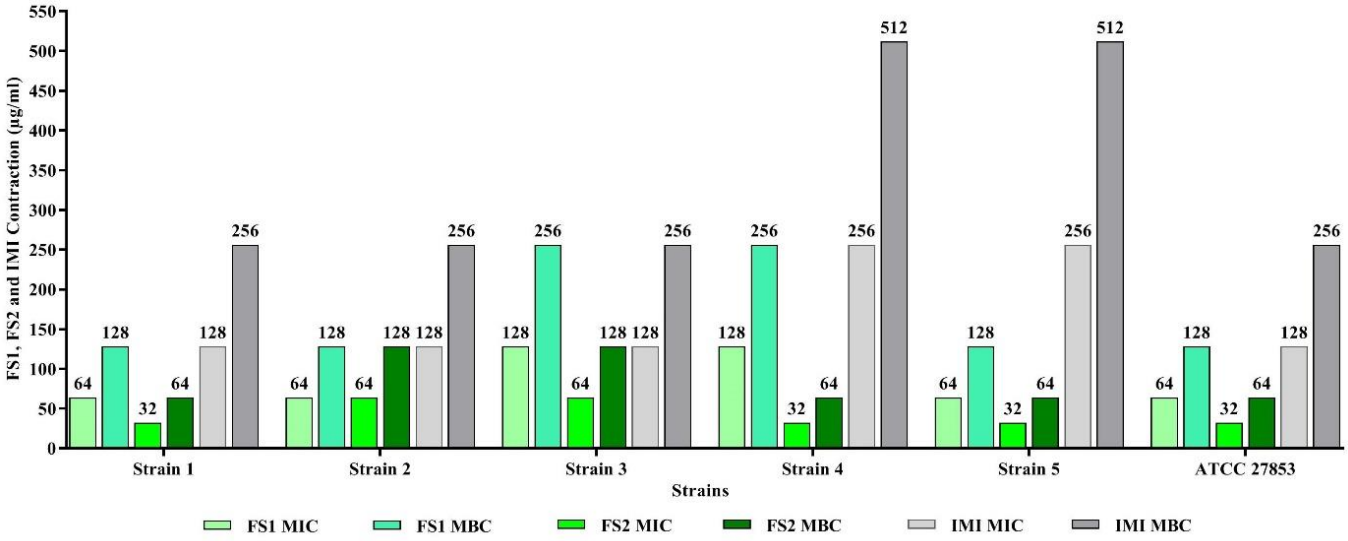

Figure 3. MICs and MBCs of the essential oil from *C. funebris* (FS1) and *C. sempervirens* (FS2), and imipenem (IMI) against *P. aeruginosa* strains.

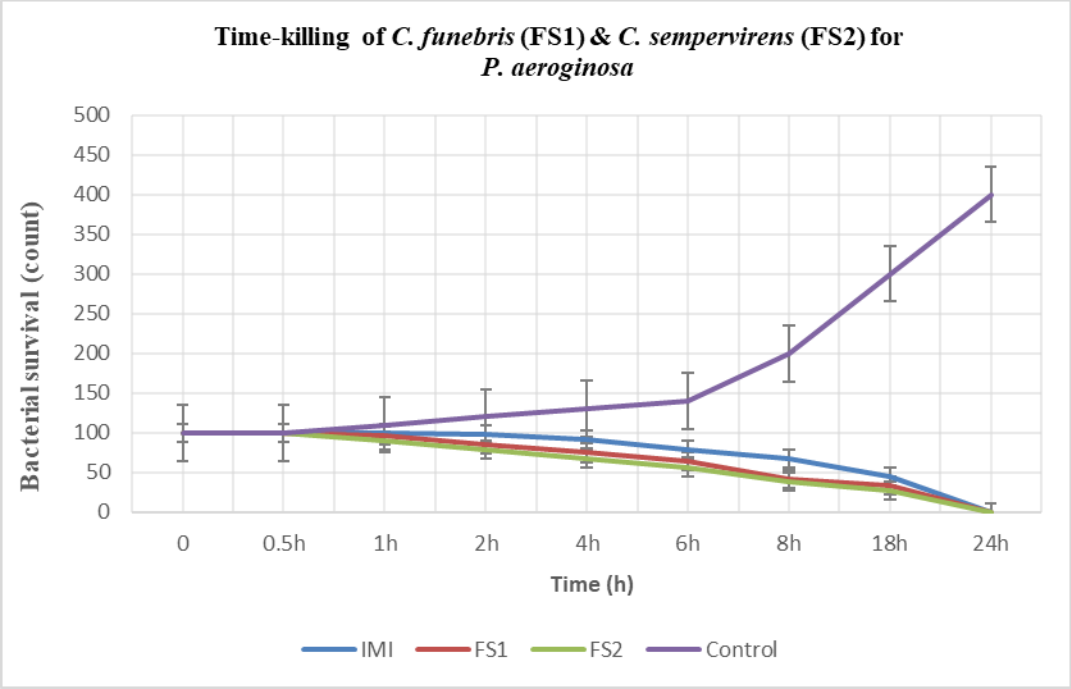

Figure 4. Time-killing assay for *P. aeruginosa* treated with the *C. funebris*, *C. sempervirens*, and imipenem. As shown, essential oils are more efficient in inhibiting bacteria growth than imipenem (as a positive control).

4. Conclusion

The high frequency of MDR strains of K. pneumoniae in different clinical sources in Isfahan, Iran, is significant. The highest frequency observed in urinary tract infections should be carefully considered by healthcare systems. The frequency of more than 90% of fimH and wabG genes in the MDR isolates should also be considered in the infection diagnostic, and future study on alternative treatment protocols that can overcome the virulence factors is proposed.

Conflict of interest

All authors declare that there are no conflicts of interest associated with this manuscript

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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