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Chemical composition analysis of essential oils of four plants from Aurès region of Algeria and their antibacterial and antibiofilm activities against coagulase-negative staphylococci

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Abstract:

Background: The altitudinal and geographical variability of the Aurès mountains of Algeria favored the existence of some endemic and rare varieties of medicinal plants. The aim of the present work is to determine the chemical composition, antimicrobial and antibiofilm properties of the essential oils (EOs) from aerial parts of four medicinal plants from Aurès region of Algeria; *Juniperus thurifera* L., *Juniperus oxycedrus* L., *Salvia officinalis* L. and *Thymus ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. on coagulase negative staphylococci (CoNS) isolates.
Methodology: Extraction of EOs from the four plant materials was carried out by hydro-distillation, and the EO yield expressed in gram of the distillate per 100 grams of dry matter. The chemical composition of the EOs was analyzed by gas chromatography-mass spectrometry (GC-MS) method. *In vitro* antibacterial and antibiofilm activities of the EOs were evaluated against CoNS previously isolated at the Anti-Cancer Center of Batna, Algeria using the agar disc diffusion assay and biofilm inhibition study, respectively. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the EOs of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* were determined by the dilution method.
Results: Twenty-seven and 41 compounds rich in monoterpene hydrocarbons were identified from *J. oxycedrus* and *J. thurifera* plants respectively, while 45 and 32 compounds, constituted mainly by oxygenated mono-terpenes, were identified from *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus*, respectively. The EOs of *T. ciliatus* ssp. *munbyanus* showed the most inhibitory activity of all the four plants on CoNS isolates (n=66) with mean inhibition zone diameter of 24.99±6.29mm, and mean MIC and MBC values of 2.65±3.77mg/ml and 5.31±7.41mg/ml respectively, followed by *S. officinalis* L., with mean inhibition zone diameter of 13.38± 6.52mm, and mean MIC and MBC values of 27.53±28.2 mg/ml and 31.97±33.19 mg/ml respectively (p<0.0001 by one-way ANOVA). Also, percentage biofilm inhibition of CoNS isolates (n=59) was high for EOs of *T. ciliatus* ssp. *munbyanus* (65.63±10.71%) and *S. officinalis* L. (53.13±5.83%), although was significantly higher for *T. ciliatus* ssp. *munbyanus* compared to *S. officinalis* L. (p<0.0001, t=7.874).
Conclusion: Essential oils from *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. could represent an alternative to classical antibiotics against planktonic cells and biofilms of CoNS.

Keywords: coagulase-negative staphylococci; chemical composition; essential oils; antibacterial activity; antibiofilm activity

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Analyse de la composition chimique des huiles essentielles de quatre plantes de la région des Aurès en Algérie et leurs

activités antibactériennes et anti biofilm contre les staphylocoques à coagulase négative

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Résumé:

Contexte: La variabilité altitudinale et géographique des montagnes des Aurès en Algérie a favorisé l'existence de certaines variétés endémiques et rares de plantes médicinales. L'objectif de ce travail est de déterminer la composition chimique, les propriétés antimicrobiennes et antibiofilm des huiles essentielles (HE) des parties aériennes de quatre plantes médicinales de la région des Aurès en Algérie; *Juniperus thurifera* L., *Juniperus oxycedrus* L., *Salvia officinalis* L. et *Thymus ciliatus* ssp. *Munbyanus* (Boiss. & Reut.) Batt. sur des isolats de staphylocoques à coagulase négative (SCN).
Méthodologie: L'extraction des HE des quatre matières végétales a été réalisée par hydro-distillation, et le rendement en HE exprimé en gramme de distillat pour 100 grammes de matière sèche. La composition chimique des HE a été analysée par la méthode de chromatographie en phase gazeuse-spectrométrie de masse (GC-MS). Les activités antibactériennes et antibiofilm *in vitro* des HE ont été évaluées par rapport à la SCN précédemment isolée au Centre anticancéreux de Batna, en Algérie, en utilisant respectivement le test de diffusion sur disque d'agar et l'étude d'inhibition du biofilm. Concentration minimale inhibitrice (CMI) et concentration minimale bactérienne (CMB) des HE de *S. officinalis* L. et *T. ciliatus* ssp. *munbyanus* ont été déterminés par la méthode de dilution.
Résultats: Vingt-sept et 41 composés riches en hydrocarbures monoterpéniques ont été identifiés chez les plantes *J. oxycedrus* et *J. thurifera* respectivement, tandis que 45 et 32 composés, constitués principalement de monoterpènes oxygénés, ont été identifiés chez *S. officinalis* L. et *T. ciliatus* ssp. *munbyanus*, respectivement. Leuile de *T. ciliatus* ssp. *munbyanus* a montré l'activité la plus inhibitrice des quatre plantes sur les isolats SCN (n=66) avec un diamètre moyen de la zone d'inhibition de 24,99±6,29 mm et des valeurs moyennes de CMI et CMB de 2,65±3,77 mg/ml et 5,31±7,41 mg/ml respectivement, suivi de *S. officinalis* L., avec un diamètre moyen de la zone d'inhibition de 13,38±6,52 mm, et des valeurs moyennes de CMI et CMB de 27,53 ±28,2 mg/ml et 31,97±33,19 mg/ml respectivement (p<0,0001 par ANOVA à un facteur). De plus, le pourcentage d'inhibition du biofilm des isolats de SCN (n=59) était élevé pour les HE de *T. ciliatus* ssp. *munbyanus* (65,63±10,71%) et *S. officinalis* L. (53,13±5,83%), bien qu'il soit significativement plus élevé pour *T. ciliatus* ssp. *munbyanus* par rapport à *S. officinalis* L. (p<0,0001, t=7,874).
Conclusion: Les huiles essentielles de *T. ciliatus* ssp. *munbyanus* et *S. officinalis* L. pourraient représenter une alternative aux antibiotiques classiques contre les cellules planctoniques et les biofilms de SCN.

Mots clés: staphylocoques à coagulase négative; composition chimique; huiles essentielles; activité antibactérienne; activité antibiofilm

Introduction:

Coagulase-negative staphylococci (Co NS) are very widespread in nature (air, soil, water), and also part of the normal flora of the skin and mucous membranes of mammals and birds. This flora plays an important role in the physicochemical balance of the skin and constitutes a barrier against bacteria of the transient flora (1). The emergence of CoNS as pathogens for various infections may be the result of the increasing use of invasive procedures such as catheters and intravascular prostheses, intensive care unit (ICU) treatment of patients with cancers, transplant recipients, immunocompromised states and premature children (2). In addition, the production of biofilm has been considered as an important factor in the pathogenesis of CoNS, protecting against antibiotics and the immune system (3). This situation has forced scientists to look for new alternative strategies to eliminate these bacteria that are resistant to antibiotics and producing biofilms (4). A possible approach is the use of medicinal plants, which are good sources of new antimicrobial chemotherapeutic agents, in particular, essential oils (EOs).

Four plant species from the Aurès mountains of Algeria are widely used for many therapeutic properties; *Juniperus oxycedrus* L. and *Juniperus thurifera* L. from the Cupressaceae family, and *Salvia officinalis* L. and *Thymus ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. from the Lamiaceae family. *Juniperus oxycedrus* L., juniper (also known as cade tree) is a tree that can reach up to 8m in height and is native to the Mediterranean region (5). The trunk has a coat of grey to reddish-brown fibrous bark in longitudinal stripes, and has many branches, spreading or ascending. The leaves are similar to needles and alternate in three turns. The needles are 1 to 2.5 cm long and 1 to 2.5 mm wide, with two furrows of white, waxy stomata above, an edge below and a thorny tip. This tree is used in traditional medicine for the treatment of various diseases such as hyperglycaemia, obesity, tuberculosis, bronchitis and pneumonia (6).

The Thuriferous Juniper (*J. thurifera* L.) known as Aiwal or Hazenzna in Berber (7) is a tree or shrub of the Cupressaceae family which grows only in isolated parts of the western Mediterranean basin; France, Italy and Spain in Europe, and Algeria and Morocco in North Africa (8-9). In Algeria, this species can be found associated with cedar and its areal is strictly limited to the Aures mountains with a number of scattered and often very large trees, which are probably the remains of more extensive juniper stands (8). It is a dioecious tree or shrub, with scale leaves and bluish black berries at maturity (9). Different species of Juniperus have been used in traditional medicine for centuries as incense, diuretics, remedies for indigestion (10), cough suppressants, anti-fertility, anti-tuberculosis, colds, dysentery, leukorrhea, rheumatic arthritis and fever (11).

Salvia officinalis L. (sage, garden sage, or common sage) is a perennial, evergreen shrub (12), grey-green and with wrinkles on the upper surface and in the lower surface are almost white with much shorter soft fluff (13), woody stems, and blue flowers with purplish colour (12-14). *S. officinalis* is part of the Lamiaceae family (14), and this species is generally cultivated, but also grows spontaneously in the wild in different geographical areas. It is encountered in the glades, forests, scrub, grasslands, steppes, plains, highlands and mountains up to 2500m altitude. Sage is characterized by a very widespread distribution, and is mainly found in Yugoslavia, Bulgaria, France, Italy, USA, India, Spain, United Kingdom, Turkey, Morocco, Greece, South Africa, South America, and South East Asia (15). *S. officinalis* has various uses, essentially as herbal remedy for a wide range of disorders and diseases by applying it internally or externally. It is used as a diuretic, tonic, pain relief styptic, antiseptic, anti-inflammatory, anti-fungal and as anti-spasmodic. It is also used as a treatment for dysentery, cough, ulcers, varicose veins, insect bites (16), obesity, diabetes, depression and cancers in ancient times (14).

Thymus (*Thymus* L.) is a large genus of the Lamiaceae family encompassing about 215 medicinal and aromatic species, and 20 species have been reported in Algeria. *T. ciliatus* ssp. *munbyanus* locally known as "Zaatar", is a fragrant subshrub, with flowers 16 to 20 mm long, pooled in false whorls; leaves are more or less contracted, with their accompanying flowers being morphologically different from those inserted on the stem, which are generally wider at the base (17). Thymus is largely used in traditional Algerian medicine for its expectorant, antitussive, anti-bronchiolitic, anti-spasmodic, anti-helminthic, carminative and diuretic properties (18). This species has been used in the Aurès region (Eastern Algeria) as a traditional remedy for bronchitis, lung infections, influenza, cough and certain gastrointestinal disorders (19).

The objective of the present study is to determine the chemical composition, antibacterial and antibiofilm activities of essential oils (EOs) extracted from *J. oxycedrus* L., *J. thurifera* L., *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. on coagulase negative staphylococci (CoNS) previously isolated at the Anti-Cancer Center of Batna, Algeria (20).

Materials and Method:

Bacterial isolates

Sixty-six previously identified clinical CoNS isolates by Zatout et al., (20) were used to test for antibacterial activity of the EO extracts of the plants, and among them, those showing biofilm production ability (59 isolates) were used to test for anti-biofilm activity of the EOs (Table 1).

Table 1: CoNS isolates used to evaluate antibacterial and antibiofilm activities of essential oils

CoNS species	Number	
	Antibacterial activity	**Antibiofilm activity**
S. epidermidis	29	27
S. haemolyticus	15	13
S. hominis	8	6
S. chromogenes	6	6
S. xylosus	4	3
S. capitis	1	1
S. saprophyticus	1	1
S. cohnii	1	1
S. simulans	1	1
Total	**66**	**59**

CoNS = coagulase negative staphylococci; S = *Staphylococcus*

Plant materials

The plant materials consist of leaves of *S. officinalis* L. and *T. ciliatus* ssp. *munby-*

anus (Boiss. & Reut.) Batt. from the Lamiaceae family, and *J. thurifera* L. and *J. oxycedrus* L. from the Cupressaceae family. The botanical identification of the four plants was carried out by Pr. Oudjehih B., Botanical Laboratory, Department of Agronomy of the University of Batna 1. The leaves of *J. oxycedrus* and *J. thurifera* were collected from the region of El Mahmel, Batna in October 2016 while the leaves of *S. officinalis* and *T. ciliatus* ssp. *munbyanus* were harvested in April 2017 from the region of El Madher and Tazoult respectively. The leaves of the four plants were washed, dried in the shade and then grinded using an electric grinder, and the powders obtained are kept away from light and moisture (21).

Extraction of essential oils

The extraction of essential oils was carried out by hydro-distillation of the plant materials in a Clevenger type device (22). During each test, 100 g of raw materials were processed. The distillation lasts approximately 180 minutes after the appearance of the first drop of the distillate at the outlet of the steam condensation tube. The recovered essential oils were treated with a few crystals of anhydrous magnesium sulfate and then stored at +4°C in the dark. Three distillations were carried out for each plant.

Determination of essential oil yield

The essential oil yield was determined as described by Bourkhiss et al., (23), expressed in gram of the distillate per 100g of dry matter, by the following relationship; EOY=[(M/Ms)x100] ± [(ΔM/Ms)x100], where EOY is essential oil yield, M is the mass of collected essential oils (g), ΔM is error on reading, and Ms is dry plant mass (g).

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography – mass spectrometry (GC-MS) analyses of the EOs were conducted using an Agilent 7890B gas chromatograph equipped with an Agilent HP-5MS, capillary column (30m×0.25 mm; coating thickness 0.25µm) and an Agilent 5977B mass, using the following working conditions; (i) injector and transfer line temperatures 220 and 240°C, respectively; (ii) oven temperature programmed from 60 to 240°C at 3°C/min; (iii) carrier gas helium at 1 ml/min; (iv) injection of 1 µl (5% HPLC grade n-hexane solution); split ratio 1:25

Identification of the constituents was based on a comparison of the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons and computer matching against commercial (NIST 14 and ADAMS) and laboratory-developed mass spectra library (24).

Antibacterial activity of essential oils of the four plants by agar disc diffusion assay

For determination of the antibacterial activity of the essential oils, the disc diffusion technique described by Rajouani et al., (25) was used. A Whatman paper (N° 03) was cut into disc sizes of 6 mm in diameter and sterilized by autoclaving in tubes. The discs were then impregnated with 10 µl of each pure EO. Pure 24-hour cultures of CoNS isolates were suspended in Mueller-Hinton (MH) broth and adjusted to 0.5 McFarland standards. The suspension was then streaked with a sterile swab on MH agar plates. The EO discs and 30µg vancomycin disc (used as positive control) were placed on the inoculated MH agar plates, which were then incubated aerobically at 37°C for 24 hours. Antibacterial activity was assessed by measuring the diameter of the inhibition zone (mm) around the discs.

Determination of MIC and MBC of essential oils of *S. officinalis* and *T. ciliatus* ssp. *munbyanus*

The determination of the minimum inhibitory concentrations (MICs) of EOs of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* was carried out according to the technique of dilution in a liquid medium described by Bazargani and Rohloff (26), with some modifications. The CoNS isolates were cultured on MH agar plates and incubated aerobically at 37°C for 12 hours. After incubation, 5 to 7 isolated colonies were inoculated in tubes containing 5ml of MH broth and incubated at 37°C for 8 to 12 hours to ensure that the bacteria were in the log phase. Each bacterial suspension was then diluted 1:100 in MH broth. The EOs were dissolved in MH broth + 0.5% Tween 80 to obtain solutions at concentrations of 400 mg/ml for *S. officinalis* L. and 200 mg/ml of *T. ciliatus* ssp. *munbyanus.*

For each microplate well, 100 µl of MH broth was added, then 100 µl of each concentration of EOs were placed in the first microplate well and diluted in the sterile MH broth. Finally, 100 µl of the diluted CoNS isolates suspension were added to obtain a final concentration in the range of 100 to 0.39 mg/ml and 50 to 0.195 mg/ml of *S. officinalis* L. and *T. ciliatus* ssp. *munbyanus* respectively. Approximately 200 µl of the MH broth + 0.5% Tween 80 served as negative control.

The plates were prepared in three replicates, and incubated aerobically for 18 to 24 hours at 37°C. The MIC was defined as the lowest concentration of EOs that produced no macroscopically visible growth of CoNS isolates. The minimum bactericidal concentration (MBC) represents the lowest concentration of EOs at which 99.9% of CoNS bacteria were killed.

Biofilm inhibition study (inhibition of bacterial cell fixation)

The EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* were evaluated at their MIC concentrations for their inhibition potential with regard to cell attachments as described by Marino et al., (27) with some modifications. 100 µl of each essential oil solubilized in the TSB+1% Glu+0.5% Tween 80 (at the MIC x 2 value) were added to each well of a 96-well microplate. The negative control contains 100 µl of TSB + 1% Glu. Finally, 100 µl of each bacterial culture suspension of CoNS (~10^6 CFU/ml) was introduced into each well (the final volume was 200 µl in each well). 200 µl of TSB + 1% Glu + 0.5% Tween 80 was included and 200 µl of EO was added in blank wells.

The microplates were incubated at 37°C for 24 hours. After incubation, the medium was aspirated, rinsed twice with phosphate-buffered saline (PBS), and rinsed once with ethanol (20%) to remove traces of EOs, then were fixed by drying at 60°C for 30 minutes and 200 ml of 1% crystal violet staining was added to the wells for 30 minutes. The contents of the wells were then aspirated, and after rinsing with distil water, 200 mL of ethanol (95%v/v) was added to the wells for spectrophotometric analysis (at OD 550 nm). The mean (average) absorbance of the samples was determined, the absorbance in the blank well was subtracted from the reading of the absorbance of each sample (experimental OD) and the percentage inhibition and efficiency were determined according to the following formula described by Bazargani and Rohloff (26) as; Inhibition percentage = (OD of negative control – OD of experimental)/OD of negative control × 100.

Statistical analysis

The SPSS version 22.0 software was used for statistical analysis. A single factor (one-way) ANOVA (for comparison of ≥ 3 means) and unpaired *t* tests, were carried out to determine the effect of diameters of inhibition zones, MICs, MBCs and biofilm inhibition of the EO extracts of the plants on CoNS isolates, and $p < 0.05$ was considered statistically significant.

Results:

Essential oils yield from the plants

The yields of the EOs were expressed relative to the weight of the plant dry matter. The extraction of the EOs by hydro-distillation showed greater extraction in *S. officinalis* L. (1.41±0.05%) and *T. ciliatus* ssp. *munbyanus* (Boiss. & Reut.) Batt. (1.25±0.03%) than in *J. oxycedrus* L. (0.81±0.04%) and *J. thurifera* L. (0.36±0.04%) (p<0.0001 by one way ANOVA test) (Table 2).

Chemical composition of essential oils

The complete identification by GC-MS of the components of the EOs is shown in Table 3. In this study, 27 compounds were identified in the EO of *J. oxycedrus*, with α-pinene (56.1%), β-phellandrene (17.9%), α-phellandrene (4.4%) and myrcene (3.3 %) being the main ones. In *J. thurifera* EO, 41 compounds were identified, and sabinene (24.2%), 4-terpineol (12.5%), methyl eugenol (8.9%), safrole (7.5%) and α-pinene (6.5%) characterized its composition. In the EO of *S. officinalis*, 45 compounds were identified, with α-thujone (16.7%) as the main constituent, followed by camphor (14.9%), 1,8-cineole (11.8%) and viridiflorol (10.1%). In *T. ciliatus* ssp. *munbyanus* EO, 32 compounds were identified, among which thymol (69.0%) was the main component, followed by γ-terpinene (5.1%), p-cymene (3.7%), carvacrol (3.7%) and β-caryophyllene (3%).

Table 2. Percentage yield of essential oils from four medicinal plant species

Medicinal plants	Yield of essential oils (%) (Mean ± SD)	*p* value (ANOVA)
Thymus ciliatus ssp. *munbyanus* (Boiss. et Reut.) Batt.	1.41 ± 0.05	<0.0001*
Salvia officinalis L.	1.25 ± 0.03	
Juniperus thurifera L.	0.81 ± 0.04	
Juniperus oxycedrus L.	0.36 ± 0.04	

SD = Standard deviation; ANOVA = Analysis of variance; * = statistically significant

Table 3: Chemical composition of the essential oil extracts of four medicinal plants from Aurès region of Algeria

Chemical compound	LRI	*J. oxycedrus*	*J. thurifera*	*S. officinalis*	*T. ciliatus* ssp. *munbyanus*
Tricyclene	928	0.1			
α-thujene	933		3.9	0.6	0.1
α-pinene	941	56.1	6.5	2.2	1.0
Camphene	955	0.3	0.1	3.1	0.3
Sabinene	977	0.4	24.2	2.9	
β-pinene	982	1.5	0.6	2.3	0.3
Myrcene	992	3.3	2.3	1.3	0.5
δ-2-carene	1002	0.4			
α-phellandrene	1006	4.4	0.1		0.1
α-terpinene	1020		3.9	0.7	0.9
p-cymene	1028	1.5	0.8	0.9	3.7
Limonene	1032		1.2	2.2	0.5
β-phellandrene	1033	17.9			0.8
1,8-cineole	1034			11.8	
(E)-β-ocimene	1051		0.1		
γ-terpinene	1063	0.1	6.5	1.3	5.1
Cis-sabinene hydrate	1070		0.5	0.2	
Terpinolene	1090	0.8	1.7	0.5	0.1
Trans-sabinene hydrate	1099		0.5		
Linalool	1101		3.3	0.6	1.1
α-thujone	1105			16.7	0.7
β-thujone	1116			3.5	
Cis-p-menth-2-en-1-ol	1123		0.5		
α-campholenal	1126	0.3			
Trans-pinocarveol	1141			0.2	
Trans-p-menth-2-en-1-ol	1142		0.3		
Camphor	1144		0.1	14.9	1.1
Borneol	1168			1.5	0.1
4-terpineol	1179		12.5	2.7	0.3
α-terpineol	1191	1.1	1.3	0.5	
Methyl chavicole (=estragole)	1197		0.6		
Methyl thymol	1234	0.2			0.9
Linalyl acetate	1259		2.4	0.5	
Bornyl acetate	1287			0.5	
Safrole	1287		7.5	1.6	
Thymol	1291		2.5	0.6	69.0
Carvacrol	1301				3.7
α-terpinyl acetate	1352	0.2	0.7	0.2	
Piperitenone oxide	1365	1.4			
Neryl acetate	1366		0.1		
β-bourbonene	1385	0.1			
Geranyl acetate	1386		0.2		

Methyl eugenol	1403		8.9	2.1	
β-caryophyllene	1419	0.3	0.2	2.0	3.0
α-humulene	1455			1.4	0.2
Cis-muurola-4(14),5-diene	1463		0.1		
γ-muurolene	1478			0.2	0.4
Germacrene D	1482	0.8	0.2	0.4	0.2
Valencene	1492				0.2
2-tridecanone	1497	0.3			
α-muurolene	1499				0.1
β-bisabolene	1508				1.2
Trans-γ-cadinene	1515		0.4	0.3	0.4
δ-cadinene	1524	0.2	1.1	0.7	1.0
α-calacorene	1543				0.1
Elemol	1550		0.9	0.5	
Germacrene B	1556		0.2		
Elemicin	1557		0.8	0.3	
Caryophyllene oxide	1582			1.3	0.4
Viridiflorol	1591			10.1	1.6
Humulene epoxide II	1607			0.9	
1-epi-cubenol	1629		0.4	0.3	
γ-eudesmol	1630		0.2	0.2	
T-cadinol	1641			0.2	
β-eudesmol	1650		0.4	0.3	
α-eudesmol	1651		0.4	0.3	
α-cadinol	1652			0.3	
(Z)-9-tetradecen-1-ol	1665	2.8			
(E, E)-farnesol	1732	1.2			
Manoyl oxide	1993	0.7			
Abietatriene	2054	0.6			
Manool	2056			4.0	
Abietadiene	2081	2.5			
Monoterpene hydrocarbons		86.8	51.9	18.0	13.4
Oxygenated monoterpenes		3.2	24.9	54.4	76.9
Sesquiterpene hydrocarbons		1.4	2.2	5.0	6.8
Oxygenated sesquiterpenes		1.2	2.3	14.4	2.0
Diterpenes		3.8	0.0	4.0	0.0
Phenylpropanoids		0.0	17.8	4.0	0.0
Non-terpene derivatives		3.1	0.0	0.0	0.0
Total identified		**99.5**	**99.1**	**99.8**	**99.1**

LRI = Linear Retention Index

Antibacterial activity of the essential oils on CoNS by the disc diffusion assay

The antibacterial activity of the EOs by the disc diffusion test showed that growth inhibition varies depending on the species of CoNS, concentrations and type of EOs (Table 4). The results as presented in the table shows that EOs of *T. ciliatus* ssp. *munbyanus* had the highest inhibitory activity on CoNS isolates (n=66), with mean inhibition zone diameter of 24.99±6.29 mm, followed by *S. officinalis* with mean inhibition zone diameter of 13.38±6.52 mm. On the contrary, low inhibitory activity was observed with the EOs of *J. oxycedrus* and *J. thurifera,* having mean inhibition zone diameters of 6.67±1.36mm and 6.40±0.82mm, respectively. The inhibitory activity of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* on the CoNS isolates was significantly higher than the inhibitory activity of *J. thurifera* and *J. oxycedrus* on the CoNS isolates and for each species (p<0.0001 by one-way ANOVA test). However, the inhibitory effects of the EOs were not significantly different among the CoNS species for *T. ciliatus* ssp. *munbyanus* (p=0.836) and *S. officinalis* (p=0.080), *J. thurifera* (p=0.989), and *J. oxycedrus* (p=0.170).

MICs and MBCs of essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L.

The MIC and MBC results showed that bactericidal activity of the EOs varies depending on the species of CoNS, the concentration and type of EOs tested (Table 5). As shown in the table, EOs of *T. ciliatus* ssp. *munbyanus*, had high bactericidal activity against the CoNS isolates (n=66), with mean MIC and MBC values of 2.65±3.77 and 5.31± 7.41 mg/ml respectively, which is significantly higher than the bactericidal activity of the EOs of *S. officinalis*, with mean MIC and MBC values of 27.53±18.2mg/ml (p<0.0001, t = 7.104) and 31.97 ± 33.19 mg/ml (p< 0.0001, t = 6.369) respectively. The mean MIC and MBC values of *T. ciliatus* ssp. *munbyanus* EOs are also significantly higher than those of *S. officinalis* across the different CoNS species except for *S. hominis*, which was not significantly different for MIC (p=0.1624) and MBC (p=0.1250). While the mean MIC values of *T. ciliatus* ssp. *munbyanus* EOs were not significantly different across the CoNS species (p=0.838), the mean MIC values of *S. officinalis* were significantly different across the CoNS species (p=0.002), with low mean MIC value for *S. hominis* (8.20±7.97mg/ml) and high MICs for *S. chromogenes* (50.0±51.44mg/ml) and *S. epidermidis* (37.01±36.58 mg/ml).

Antibiofilm activity of the essential oils on CoNS isolates

The evaluation results of the EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* on biofilm producing CoNS isolates (n=59) indicated that the percentage biofilm inhibition was significantly higher for *T. ciliatus* ssp. *munbyanus* (65.63±10.71%) than *S. officinalis* (53.13%±5.83) EOs (p<0.0001, t=7.874) (Table 6). The percentage biofilm inhibition by the EOs of *T. ciliatus* ssp. *munbyanus* was also significantly higher than that of *S. officinalis* for the different CoNS species except for *S. hominis* (p=0.9691) and other CoNS species (p=0.2112), which were not significantly different. While the percentage biofilm inhibition by the EOs of *T. ciliatus* ssp. *munbyanus* was not significantly different for each CoNS species (p=0.997 by one-way ANOVA test), that of *S. officinalis* was significantly different (p=0.000133 by one-way ANOVA), with high percentage inhibition for *S. hominis* (65.00±4.03%) and low percentage inhibition for *S. chromogens* (43.79±3.50%).

Table 4: Mean (Average) inhibition zone diameters of essential oil extracts of four medicinal plants on CoNS species

CoNS isolates	*T. ciliatus* ssp. *munbyanus*	*S. officinalis* L.	*J. thurifera*	*J. oxycedrus*	*p* value (one way ANOVA)
S. epidermidis (n=29)	26.35± 6.37	14.56±6.30	6.43±0.76	6.32±0.98	<0.0001*
S. haemolyticus (n=15)	24.67±5.19	14.23±6.28	6.32±0.84	6.97±1.67	<0.0001*
S. hominis (n=8)	25.14±5.82	17.58±7.10	6.52±1.07	7.39±1.97	<0.0001*
S. chromogenes (n=6)	23.36±6.14	7.91±1.76	6.33±0.76	6.00±0.00	<0.0001*
Other species of CoNS (n=8)	25.43±8.02	12.62±6.51	6.41±0.76	6.70±1.33	<0.0001*
***p* value (one way ANOVA)**	0.836	0.080	0.989	0.170	
Total (n=66)	**24.99±6.29**	**13.38±6.52**	**6.40±0.82**	**6.67±1.36**	**<0.0001***

CoNS = coagulase negative staphylococci; n= number; ANOVA=analysis of variance; * = statistically significant

Table 5: MICs and MBCs of essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. on CoNS species

CoNS isolates	**MIC (mg/ml)**		***p* value (unpaired *t* test)**	**MBC (mg/ml)**		***p* value (unpaired *t* test)**
	T. ciliatus* ssp. *munbyanus	***S. officinalis***		***T. ciliatus* ssp. *munbyanus***	***S. officinalis***	
S. epidermidis (n=29)	3.31±4.43	37.01±36.58	<0.0001* (t=4.925)	6.84±8.96	37.36±42.09	0.0003* (t=3.819)
S. haemolyticus (n=15)	2.55±3.21	26.33±27.69	0.0026* (t=3.304)	4.61±6.12	43.15±41.43	0.0013* (t=3.564)
S. hominis (n=8)	3.49±4.25	8.20±7.97	0.1624 (t=1.475)	6.02±7.42	15.36±14.39	0.1250 (t=1.632)
S. chromogenes (n=6)	1.59±0.87	50.00±51.44	0.9821 (t=0.2305)	4.40±2.30	-	
Other CoNS species (n=8)	2.34±2.45	16.14±17.35	0.0428* (t=2.228)	4.71±4.87	32.03±34.88	0.0456* (t=2.194)
***p* value (one way ANOVA)**	0.838	0.002*		0.674	0.215	
Total CoNS (n=66)	**2.65±3.77**	**27.53±28.2**	**<0.0001* (t=7.104)**	**5.31±7.41**	**31.97±33.19**	**<0.0001* (t=6.369)**

CoNS=coagulase negative staphylococci; n= number; MIC = minimum inhibitory concentration; MBC=minimum bactericidal concentration; ANOVA = analysis of variance; - = no inhibition; * = statistically significant

Table 6: Antibiofilm activity of the essential oils of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* L. on CoNS isolates

CoNS isolates	**Percentage inhibition of biofilm formation**		***p* value (unpaired *t* test)**
	***T. ciliatus* ssp. *munbyanus* Mean (Average) (%)**	***S. officinalis* L. Mean (Average) (%)**	
S. epidermidis (n=27)	65.78±10.98	48.22±8.51	<0.0001* (t=6.568)
S. haemolyticus (n=13)	66.77±10.63	49.22±4.93	<0.0001* (t=5.400)
S. hominis (n=6)	64.78±12.94	65.00±4.03	0.9691 (t=0.03976)
S. chromogenes (n=6)	65.47±10.44	43.79±3.50	0.0007* (t=4.823)
Other CoNS species (n=7)	65.36±8.54	59.45±8.20	0.2112 (t=1.321)
***p* value (one way ANOVA)**	0.997	0.000133*	
Total (n=59)	**65.63±10.71**	**53.13±5.83**	**<0.0001* (t=7.874)**

CoNS = coagulase negative staphylococci; n= number; ANOVA=analysis of variance; * = statistically significant

Discussion:

Previous studies on Thymus reported variable EO yield values; Kabouche et al., (19) reported 2.1%, Heni et al., (28) 2.5%, Amarti et al., (29) 1.2% and Ouknin et al., (30) 1.7%. For *S. officinalis*, reported EO yields also varies; Soković et al., (31) 2.2%, Meziou-Chebouti et al., (32) 1.06% and Golparvar et al., (33) 2.4%. For *J. thurifera*, Bahri et al., (34) reported 1.03% yield, while for *J. oxycedrus*, Angioni et al., (35) and Marongiu et al., (5) reported 0.04% and 0.20% yield, respectively. The difference in percentage yield reported in the literature in comparison with our results could be attributed to different factors such as the nature of the soil, the genetic variation of the plant, the mode of extraction of the oil (36), climate, collection period, age (28-36), altitude, as well as by the interaction of various factors (37), the part of the plant extracted, and the specific geographic location of the plants (28).

Different chemical composition of EOs have also been reported from GC-MS of *J. oxycedrus*, Angioni et al., (35) mainly found α-pinene (85.95%), δ−3-carene (2.81%) and myrcene (1.20%), and Boudjedjou et al., (38) reported the main components of EOs of *J. thurifera* to be m-mentha-6,8-diene (15. 43%), β-pinene (10.59%), elemol (8.31%) and 4-terpineol (7.44%). Other researchers have reported different compositions for *S. officinalis* EOs, Nikolić et al., (39) identified cis-thujone (32.7%), camphor (17.2%), 1,8-cineole (10.1%), α-pinene (8.6%), trans-thujone (7.7%) and camphene (7.3%), while Golparvar et al., (33) identified α-thujone (37.18%), 1,8-cineole (12.71%), β-thujone (9.10%), camphene (5.54%) and viridiflorol (5.33%). Ouknin et al., (30) described a different composition for the EO of *T. ciliatus* ssp. *munbyanus*, with carvacrol (31.8%), γ-terpinene (21.9%), p-cymene (14.7%), thymol (7.6%), linalool (4.3%), borneol (3.9%) and α-terpinene (2.1%) as principal constituents. The differences in the chemical compositions reported in our current study and those of other studies may be due to different growth stages, ecological conditions, method of extraction (16), variation in population, organs of the plant and stress conditions (40).

The antibacterial activity of EOs depends on their chemical composition. The most active ones are often characterized by two or three main components at fairly high concentrations (up to 80%) together with other minor compounds (41). Khadir et al., (42) reported similar results using *T. ciliatus* ssp. *munbyanus* on 19 MRSA isolates, with a diameter of 25.8mm. Different results were obtained from *S. officinalis* by Meziou-Chebouti et al., (32) on clinical isolates of *S. aureus*, with an inhibition diameter of 35mm. Also, Bahri et al., (34) obtained different results (inhibition diameter of 27.0mm) with EOs of *J. thurifera* on *S. aureus* (ATCC 33862). Zeraib et al., (9) testing the EOs of male and female leaves of this plant on MRSA reported diameters of 12.66±1.15mm and 13.33±1.15 mm, respectively. The results obtained by Bousmaha-Marroki et al., (43) from the EOs of *T. ciliatus* ssp. *euciliatus* on clinical *S. aureus* were also different with a MIC of 920 μg/mL (0.92mg/mL).

The main mode of antibacterial action of thymol is not completely understood, but researchers have thought that it involves a disturbance of internal and external membranes and interaction with membrane proteins and intracellular targets (44) or disruption of bacterial enzyme systems (45). Another component which is carvacrol is a terpene known for its antimicrobial activity against a wide range of bacteria (43-46). It is also considered as biocide, with its precursor, p-cymene weakly antibacterial, but probably acts in synergy by the expansion of the membrane, causing its destabilization (46). Soković et al., (31) reported a strong activity of *S. officinalis* on *S. epidermidis* (ATCC 12228) with MIC and MBC values of 6.0 and 6.0 μg/ml, respectively. Pierozan et al., (47), testing on *S. aureus* the EOs obtained from two different plants, *S. officinalis* 1 and *S. officinalis* 2, obtained MICs of 3.42 and 2.87 mg/ml respectively. The antimicrobial activity of *S. officinalis* has been recognized for several decades and has been attributed to the presence of 1,8-cineole, α-thujone, camphor (48), as well as to β-thujone, borneol, p-cymene, and others (47). Infact, a synergistic effect may be observed between major and minor constituents (25).

Several studies have described that thymol and carvacrol are able to inhibit the growth of preformed biofilm and interfere with biofilm formation during planktonic growth. Memar et al., (49) reported that carvacrol and thymol attenuated biofilm formation in *S. aureus* and *S. epidermidis* on polystyrene microplates. Thymol can prevent the early stages of biofilm formation and interfere with the formation of mature biofilms due to metabolic activity in biofilms. All of these events can lead to a major membrane and block the production of filamentous forms at the start of biofilm formation. Biofilms formation, being a multifactorial event, can be affected by thymol with various mechanisms at different stages of their development. Furthermore, Karpanen et al., (50) demonstrated increased susceptibility of staphylococci in a biofilm mode of growth to an EO-based formulation, compared with planktonic cells. Thymol and carvacrol are phenolic compounds having both hydrophilic and hydrophobic properties, which may enhance their diffusion in a biofilm and allow their access to bacterial cells where they can alter the permeability of the plasma membranes.

Conclusion:

Chemical analyses by GC-MS allowed the identification of 99.1 to 99.8% of the EO composition in our study. The major constituents were α-pinene (56.1%), sabinene (24. 2%), α-thujone (16.7%) and thymol (69.0%) for *J. oxycedrus*, *J. thurifera*, *S. officinalis* and *T. ciliatus* ssp. *munbyanus*, respectively. From this study, EOs of *T. ciliatus* ssp. *munbyanus* and *S. officinalis* clearly showed high antibacterial and antibiofilm activities, but the activities of *T. ciliatus* ssp. *munbyanus* were significantly higher than those of *S. officinalis*. EOs of these two plants could serve as alternatives to classical antibiotics against planktonic and biofilm forms of CoNS isolates in view of their high antibacterial and antibiofilm formation capabilities.

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Contributions of authors:

AZ designed the study and contributed to specimen collection, laboratory isolation of bacterial pathogens, extraction of EOs, antibacterial and antibiofilm detection, and manuscript writing. RD, FG, AR, BC, MHE, BF, MS and KA contributed to the study protocol and manuscript revision. All authors read the final manuscript.

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