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SCREENING FOR BIOLOGICAL ACTIVITIES OF ESSENTIALS OIL PROFILES (GC-FID) AND ETHANOLIC EXTRACTS FROM *PINUS BRUTIA* (TEN) AND *PINUS CANARIENSIS* (C. SMITH)

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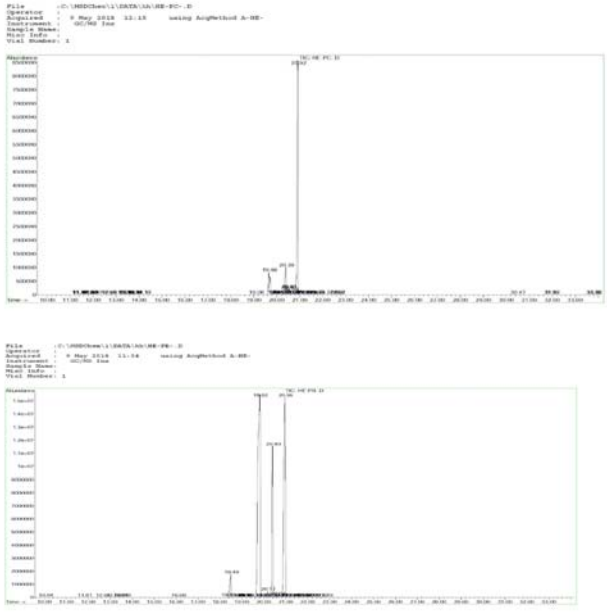
Interest in natural products derived from plants and their biological potentialities has increased as a result of environmental concerns. In this study, the phytochemical characterisation of essential oils and ethanolic extracts originated from *Pinus brutia* (Ten) and *Pinus canariensis* (C. Smith) needles along with their antimicrobial activities were achieved. The essential oils from both pine *species* were characterized using GC-FID and GC-MS and the chemical profiles included mainly *trans*-caryophyllene (49.82%), δ-cadinene (32.50%) and isoterpinolene (2.99%). The essential oils of both *Pinus canariensis* and *Pinus brutia* exhibited notable antimicrobial activities against the clinical gram-negative bacteria *Enterobacter cloacae* and *Escherchia coli*. The biological activities of both studied essential oils recommended their use in the formulation of environmentally-friendly and biocompatible pharmaceutical drugs.



INTRODUCTION

The use of essential oils to control many diseases and their effective usage as antimicrobial agents,[1] in addition to their use as functional ingredients in foods, drinks, toiletries and cosmetics are gaining momentum, due to the growing interest of consumers in ingredients from

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natural sources, and to the increasing concern about potentially harmful synthetic additives.[2] Essential oils have been known as complex mixture of natural compounds, mostly of plant origin, extremely volatile and with an intense odour. Even if they represent only a small fraction of the plant from which they were derived, they give the whole plant the characteristic of aromatic smell. As a results, these plants were used in drugs, food and perfume industries.[3] The *species* that show the largest contents of essential oils belong to many families namely: *Asteraceae, Lamiaceae, Apiaceae, Rutaceae, Lauraceae, Myrtaceae, Magnoliacea* and *Pinaceae*.[4] The *Pinaceae* family is a common ornamental plant *species* cultivated around the world, particularly in South America, the Mediterranean basin as well as North Africa. Pine *species* were considered as a natural source of antioxidant compounds. Most pine *species* were reported to have a high antioxidant capacity (*Pinus halepensis*, *Pinus pinea*, *Pinus sylvestris*, *Pinus nigra*…). The genus *Pinus* belongs to the *Pinaceae* family and comprises about 250 species.[5] Many pine needle oils were used industrially by soap and perfume manufacturers to add pleasant smells to their products. They are also used as a fuel due to the high heating power of pine charcoal. The chemical composition of various pine *species* volatiles have been the subject of numerous studies. The majority of the works were focused on North American and Central European *species*. Whereas, only limited number of chemically reports deal with Mediterranean pine *species*. Rare data was known about the chemical composition of the volatile metabolites of *Pinus canariensis*. In this context, few studies describe the composition of the essential oil of *Pinus canariensis,* growing in Greece.[6] Therefore, the main objective of this work, was to identify the chemical composition of the essential oils and ethanolic extracts from the aerial parts of *Pinus canariensis* (C. Smith) and *Pinus brutia* (Ten), to determine their antimicrobial activities, in order to contribute to the use of these extracts as alternative products.

MATERIALS AND METHODS

Plant material

Pinus brutia and *Pinus canariensis* needles were obtained from Hinchir Naam's arboretum, North Est Tunisia; latitude 36°34″38.22″ (N); longitude 10°51″29.63″ (E); altitude 637 m. The voucher specimens PB2019 (*Pinus brutia*) and PC2019 (*Pinus canariensis*) were deposited at the Laboratory of Management and Valorization of Forest Resources. The harvested pine needles' (3 kg for each sample) were transported in the same day to the laboratory in ventilated plastic boxes. The plant materials were air-dried at room temperature for one week. Then, the needles were finely grounded using an electric grinder to get a fine powder that was stored in closed vials until further analyses.

Sample extraction

1. Extraction of phenolic compounds

Phenolic compounds were extracted from dry needles (5 g) by maceration at room temperature using ethanol (1/10: w/v) as solvent. After shaking for 24 h, the mixture was centrifuged at 3500 rpm for 20 min. The supernatant was then concentrated under reduced pressure at 40°C. The pellets were re-suspended in the appropriate solvent (1 mL of ethanol or DMSO). The extracts were stored at 4°C for further uses.[7]

2. Essential oil extraction and analysis

For each pine *species*, 300 g of the powdered needles were hydro-distilled for 4 h using a Clevenger apparatus. The obtained volumes of Essential Oils (EOs) were measured directly in the extraction burette. The amount of the obtained oil (%) was calculated as volume (mL) of EOs per 100 g of dry plant material. The Essential oils were dehydrated over anhydrous Na_2SO_4 and stored in a cool and dark place prior to analysis.[8] The identification and quantification of the studied essential oils were carried out by combination of gas chromatography (GC-FID) and gas chromatography–mass spectrometry (GC–MS). GC analysis was performed using a Hewlett-Packard 6890 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector (FID) and split-split less injector attached to HP-INNOWAX polyethylene glycol capillary column (30 m × 0.25 mm). On the other hand, GC–MS analysis has been carried out using HP model 5975B inert MSD (Agilent Technologies, J&W Scientific Products, Palo Alto, CA, USA) equipped with an Agilent Technologies capillary DB-5MS column (30 m length; 0.25 mm: i.d; 0.25 µm film thickness) and coupled to a mass

selective detector (MSD5975B, ionization voltage 70 eV; all Agilent, Santa Clara, CA). The Helium was used as carrier gas with a flow rate equal to 1.0 ml/min. Besides, temperature of the injector and the detector were set at 250 and 230 °C, respectively. The GC oven temperature started at 100 °C and then held for 1 min at 260 °C and then for 10 min with a program rate of 4°C.min^{-1}. A sample of one µl was injected, using split mode (1:100).[8] Compounds of the volatile oil were identified by both their retention indices and their mass spectra. The Retention indices were calculated by linear interpolation relative to retention times of C_8–C_{24} of n-alkanes and compared with those of reference compounds included in our laboratory database or literature data. And the Mass spectra were matched with reference spectra from Wiley/NIST database, published data and spectra of authentic compounds.[8] Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

Determination of bioactive compounds contents

Determination of total phenols content (TPC) was assessed by using a modified colorimetric Folin–Ciocalteu method.[9,10] To 50 µL of the ethanolic extract, 125 µL of Folin–Ciocalteu reagent were added. After 5 min of incubation at room temperature in the dark, 125 µL of Na_2CO_3 (20 %) were added and immediately, the mixture volume's was made up to 1 mL with distilled water. After 60 min in the dark at room temperature, the absorbance of each sample was read at 760 nm. The results were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total flavonoids content (TFC) was estimated according to the method of Rigane *et al.,*[9] with some modifications and expressed in mg of rutin equivalent per gram of dry weight (mg RE/ g DW). Briefly, 30 µL of each ethanolic extract was mixed with 300 µL of $NaNO_2$ (16 %). After 60 min of incubation at room temperature, 200 µL of NaOH (1 M) solution, 60 µL of $AlCl_3$ (10 %) and 700 µL of H_2O were added to the solution. The mixture absorbance was measured at 510 nm. The quantification of bioactive compounds (TPC and TFC) has been performed in triplicate using a UV-Visible spectrometer (BECKMAN DU 800).

Antimicrobial activity

1. Microorganisms and culture conditions

The essential oils and ethanolic extracts from both studied *species* were dissolved in DMSO and sterile water, respectively, in order to test them against a list of pathogenic Tunisian clinical strains. Therefore, two gram-negative bacteria (*Escherchia coli* and *Enterobacter cloacae*), two gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis),* three yeast strains *(Candida albicans, Candida parapsilosis* and *Candida sake)* and three mould strains (*Penicillium spp.*, *Aspergillus niger* and *Alternaria spp.*) were used. Before use, each essential oil and ethanolic extracts were diluted in its corresponding solvent to 10^{-1}, and then sterilized by filtration through a 0.2 µm pore size filter according to Essghaier *et al.*[11]

2. Agar diffusion method

Antibacterial and antifungal tests were performed by agar well diffusion method as described before by Dharajiya *et al.*[12] and Thakur *et al.*[13] For broth micro-dilution assay, sterile Mueller–Hinton media (BioRad, France) for bacterial strains and yeast malt extract agar YMA (Bio-Rad, France) for antifungal tests were used. A freshly cell suspension (0.1 mL) adjusted to 10^7 CFU/mL for bacteria and 10^5 spores/mL for fungus were inoculated onto the surface of agar plates. Afterwards, wells with 6 mm diameter were punched in the inoculated agar medium and 20 µL of the ethanolic extract and 10 µL of the essential oil were added to each well. Negative controls are consisted of 10 µL of DMSO and 20 µL of sterile water. Two positive controls were also used namely ceftazidime CAZ 30 as antibiotic and voriconazole VCZ as fungicide. The plate was allowed to stand for 30 min at 4°C and room temperature to permit, respectively, the diffusion of the ethanolic extracts and the essential oil followed by incubation at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the zones of inhibition (clear zone around the well) against the test micro-organisms. All assays were repeated three times.[11]

3. Determination of Minimum Inhibitory Concentration (MIC) and Minimal bactericidal concentration (MBC)

The minimum inhibitory concentrations (MIC) of the tested ethanolic extracts and essential oils

against pathogenic microorganisms were determined using the micro-dilution broth method. MIC was estimated visually (absence of turbidity) and was determined by three independent measurements.[13] Minimal bactericidal concentrations (MBC) were determined from the micro-dilution plates used in the MIC assay.[11] Aliquots (10 µL) without visible growth were transferred to plates containing the corresponding media culture, and then incubated at 37 °C for 24 h then colony growth was verified. All assays were performed in triplicate.

4. Resolution of bactericidal activity

The antimicrobial activity of the tested samples was expressed in arbitrary units per mL (AU/mL) and was established by an agar diffusion assay as described by Graciela *et al.*[14] Briefly, a serial two fold dilution of essential oil in DMSO, and ethanolic extracts in sterile water were prepared. Then 40 µL of each extract dilution and 10 µL of essential oil dilution were spotted onto each well in culture medium plate previously seeded with 10^7 CFU/mL of pathogen bacteria. The AU/mL was calculated as:

$$\mathbf{AU/mL} = \frac{1000\,D}{A}$$

where: A was the volume of the tested sample spotted on well agar plate 40 µL for extract in this case and 10 µL for essential oil; D was the reciprocal of the highest dilution showing a clear inhibition of the indicator strain.

Statistical analysis

All data were expressed as the average ± standard deviation of the measurements. Quantitative differences were assessed by Tukey's test (at $p < 0.05$) followed by Dunn's post–hoc multiple comparison test (SPSS. v15).

RESULTS AND DISCUSSION

Total Phenols and Flavonoids Contents

The total phenolics in the ethanolic extracts varied significantly ($p < 0.05$) between both described pine *species*. The total phenol content was 65.75 ± 0.63 (mg GAE/g DW) and 20.63 ± 1.55 (mg GAE/g DW) for *Pinus brutia* and *Pinus canariensis*, respectively. The results of Dhibi *et al.* [15] revealed a value of 0.72 ± 0.06 (g/kg of fresh weight) of the polyphenols contents from the Aleppo pine seeds from Siliana (Tunisia). On the other hand, the polyphenol contents found by Kadri *et al.*[16] were around 79.23 mg GAE/g DW for Maritime pine and 3.71 mg GAE/g DW for Aleppo pine seeds. For the study made by Ustun *et al.*[17] Aleppo pine seeds of Turkish origin showed 72.77 mg GAE/g DW for ethanolic extract against 102.56 mg GAE/g DW by using the ethyl acetate as a solvent. Compared to other pine *species* such as Siberian pine "*Pinus sibirica*" and Korean pine "*Pinus koraiensis*"[18,19] this both *species* were much richer in phenolic compounds with levels of 266 and 264 mg/g, respectively. Among the two studied pine *species* here, the content of flavonoids, varied significantly ($p<0.05$). *Pinus Brutia* extract presented the highest flavonoid content (23.4 ± 0.468 mg RE/g DW). The analyses achieved by Kadri *et al.*[16] have revealed amounts of 0.80 and 1.42 mg quercetin equivalent/g for Aleppo pine and maritime pine, respectively. Our results were within the range of those reported by Apetrei *et al.*[20] for the Cembran pine (19.84 ± 0.57 mg catechin/g extract).

Essential oil composition

The essential oil yield in needles of *Pinus brutia* and *Pinus canariensis* were 0.31% (w/w) and 0.24% (w/w), respectively. Our results have been in accordance with those reported by Hanana *et al.*[20] who mentioned that the essential oil yield from Tunisia *Pinus bruti* was 1.1%. The chromatograms of the two studied *Pinus species* showed that the essential oils were a mixture of numerous compounds; some of them were present in trace amounts (Fig. 1). The major components in the essential oil extracted from *Pinus brutia* needles were as follows: *trans*-caryophyllene (49.82%), δ-cadinene (32.50%) and isoterpinolene (2.99%). Other compounds were identified including α-humulene (0.46%) and α-amorphene (0.10%). The obtained results were not in agreement with other investigations. Hanana *et al.*[20] reported that α-pinene (29.1%) was the major constituent of the essential oil of *Pinus brutia*. Moreover, the principal constituents of *Pinus canariensis* needles were δ-cadinene (81.83%), amorphene (12.14%). However, minor constituents have been identified, namely D-β-cubeben (0.62%), l-β-cubeben (0.31%), aromadendrene

(0.01%) and camphene (0.01%). In a previous study, 116 substances were detected in the essential oil of *Pinus canariensis* (C. Smith) growing in Austria, of which 75 (comprising 93.9% w/w of the sample) were positively identified and a further 41 partially characterized. One hundred and eight substances could be assigned to terpenoids: 33 monoterpenes (42.7%), 46 sesquiterpenes (52.1 %), and 29 diterpenes (4.8%). Minor substances were alkane derivatives as well as the benzyl esters of benzoic and salicylic acid.[6] On the other hand; in 2005; Dob *et al.* [22] reported that the major compounds in the essential oil of *Pinus halepensis,* growing in Algeria, were β-caryophyllene (40.31%), β-pinene (10.5%), β-caryophyllene (5.3%) and β-thujone (4.8%).

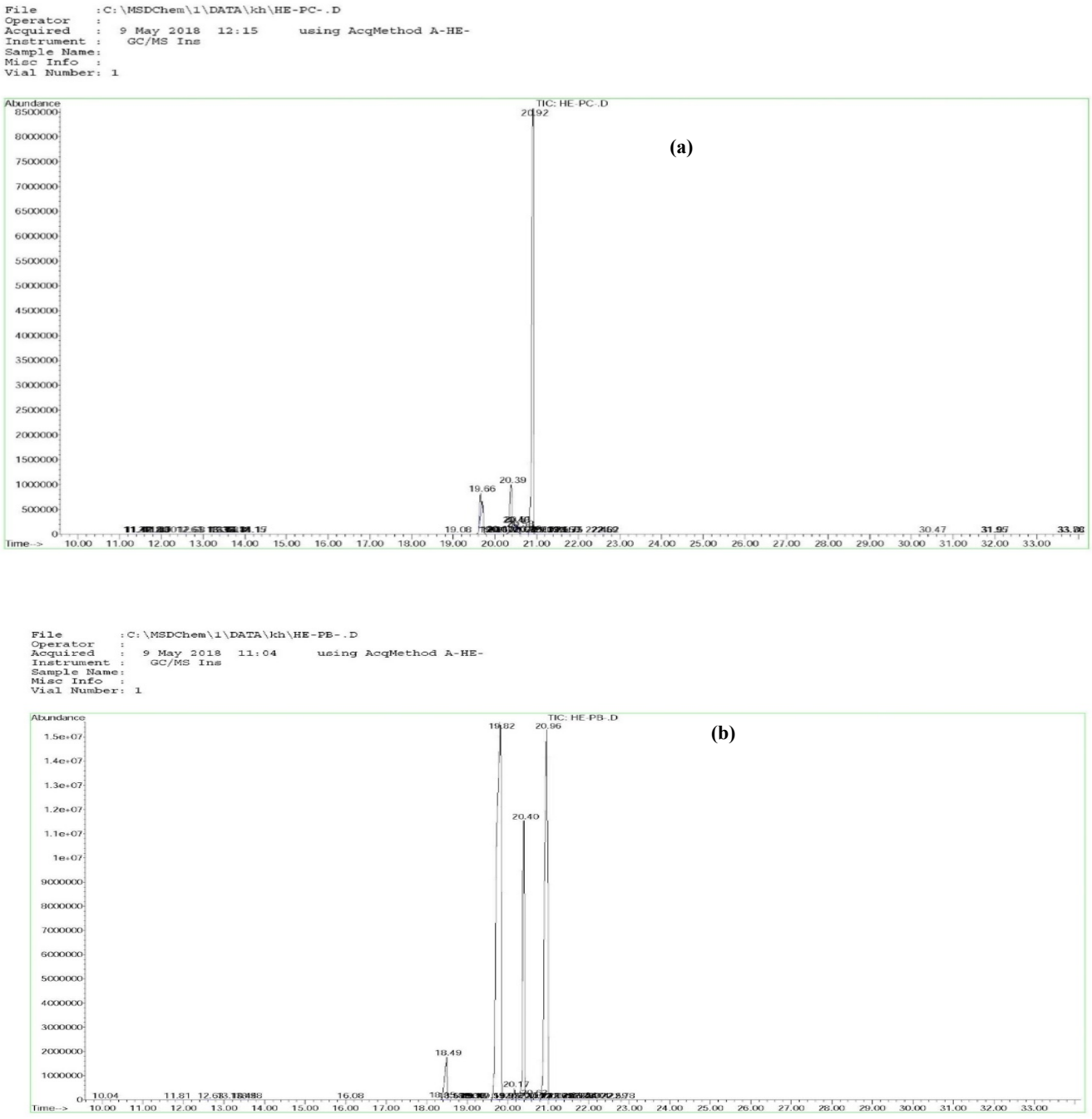

Fig. 1 – GC-FID chromatograms of *Pinus canariensis* (a) and *Pinus brutia* (b) esssential oils.

Antimicrobial activity

The results of the antibacterial ability measured using the agar diffusion method showed that the diameter of the inhibition zones given by both ethanolic extracts and the essential oils varied from 9 to 28.5 mm. These values have revealed the variations related to micro-organisms *species* tested here. Similar results were previously obtained by EO derived from corn and flower of *Pinus brutia* against bacteria and *Candida* strains.[23] The highest inhibition diameter was exhibited by the essential oil from *Pinus brutia* (Ten) with 28.5 mm against *Enterobacter cloacae.* It should be noted that these value was higher than those obtained by the positive control ceftazidime CAZ30 against the same bacteria *specie* with 27 mm. The most effective treatment against *Escherchia coli* was the ethanolic extract from *Pinus brutia* (Ten) with 26 mm compared to others one especially compared to the positive control ceftazidime CAZ30 with an inhibition zone of about 24 mm. Unlike the other treatments were fewer effective not exceeding 11 mm, with only 5 mm of the diameter inhibition zone (Fig. 2). Both ethanolic extracts and the essential oils from *P. canariensis* were more active against *Enterobacter cloacae* with 23.66 mm. All others inhibition diameters did not exceed 23 mm, given by the positive control ceftazidime CAZ30. Markedly the oil *P. canariensis* was the most active against *Staphylococcus aureus* with 21.5 mm compared to other diameters which were unable to exceed 15mm for all tested treatments. In literature, the antifungal potential investigated by the EO produced by pine species[24, 25] and Chutia *et al.* [26] proved that α-pinène and limonene showed antifungal activities against *Verticillium fungicola* and *Trichoderma harzinum* monoterpenes. It is known that monoterpenes were able to penetrate and altered the fungi cell.[27] Finally, we suggest that the synergy effect between the chemical constituent of the EO can also play a major role in the antimicrobial activities, as reported by other studies.[21] The antifungal activities of the ethanolic extracts from pine *species* were investigated against three *Candida* isolates, using a standard agar well diffusion method and voriconazole VCZ as positive control. In the whole, the positive antifungal control voriconazole VCZ was more effective against our ethanolic extracts and the essential oils from both pine *species* studied here, with diameter of inhibition varied from 30 to 35.5 mm. Both ethanolic extracts exhibited minor variations in their inhibitory activities against three tested *Candida species* and their diameters not exceed 27 mm. The essential oil from *P. canariensis* was the most active compared to others tested extracts and essential oils with 27 mm zone inhibition against *Candida parapsilosis* and with 22.5 mm against *Candida sake* (Fig. 3). Our results have shown that both ethanolic extracts and essential oils from pine *species* studied here appear to be effective as natural antibacterial and antifungal agents, and could be superior to the positive controls voriconazole VCZ and ceftazidime CAZ30 for some clinical pathogen *species* tested in the present work. The results have also shown that the antimicrobial efficiency is related to the microorganism *species* used here. Moreover, the MIC and MBC values of various tested extracts and essential oils presented in Table 1, showed that the ethanolic extracts were more efficient with high dilution value of 0.001µg/mL compared to both essential oils with very weak dilution value of 3.1 µg/mL for *P. brutia* EO and 2.4 µg/mL for *Pinus canariensis* EO. Similar results have been given with MBC value since these latter were correlated positively with the MIC values respectively with 1µg/mL for the ethanolic extracts and 3.1 µg/mL and 2.4 µg/mL respectively for EO produced from *Pinus brutia* and *Pinus canariensis*. The highest bactericidal activities presented by the ethanolic extracts were given against bacterial *species* belonging to *Enterobacter cloacae* and *Staphylococcus aureus* respectively with 2.5 10^9 and 2.5 10^8 UA/mL for *P. canariensis* extract and same value of 2.5 10^8 were given by the second ethanolic extract from *Pinus brutia.* These important bactericidal activities highlight the antibacterial activities of the ethanolic extracts compared to the essential oil from both *species* with value not exceed 5 10^3 (Table 2). In the present work, no antifungal activities were observed against the three tested moulds belonging to *species Penicillium spp*, *Aspergillus niger*, and *Alternaria spp* neither by the ethanolic extracts nor the essential oil from both *Pinus species* tested by the application of the agar diffusion method. Our results have shown markedly the efficiency of extracts and EO produced from both pine *species* since we have detected high MIC and MBC values compared to those reported by other works like for the EO produced by flower and corn of *Pinus brutia* with MIC values exceeded 6 µl/mL.[23] The antimicrobial

efficiency of our extracts pine *species* were also confirmed compared to previous reported results of Mohareb *et al.*[28] who have described that all tested extracts exhibiting promising antibacterial potency with varying MIC values 430–1800 mg/mL. Based on a reported study of Hanana *et al.*,[21] which focused on the evaluation of *pine species* (*Pinus coulteri*, *Pinus brutia* and *Pinus caribaea*) located in Tunisia that validated their antifungal and herbicidal potentialities,[21] our work has encouraged the application of *Pinus species* as biopesticides based on pine essential oils to reduce pathogen.

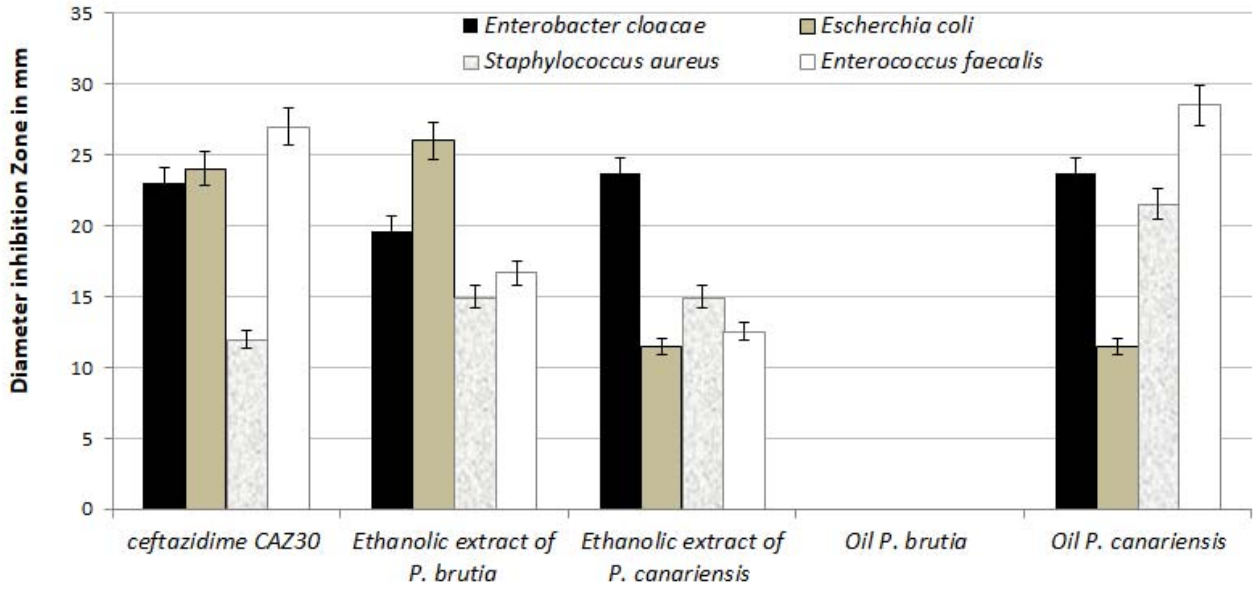

Fig. 2 – Antibacterial effectiveness of the ethanolic extracts and the essential oils from *Pinus brutia* (Ten) and *Pinus canariensis* (C. Smith) used at 10^{-1} (µl/mL) compared to the antibiotic positive standard ceftazidine CAZ30 disc (30 µg) based on the diameter of zone inhibition expressed in mm.

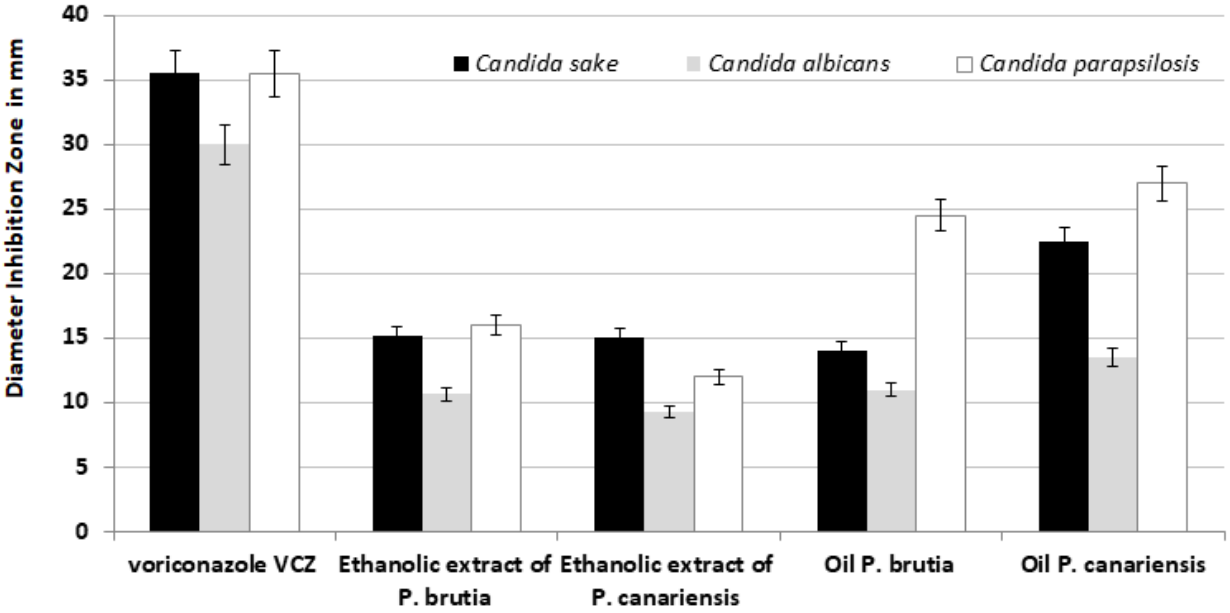

Fig. 3 – Antifungal activities of the ethanolic extracts and the essential oils from *Pinus brutia* (Ten) and *Pinus canariensis* (C. Smith) used at 10^{-1} (µl/mL), compared to a positive antifungal control voriconazole VCZ (1µg). Values means diameter inhibition zone measured in mm.

Table 1

MIC (µg/mL) and MBC (µg/mL) values of ethanolic extracts and essential oils extracted from *Pinus brutia (Ten) and Pinus canariensis (C. Smith)*

Pathogens	***Pinus canariensis***				***Pinus brutia***			
	Ethanolic extract		Essential oil		Ethanolic extract		Essential oil	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Enterobacter cloacae	0.001	0.001	0.0024	0.0024	0.01	0.001	-	-
Escherchia coli	0.1	0.01	0.024	0.0024	0.1	0.01	-	-
Staphylococcus aureus	0.01	0.001	0.0024	0.0024	0.01	0.001	-	-
Enterococcus faecalis	1000	100	0.0024	0.0024	1	0.1	-	-
Candida sake	1000	1000	0.024	0.0024	1000	100	0.031	0.031
Candida albicans	10	1	0.024	0.0024	1	0.1	0.0031	0.00031
Candida parapsilosis	10000	10000	0.024	0.0024	10000	1000	0.0031	0.00031

Table 2

Bactericidal and fungicidal activities of the ethanolic extracts and the essential oils extracted from *Pinus brutia* (Ten) and *Pinus canariensis* (C. Smith)

Microorganisms	***Pinus canariensis*** (UA/mL)		***Pinus brutia*** (UA/mL)	
	Ethanolic extract	**Essential oil**	**Ethanolic extract**	**Essential oil**
Enterobacter cloacae	**$2.5\ 10^9$**	**$5\ 10^3$**	**$2.5\ 10^8$**	-
Escherchia coli	$2.5\ 10^7$	$5\ 10^2$	$2.5\ 10^7$	-
Staphylococcus aureus	**$2.5\ 10^8$**	**$5\ 10^3$**	**$2.5\ 10^8$**	-
Enterococcus faecalis	$2.5\ 10^3$	**$5\ 10^3$**	$2.5\ 10^6$	-
Candida sake	$2.5\ 10^3$	$5\ 10^2$	$2.5\ 10^3$	$5\ 10^2$
Candida albicans	$2.5\ 10^5$	$5\ 10^2$	$2.5\ 10^6$	**$5\ 10^3$**
Candida parapsilosis	$2.5\ 10^2$	$5\ 10^2$	$2.5\ 10^2$	**$5\ 10^3$**

CONCLUSION

This investigation revealed significant variations in the essential oils chemical composition and total phenolic and flavonoids contents of the two *species Pinus brutia* (Ten) and *Pinus canariensis* (C. Smith). Moreover, the obtained results supported the antimicrobial properties of the tested essential oils and ethanolic extracts. They could be used as antimicrobial supplement in the developing countries towards the development of new therapeutic agents. Additional *in-vivo* studies and clinical trials would be needed to justify and further evaluate the potential of these oils as antimicrobial agents.

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