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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *EUCALYPTUS* ESSENTIAL OIL: A COMPARATIVE STUDY BETWEEN *EUCALYPTUS MARGINATA* L. AND *EUCALYPTUS PAUCILORA* L.

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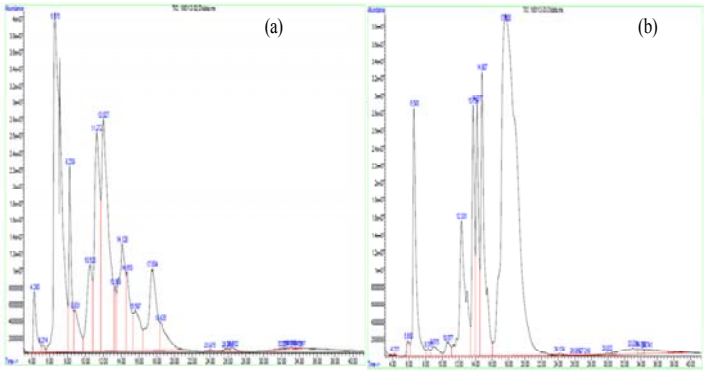
This study is the first to investigate the chemical composition of essential oil (EO), secondary metabolites as well as its biological activities of *eucalyptus marginata* L. and *eucalyptus pauciflora* L. leaves extracts. EOs have been analyzed using GC-MS apparatus. The chemical profile of the EOs of *eucalyptus marginata* L. and *eucalyptus pauciflora* L. mainly included 1,8-cineole (27.47 %) for the first *specie* while globulol (54.86 %) and α-Gurjunene (24.11 %) for the second one. Antioxidant and antimicrobial activities were also studied. The EOs of *E. marginata* and *E. pauciflora* exerted notable antibacterial activities especially against *Staphylococcus aureus*, and antifungal activity against *Candida parapsilosis*. The obtained EOs have a significant antioxidant potential. The biological activities and potentialities of EOs recommend their use in the formulation of natural and environmentally friendly pharmaceuticals.



INTRODUCTION

The family *Myrtaceae* is composed of at least 3.000 *species* in 130-150 genus.[1] They have a wide distribution in tropical and sub-tropical areas, and were cultivated in many other climates.[2] *Eucalyptus* is a native Australian tree. It is represented by more than 900 *species*.[3] It has been introduced worldwide, including in Tunisia. *Eucalyptus* is mainly cultivated for its timber, pulp and essential oils that present medicinal properties and therapeutic uses. It was considered as an important source of EOs used in traditional medicine. *Eucalyptus* EO was used to relieve head colds, rheumatism, muscular pain, and as an expectorant in cases of bronchitis. [4.] It has been used in folk medicine throughout the world as anti-inflammatory, analgesic and antipyretic remedies for the symptoms of respiratory infections, such as cold, flu, and sinus congestion. [3,4] EOs from e*ucalyptus species* have been approved as food additives, and the

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extracts also widely used in modern pharmaceutical, and cosmetic industries.[5] In addition, the oil possessed a wide spectrum of biological activity including antimicrobial, fungicidal, insecticidal/ insect repellent, herbicidal, acaricidal and nematicidal.[6]

There were a larger number of recent studies focusing on *eucalyptus species* all over the world, but the present study was the first comparative investigation between *eucalyptus marginata* L. and *eucalyptus pauciflora* L. essential oils as well as its ethanolic extract. Therefore, we aimed to analyze qualitatively and quantitatively the constituents of *eucalyptus marginata* L. and *eucalyptus pauciflora* L. leaves' EO and its ethanolic extracts. Antimicrobial and antioxidant activities were also investigated. Correlations between chemical composition and biological activities were studied. The ultimate objectives of this work were to find new potential sources of natural antioxidants and antimicrobial agents in food industry.

MATERIALS AND METHODS

Plant Material

Eucalyptus marginata L. and *Eucalyptus pauciflora* L. leaves were collected in May 2014 from Souiniet arboretum in Northwest of Tunisia (8 ° 48 E, 35 ° 54N, 492 m). The identification of the plant material was done by Professor Mohamed Laarbi Khouja and a voucher specimen (EM2018 and EP2018, for *Eucalyptus marginata* L. and *Eucalyptus pauciflora* L., respectively) were deposited at the Herbarium of INRGREF (Tunisia).

Sample preparations

Extraction of phenolic compounds. Each plant powdered material (5 g) were extracted by maceration in ethanol (3 x 50 mL) at room temperature for one hour. The extract was filtered through Whatman no. 4 paper. The ethanolic extracts were evaporated at 35 °C to dryness then stored in the dark at 4 °C until use. Before testing, the dryness extract was freshly re-dissolved in ethanol at a final concentration of 100mg/mL of ethanolic extract.

Extraction of condensed Tannin (CT). Each plant materials (40 g dry weight) were soxhlet-extracted with *n*-hexane for six hours to remove lipids and lipophilic substances. Subsequently, the CT was isolated from the solid residue by an acetone/water solution (70:30, v/v) after addition of ascorbic acid (0.1 %, w/v). Subsequently, acetone were evaporated using a rotary evaporator, and the aqueous phase was washed successively with chloroform and ethyl acetate in order to remove chlorophyll, carotenoids, low molecular weight phenolics, and tannin monomers.[7]

Total phenols, flavonoids and tannins contents

Total phenols were determined with Folin–Ciocalteu reagent according to the procedure described by Singleton and Rossi[8] with slight modifications.[7] Gallic acid was used as a standard for calibration curve and results were expressed as mg of gallic acid equivalent.g^{-1} DW (mg GAE.g^{-1} DW). While, total flavonoids content was based as described before by Yahyaoui *et al.*[7] with some modifications, the results were expressed as milligram of rutin equivalents per 1 g of dry weight (mg RE.g^{-1} DW). On the other hand, the condensed tannins were tested colorimetrically as described previously.[9] The content of condensed tannins in the ethanolic extracts was expressed as mg catechin equivalents per g of dry weight (mg CE.g^{-1} DW).

Essential oil isolation and analysis

Biologic material/leaves from the two *eucalyptus species* were subjected to hydro-distillation in a Clevenger's type apparatus for 3 h in order to isolate essential oil (EO). The EOs were measured directly in the extraction burette and the amount of oil obtained (%) was calculated as volume (ml) of EO per 100 g of dry plant material. The oils were dehydrated over anhydrous Na_2SO_4 and kept in a cool and dark place prior to analysis. Analysis of the EOs were carried out by combination of gas chromatography (GC-FID) and gas chromatography–mass spectrometry (GC–MS) according to a standard analytical procedure using a DB-5MS column (30 m, 0.25 mm and 0.25 µm film thickness). The flow rate of the carrier gas (helium) was 1.0 mL/min. 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}. Besides, the injector and detector temperatures were set at 250 and 230 °C, respectively. The mass range was scanned from 50 to 550 amu. A sample of 1.0 µL was

injected, using split mode (1:100). Compounds of the EO were identified by both their Kovats indices and mass spectra. Kovats indices were calculated by linear interpolation relative to retention times of *n*-alkanes (C_8–C_{24}). Mass spectra were matched with reference spectra from Wiley/NIST database, published data and spectra of authentic compounds. Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction. [10–12]

Antioxidant activities

DPPH assay. The free radical-scavenging capacity was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method described in a previous study.[13,14] All tests were carried out in triplicate. Sample concentrations providing 50 % inhibition (IC_{50}) were calculated and expressed as mg/mL of ethanolic extract or EO.

ABTS assay. ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonate]) was estimated following the procedure originally described by Re *et al.*[15] Tests were carried out in triplicate. Sample concentrations providing 50 % inhibition (IC_{50}) were calculated and expressed as mg/mL of ethanolic extract or EO.

Antimicrobial activity screening

The EO and the ethanolic extracts from *E. marginata* L. and *E. pauciflora* L. leaves were tested against a list of pathogenic Tunisian clinical strains belonging to the laboratory culture collection Mycology Pathology and Biomarkers, Faculty of Sciences of Tunisia. These pathogenic strains used in this work were obtained after Tunisian various human clinical samples isolation and identification by phenotypic and genotypic criteria as previously reported.[16,17] Two gram-negative bacteria (*Escherchia coli* and *Enterobacter cloacae*), two gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis)* and three yeast strains *(Candida albicans, Candida parapsilosis* and *Candida sake)* were used in this study. Before use, each EO and ethanolic extracts were diluted in DMSO and ethanol, respectively, to 10 % v/v, and sterilized by filtration through a 0.2 μm pore size filter. Antibacterial and antifungal tests were performed by agar well diffusion method as described and broth microdilution assay using sterile Mueller–Hinton media (Bio Rad, France) for bacterial strains and yeast malt extract agar YMA (Bio-Rad, France) for antifungal tests.[18,19]A freshly cell suspension (100 μL) adjusted to 10^7 CFU/mL for bacteria and 10^5 spores/mL for fungus were inoculated into the surface of agar plates. Thereafter, wells with 6 mm in diameter were punched in the inoculated agar medium and 20 μL of the ethanolic extract or essential oil were added to each well. Negative controls consisted of 20 μL ethanol, used to dissolve the ethanol or the essential oil. Two positive controls were also used ceftazidime CAZ30 as antibiotic and voriconazole VCZ as antifungal.

The plate was allowed to stand for 2 h to allow the ethanolic extract or EO diffusion 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 tests were repeated three times.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the tested ethanolic extract or EO against pathogenic microorganisms was determined using the series micro-dilution method by using the solvent ethanol for extract dilution from the stock solution of 100 mg/mL to 0.01 mg/mL and the solvent DMSO was used for EO dilution from a stock solution of 50 mg/mL to 0.05 mg/mL. MIC expressed in mg/ml were estimated visually (absence of turbidity) and were determined with three independent measurements. [19]

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

Oil Yield and Chemical Constituents

The EOs yield was calculated on a dry-weight basis (w/w). The highest (0.25 %) and lowest (0.23 %) yields were obtained from *E. marginata* L. and *E. paucilora* L., respectively. These results

indicated that the yield of obtained EO varied significantly (p<0.05) with *Eucalyptus species* in the studied area. Our studies based on the comparison of the averages oil yield by Duncan test with other *Eucalyptus species*. Biljana *et al.*[20] reported that an EO yield was 1.8 % for *E. globules* leaves from Montenegro. Indeed, EO content can be mainly affected by environmental and genetic factors. [21] Moreover, it may also be influenced by the method and the extraction conditions. [22]

The chemical composition of the obtained EOs was analyzed by GC and GC-MS, which allowed identification of 98.29 % (*E. marginata* L.) and 99.25 % (*E. paucilora* L.) of oil constituents (Table 1). The main compounds from *E. marginata* oil were 1,8-cineole (27.74 %), propanal, 2-methyl-3-phenyl (18.40 %), cryptone (12.64 %), 2,5-diethylphenol (7.57 %), 4-terpineol (5.25%), *p*-cymene (5.17 %), however, the minor identified compounds were α–pinene (2.1 %), β-thujone (2.55 %) and octadecan (0.12 %). While globulol (54.86 %), α-Gurjunene (24.11 %), 2-Isopropyl-5-methyl-3-cyclohexen-1-one (8.58 %) and *p*-cymene (8.13 %) were the major compounds obtained from *E. paucilora* L., while, 1-Iodooctadecan (1.18 %), linalool (0.73 %), 4-terpineol (0.73 %) and α-phellandrene (0.44 %) were presented as minor compounds (Table 1).

No data was reported in the literature regarding the chemical composition of *E. marginata* L., and *E. paucilora* L. EOs' but we compared it to other *species*. Previous studies from Tunisia and Taiwan reported that GC and GC-MS analysis showed that chemical composition varied significantly with *Eucalyptus* species and seasons.[23–25] The difference in the chemical composition of the EO might be attributed to harvest period, climatic, soil, seasonal and geographic conditions, genetic differences and isolation technique.[26,27] For *Eucalyptus species*, many factors may influence monoterpene emissions, especially seasonal and diurnal emission activity cycles.[28]

Table 1

Chemical composition of EO from *E. marginata* L. and *E. pauciflora* L. leaves

N°	Compounds	*E. marginata*	*E. pauciflora*	RI
1	α-pinene	2.1	0.01	855
2	β -pinene	0.25	-	865
3	α-Phellandrene	-	0.44	875
4	1,8-cineol	27.74	-	887
5	*p*-cymene	5.17	8.13	888
6	α-Dimethyl styren	-	0.25	917
7	β-Thujone	2.55	-	925
8	Linalool	-	0.73	928
9	4-Terpineol	5.25	0.73	956
10	Cryptone	12.69	-	918
11	Propanal, 2-methyl-3-phenyl	18.40	-	979
12	2-Isopropyl-5-methyl-3-cyclohexen-1-one	-	8.58	984
13	2,5-Diethylphenol	7.57	-	1014
14	*p*-Cymen-7-ol	3.58	-	1023
15	α-Gurjunene	-	24.11	1259
16	Eugenol	3.15	-	1039
17	(-)-Spathulenol	7.22	-	1315
18	Globulol	-	54.86	1318
19	Calarene	2.28	-	1334
20	Phytol isomer	-	0.12	1676
21	Octadecan	0.12	0.01	1636
22	2-(2,2,4-Trimethyl-3,4-dihydro-2H-chromen-4-yl)phenol	0.22	-	1646
23	Crotocine	-	0.1	1777
24	1-Iodooctadecan	-	1.18	1829
	Total	98.29	99.25	

Notes: RI: retention indices calculated in regard to standards mixture of hydrocarbons (C_8-C_{28}) for 4.790-48.969 min. %: Percentage calculated by GC-FID on HP-INNOWax. RI: retention index

Table 2

Total phenols, flavonoids, and tannins contents in *E. marginata* L. and *E. pauciflora* L. ethanolic leaves' extracts

	Total phenol (mg GAE/ g DW)	**Total Flavonoid (mg RE /g DW)**	**Condensed tannins (mg CE/g DW)**
***E. marginata* L.**	65.89 ± 0.1[a]	7.11 ± 0.02[a]	1.42 ± .03[a]
***E. pauciflora* L.**	45.43 ± 0.04[b]	12. 29 ± 0.1[b]	1.07 ± 0.5[b]

Means with different letters in the same column were significantly different at $p<0.05$. Results are expressed as mean± standard deviation of 3 determinations.

Table 3

Antioxidant activity of EOs and ethanolic extracts of *E. marginata* L. and *E. pauciflora* L. leaves

	***E. marginata* L.**		***E. pauciflora* L.**	
	Extract	EO	Extract	EO
DPPH	41 ± 1[a]	29.3 ± 0.34[a]	35.9 ± 0.65[a]	21.16 ± 0.01[a]
ABTS	56 ± 0.4[b]	30 ± 0.1[b]	48 ± 0.32[b]	23.5 ± 0.01[b]

Means with different letters were significantly different at $p<0.05$. Results are expressed as mean± standard deviation of 3 determinations.

Total phenols and flavonoids contents

In this study, we aimed to highlight the differences in secondary metabolites contents between two *species* (Table 2). These results showed that phenols content in ethanolic extract varied significantly ($p<0.05$) between the two studied *species*. The *E. marginata* L. leaves contained the highest phenols content (65.89 mg GAE·g^{-1} DW) followed by *E. pauciflora* L. leaves' extract (45.43 mg GAE·g^{-1} DW). The *E. pauciflora* L. ethanolic extract presented the highest content in flavonoids compounds (12.29 mg RE·g^{-1} DW) while only 7.11 mg RE·g^{-1} DW were reported in extract from *E. marginata* L. leaves'. The highest tannin contents were quantified in *E. marginata* L. leaves' extract (1.42 mg CE·g^{-1} DW), followed by *E. pauciflora* L. (1.07 mg CE·g^{-1} DW). There were no previous citations according the secondary metabolites contents in *E. marginata* L. and *E. pauciflora* L. In addition, Yaya Koudoro *et al.*[29] showed that the contents of polyphenolic compounds from ethanolic and hydroethanolic extracts of *E. citriodora* were respectively 4.52 mg GAE.g^{-1} DW and 4.38 mg GAE ·g^{-1} DW for total polyphenols, 78.76 mg RE·g^{-1} DW and 81.56 mg RE ·g^{-1} DW for total flavonoids while only, 62.62 mg CE·g^{-1} DW and 67.09 mg CE·g^{-1} DW for condensed tannins.

Determination of Antioxidant activity

The ABTS and DPPH assays were used in this study to examine the antioxidant activities of the two studied *species*.[30,31] The results were shown by IC_{50} value, which is the concentration of a compound that will induce half of the maximum action (Table 3). There was a significant variation in free radical scavenging activities of EOs among *species* and the inhibitory concentrations IC_{50} ranged from 21.16 to 29.3 mg/mL. The EOs from *E. paucilora* L. exhibited the highest radical scavenging activity (IC_{50}=21.16 mg/mL) followed by *E. marginata* L. (IC_{50}=29.3 mg/mL). Using ABTS radicals for evaluating the antioxidant activity of the phenolic compounds in plant extract and EO, we have noted that all studied extracts (EOs and ethanol extract) possessed significant antiradical activities ($p<0.05$). However, EOs of the two *species* have a better antiradical activity than ethanolic extract. In previous studies, Gonzalez *et al.*[32] and Anwar *et al.*[33] reported that EOs of some *Eucalyptus species* were an effective radical scavenger. In accordance with the obtained data from this study, Aissi *et al.* [34] mentionned that the effect of geographic origin can influence the antioxidant activity of EOs. These variations were probably due to differences in phenolic compound content. Indeed, antioxidant activity was attributed to plant phenolic compounds.[35] Phenolics, due to their hydroxyl groups that allow them to inhibit DPPH free radicals, were considered as the major factor contributing to antioxidant activity of plants.[36,37] Several studies showed a positive correlation between total phenolic compound content and antioxidant activity.[38,39]

Determination of Antimicrobial activity

In this work, the ethanolic extract from *eucalyptus species*: *E. marginata* L. and *E. pauciflora* L. leaves were able to inhibit growth of tested pathogenic bacteria and *Candida*. The results were discussed on the basis of the diameter of zone of inhibition in mm.

Besides, the value of the zone inhibition ranged from 19.66 mm to 9 mm. The ethanolic extract from *E. pauciflora* was more active than those from *E. marginata* against three bacterial species *Enterobacter cloacae, Enterococcus faecalis,* and *Escherchia coli.* On the other hand, the EO from *E. marginata* was more effective against all tested bacteria strains with diameter zone inhibition above 15 mm. Against *Staphyloccoccus aureus*, both EOs from *E. marginata* L. and *E. pauciflora* L. were most effective with diameter zone inhibition of about 18.33 and 20 mm, respectively, compared to both ethanolic extract from *E. marginata* L. and *E. pauciflora* L. While the positive control with diameter zone inhibition did not exceed 13.33 mm given by ethanolic extract from *E. marginata* (Fig. 1). In general, gram-positive bacterial strains were more sensitive to *Eucalyptus* EOs than the gram-negative ones.[40,41] This can be rationalized considering that gram-negative bacteria possess a lipopolysaccharide membrane which is restrictive to the diffusion of hydrophobic compounds. In addition, the direct contact between the hydrophobic components of the EOs and the phospholipid bilayer of the cell membrane can occur in gram-positive bacteria. As a consequence, the components exert their effects such as increase in the permeability to ions, leakage of vital intracellular components or compromise bacterial enzymes.[42]

The antifungal activities of both ethanolic extracts from *Eucalyptus* species were investigated against three *Candida* isolates using a standard agar well diffusion method and voriconazole VCZ as positive control. Both ethanolic extracts exhibited poor variations in their inhibitory activity against these tested *Candida* isolates: *E. pauciflora* was more efficient against *Candida parapsilosis* with 14 mm and *E. marginata* L. more effective against *Candida albicans* with a diameter zone inhibition of about 12.5 mm. Merely only *Candida sake* was resistant to ethanolic extract from *E. marginata* L. The *E. pauciflora* L. EO was more efficient against *Candida species* than EO from the second *E. marginata* L. which exhibited diameter zone inhibition near to those obtained by the positive antifungal with values ranging from 30.33 mm to 34.33 mm, respectively, against *C. sake* and *C. parapsilosis* (Fig. 2). Our results showed that, on one hand, the EOs and the ethanolic extracts from *Eucalyptus* species studied here appear to be effective as natural antibacterial and antifungal agents, but not superior to the positive controls voriconazole VCZ and ceftazidime CAZ30. On the other hand, and in general, the ethanolic extract from *E. pauciflora* was more active than *E. marginata* L., these differences may be related to the variations in their chemical composition as proved in Table 1. EOs were more active against *Candida* strains. Results showed that the EO from *E. marginata* L. was more effective against *C. albicans* and *C. parapsilois* with same MIC values of 0.05 mg/mL (Table 4). Moreover the ethanolic extract of *E. marginata* L. was more effective as an antibacterial agent since its MIC values ranging from 0.01 mg/mL to 0.1 mg/ml against all tested bacteria strains. Unlike both EOs were not well efficient against bacteria strains with MIC value of 5 mg/mL and 0.5 mg/mL. Difference results against *Candida species* presented by each ethanolic extract, data show that *Eucalyptus pauciflora* L. was more active against *Candida sake* with MIC 0.01 mg/mL and *Eucalyptus marginata* L. ethanolic extract was more active against *Candida albicans* with the same MIC value 0.01 mg/mL.

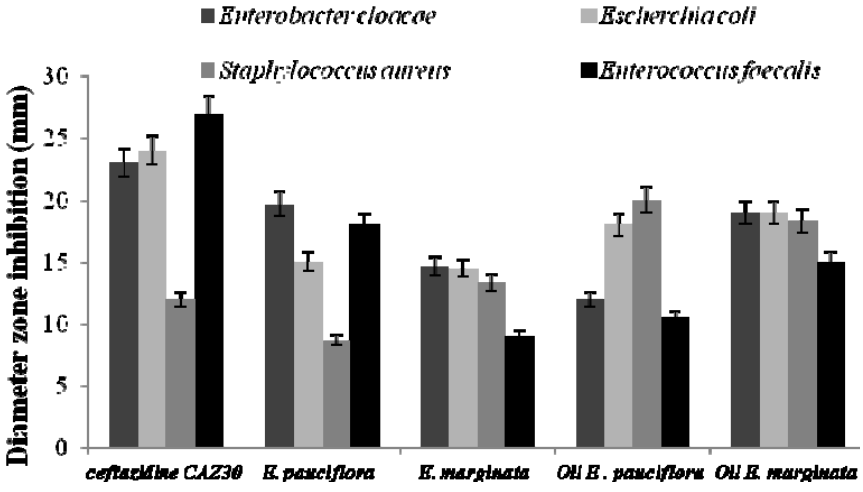

Fig. 1 – Antibacterial effectiveness of the ethanolic extracts and EOs from e*ucalyptus marginata* L. and *eucalyptus pauciflora* L. leaves in ethanol and DMSO, respectively (10 %, v/v) compared to the antibiotic positive control ceftazidine CAZ30 based on the diameter of zone inhibition expressed in mm.

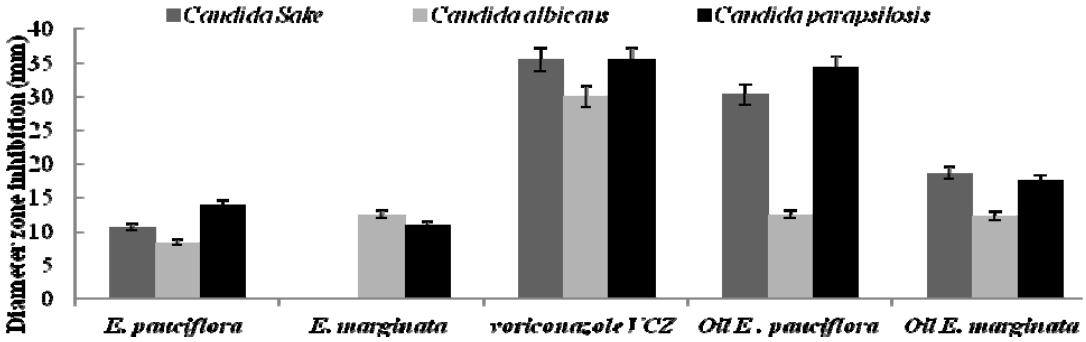

Fig. 2 – Antifungal activities of the ethanolic extracts and EOs from *eucalyptus marginata* L. and *eucalyptus pauciflora* L. leaves used in ethanol and DMSO, respectively, 10 % (v/v) compared to positive control VCZ measured in mm by agar diffusion method.

Table 4

Comparative MIC values expressed in mg/ml against bacteria and *candida species*

	***E. pauciflora* L.**		***E. marginata* L.**	
Pathogenic strains	Ethanolic extract	EO	Ethanolic extract	EO
Enterobacter cloacae	1	5	0.01	0.5
Escherchia coli	0.01	5	0.01	5
Staphylococcus aureus	1	5	0.1	0.5
Enterococcus faecalis	1	5	0.01	5
Candida albicans	1	5	0.01	0.05
Candida parapsilosis	1	0.5	1	0.05
Candida sake	0.01	5	-	0.5

CONCLUSION

Our finding revealed significant variations in the yields, chemical content, antimicrobial and antioxidant activity of *E. marginata* L. *and E. pauciflora* L. This study suggests that two *species* will be a good resource as natural antioxidant and antimicrobial agent.

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