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Chemical Constituents and Antimicrobial Activity of the Leaves of Eucalyptus (*Eucalyptus camaldulensis* Dehnh.), An Endemic Plant from West Anatolia

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The extracts of methanol–water, hexane and chloroform eucalyptus leaves (*Eucalyptus camaldulensis* Dehnh.) were screened *in vitro* for their antimicrobial activity against some pathogenic gram negative, gram positive bacteria and one yeast. Disk diffusion method was used for antimicrobial activity of extracts and the microwell dilution assay was used for the determination of minimal inhibitory concentration (MIC) of each extract. In addition, the composition of chloroform extratc was determined by means of GC/MS analysis. The methanol/water, hexane and chloroform exracts of *E.camaldulensis* leaf showed various antimicrobial activity 7-9mm, 8-11mm and 11-27 mm inhibition zone, respectively. The chloroform extracts was found to be the most effective antimnicrobial agent as compared to the other extracts. While the most sensitive bacteria was *Bacillus cereus* CCM 99 (27mm inhibition zone), the most resistant bacteria was *Esherichia coli* ATCC 35218. The methanol/water, hexane and chloroform extracts of *E. camaldulensis* leaf showed 1.25-5.0<μg/ml, 0.625-5.0<μg/ml and 0.039-0.625μg/ml MIC value, respectively. While the chloroform extract displayed the best activity (MIC 0.039 μg/ml) against *B.subtilis* ATCC 6633 and *B.cereus* CCM 99 and *C.albicans* ATCC 10239, it showed the least activity (MIC 0.625 μg/ml) against *E.coli* ATCC 35218. The GC/MS analyses allowed 11 compounds to be determined; the main constituents of the Eucalyptus leaves extract were chloroform (68.5%), hexane(10.85%), ethylacetate (9.12%), ethanol (8.25%), γ terpiene (0.78%), bromochloromethane (0.59%), 1,8 cineole (0.52 %). The data of this work indicate that extracts of eucalyptus leave from West Anatolia, especially the chloroform extracts, could be formed the basis of many applications including raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies.

Key words: *Eucalyptus camaldulensis* Dehnh., leaf extract, Antimicrobial activity, Chemical composition, GC-MS analysis.

Food borne infections have been one of the major public health concerns worldwide and account for considerably high cases of illnesses attacking humans and animals. More than 250 different food borne diseases have been described. Most of these diseases are due to microbial infections[1,2]. Antimicrobial activity of spices and herbs has been known and desribed for several centuries[3-6]. Natural products can be selected for biological screening based on ethnomedical use of plants, because many infectious diseases are known to have been treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries[7].

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The Eucalyptus plants (Myrtaceae) are used to control several diseases derived from microbial infections[8]. Several species of eucalyptus are used in folk medicine as an antiseptic and against infections of the upper respiratory tract, such as cold, influenza and sinus congestion[9]. Leaf extracts of *Eucalytus* have also been approved as food additives[10]. A number of studies have demonstrated the antimicrobial properties of *Eucalytus* species leaf extracts against a wide-range of microorganisms[11-13]. Only a few studies investigated their activity against, pathogenic and food spoilage bacteria[14]. The antibacterial activity of the leaf extracts of *Eucalyptus camaldulensis* can be recognized due to the phytochemical compounds it contians, especially saponins, tannins, volatile oils and balsam[11]. The presence of these compounds in the family Myrtaceae to which *E.camaldulensis* belong has been reported by Pamplona-Roger[15].
This study is the first report on the antimicrobial activity of West Anatolian eucalyptus leaves against some pathogenic microoorganisms. The objectives of this work were therefore to investigate the antimicrobial activities of three different solvent extracts from West Anatolian eucalyptus leaves and to determine the chemical compounds content to find out the relationship between antimicrobial activity and the compound content. Therefore, we have tested antimicrobial effect against some microorganisms including opportunistic pathogens: (*Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Streptococcus faecalis* ATCC 8043, *Chryseomonas luteola* TEM 05 (from Biology Department of Ege University, Izmir, Turkey), *Staphylococcus aureus* ATCC 6538/P) and gram – (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583, *Escherichia coli* ATCC 35218, *Enterobacter cloacea* ATCC 13047, *Aeromonas hydrophila* ATCC 19570, *Klebsiella pneumoniae* CCM 2318) bacteria and *Candida albicans* ATCC 10239. The antimicrobial activity was measured by using disk diffusion method and minimal inhibitory concentration (MIC).

MATERIALS AND METHODS

Collection of leaves

Eucalyptus leaves were obtained from Cine region that were collected on September. West Anatolian of Turkey (N:37-32' 30.1", E:28° 08' 35.6 altitude: 520m). The orchard has a planting density of 5,5x20m. The trees are twenty-five years old, being pruned when necessary. No phytosanitary treatments were applied.

Plant material and extraction

The leaves were air dried over a period of one week. 50.0g each of the dried leaves were used for the extraction. The methods of Babayi *et al.*[11] and Egwaikhide *et al.*[16] were used to obtain the leaf extracts. The leaf extracts of eucalyptus were prepared as follows. The leaves (10 g) were dried in vacuo after air drying and immersed in 200 ml 10% aquous methanol, hexane and chloroform at room temperature for 2 days. The plant extracts were filtered through Whatman filter paper No. 1 (Whatman) and concentrated to give methanol–water, hexane and chloroform extracts. The air dried extracts were stored for 2 days in sterile universal bottles at room temperature. The sterilities of the extracts were tested before use.

Test microorganisms

Test microorganisms, gram positive (*Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Streptococcus faecalis* ATCC 8043, *Chryseomonas luteola* TEM 05 (from Biology Department of Ege University, Izmir, Turkey), *Staphylococcus aureus* ATCC 6538/P) and gram negative (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583, *Escherichia coli* ATCC 35218, *Enterobacter cloacea* ATCC 13047, *Aeromonas hydrophila* ATCC 19570, *Klebsiella pneumoniae* CCM 2318) bacteria and *Candida albicans* ATCC 10239 were used in this study.

Culture media and inoculum

Sabouraud Dextrose (SD) and Nutrient Broth (NB) media (Merck) were used for *C. albicans* and test bacteria, respectively. Microbial cultures, freshly grown at 37°C or 30°C were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 10^5 CFU:ml.

Antimicrobial assays

The disc diffusion method[11] as adopted earlier Collins *et al.*[17] was used; 0.2g of the extracts were reconstituted in 5ml sterile distilled water and vortexed for homogeneity. 1ml of the reconstituted extract was added to Petri dishes having sterile

molten Mueller Hinton Agar for bacteria and Potato-Dextrose Agar for *C. albicans* to make a final concentration of 2000 µg/ml. The plates were prepared in duplicates and allowed to set at room temperature. A loopful each of the standardized culture of test organisms was streaked on the solidified medium. The plates were incubated for 18 h at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Control plates comprising extract without inoculum and inoculum with extract were made in parallel. Tobramycin (10 µg/disc) (Oxoid), ampicillin (10 µg/disc) (Oxoid) and nystatin (30 µg/disc) (Oxoid) were used as positive controls and paper discs treated with methanol:water (9:1), hexane and chloroform were used as negative controls.

Determination of minimum inhibitory concentrations (MICs)

MICs were defined as the lowest concentration of test samples that inhibited visible growth of micro-organisms. The MIC values of the plant extracts was determined on solid medium (Mueller Hinton Agar for bacteria and Potato-Dextrose Agar for *C. albicans*) using the method of Collins *et al*.[17] with some variations. The range of concentration used was 0.039 – 5.0µg/ml. The antibacterial agents (ampicillin and oxacillin) (Oxoid) and antifungal agent (nystatin) (Oxoid) were dissolved in sterilized distilled water and MICs were determined according to the procedure described above.

GC/MS analysis

The steam-distilled components were analysed by GC/MS. A HP 6890 gas chromatograph equipped with a HP-PTV and a 0.32mX0.60m HP-Innowax capillary column (0.5µm coating) was employed for the GC analysis. GC/MS analysis was performed on a HP-5973 mass selective detector coupled with a 6890 gas chromatograph, equipped with a HP 6890 gas chromatograph, equipped with HP-1capillary column. The column temperature was programmed from an initial temperature of 60 °C to a final temperature of 250 °C at 15°C/min. The carrier gas was helium (14.1mL/min). Identification of the individual components was performed by comparison of mass spectra with literature data and by a comparison of their retention time (Rt) relative to a C_{8}-C_{32} *n*-alkanes mixture[18]. A computerized search was carried out using the Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples.

RESULTS AND DISCUSSION

Plant essential oils and extracts have been used for many thousands of years[19] in food preservation, pharmaceuticals, alternative medicine and natural therapies[8,20].

In the present study, the methanol/water, hexane and chloroform extracts of *E.camaldulensis* leaf showed various antimicrobial activities 7-9mm, 8-11 mm and 11-27 mm inhibition zone, respectively (Table 1). The chloroform extract was found to be the most effective antimnicrobial agent as compared to the other extracts. All tested microorganisms were inhibited by the chloroform extracts. While the most inhibited bacteria was *B. cereus* CCM 99 (27mm inhibition zone), the least inhibited bacteria was *E. coli* ATCC 35218 (11mm inhibition zone). All test microorganisms were inhibited by the chloroform extracts.

In a similar study, El-Mahmood[13] reported that the antibacterial activity of the crude leaf extracts of *Eucalyptus camaldulensis* were determined using the agar well diffusion method against clinical isolates of *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Proteus mirabilis* and *Klebsiella pneumoniae.* At an extract concentration of 50 mg/ml, the growths of all the pathogenic bacteria were arrested, though to varying degrees. The least activity interms of zones of growth inhibition was shown by aqueous extract 7-13 mm against all tested bacteria, while the highest was demonstrated by the acetone extract, with a recorded zone diameter for 12-15 mm against all tested bacteria. Minimum inhibitory concentration (MIC) values ranged from 6.25-50 mg/mL.

Ayepola and Adeniyi[12] revealed that the antibacterial activity of the leaf extracts of *Eucalyptus camaldulensis* was studied against *Klebsiella spp, Salmonella typhi, Yersinia enterocolitica, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* by the agar diffusion method. The methanol extract, dichloromethane fraction and methanol residue at 10mg mL^{-1} displayed broad spectrum activity against all the test organisms but the petroleum ether fraction showed no activity. The antibacterial

activity of the extracts was compared to the drug gentamycin. The minimum inhibitory concentrations of the methanol extract and dichloromethane fraction determined by the agar dilution method ranged between 0.04 and 10mg mL^{-1} with that of *Bacillus subtilis* being the least.

Tosun *et al.*[21] reported that ethanolic extracts of *E. camaldulensis* Dehnh. showed no anti-mycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Ra$.

In another similar work, some researchers tested antimicrobial activity of essential oils of

Table 1. Antimicrobial activities of *Eucalyptus camaldulensis* leaf extracts against test microorganisms by disc diffusion method

Microorganism	Zone of Inhibition (mm)								
	Methanol:water (2000 µg/ml)		Hexane (2000µg/ml)	Chloroform	Tobramycin (2000µg/ml)		Ampicillin (10 µg/disc)	Nystatin (10 µg/disc)	(30 µg/disc)
	C	E	C	E	C	E			
E.coli ATCC 35218	-	9	-	8	-	11	10	12	NT
P. aeruginosa ATCC 27853	-	9	-	9	-	12	11	9	NT
E. cloaceae ATCC 13047	-	8	-	8		13	8	11	NT
B. subtilis ATCC 6633	-	7	-	10	-	24	15	10	NT
B. cereus CCM 99	-	7	-	11	-	27	16	12	NT
S. typhimurium CCM 583	-	8	-	9	-	13	10	12	NT
S. faecalis ATCC 8043	-	-	-	-	-	12	9	14	NT
C. luteola TEM 05	-	-	-	10	-	16	8	14	NT
A. hydrophila ATCC 19570	-	7	-	10	-	12	11	10	NT
K.pneumoniae CCM 2318	-	7	-	-	-	12	11	9	NT
S. aureus ATCC 6538/P	-	-	-	8	-	17	13	15	NT
C. albicans ATCC 10239	-	-	-	10	-	22	NT	NT	18

C, Negative control (methanol:water (9:1), hexane or chloroform); E, Extract (methanol:water (9:1) extract, hexane extract or chloroform extract)
-, no inhibition; NT, not tested

Table 2. MIC values of *E.camaldulensis* leaf extracts, ampicillin and oxacillin against test microorganisms

Microorganism	MIC (µg/ml)					
	E. camaldulensis leaf extracts			Ampicillin	Oxacillin	Nystatin
	Methanol:Water extract	Hexane extract	Chloroform extract			
E. coli ATCC 35218	1.25	2.5	0.625	1.25	0.312	NT
P. aeruginosa ATCC 27853	1.25	1.25	0.312	0.625	1.25	NT
E. cloaceae ATCC 13047	2.5	2.5	0.156	2.5	0.625	NT
B. subtilis ATCC 6633	5.0	1.25	0.039	0.078	1.25	NT
B. cereus CCM 99	5.0	0.625	0.039	0.078	0.312	NT
S. typhimurium CCM 583	2.5	1.25	0.156	1.25	0.312	NT
S. faecalis ATCC 8043	5.0<	5.0<	0.312	1.25	0.156	NT
C. luteola TEM 05	5.0<	1.25	0.078	2.5	0.156	NT
A. hydrophila ATCC 19570	5.0	1.25	0.312	0.625	1.25	NT
K. pneumoniae CCM 2318	5.0	5.0 <	0.312	0.625	1.25	NT
S. aureus ATCC 6538/P	5.0<	2.5	0.078	0.156	0.078	NT
C. albicans ATCC 10239	5.0<	1.25	0.039	NT	NT	0.078

5.0<, no activity

E. camaldulensis, for example Akin *et al.*[22] reported that water-distilled essential oils from leaves of *E. camaldulensis* Dehnh. showed 14 mm inhibition zone against only *S.aureus* among the tested microorganisms (*E.coli* and *P.aeruginosa*).

The degree of antimicrobial activity was considered from the MIC values against test microorganisms. The MIC values were used as guide for the treatment and battle against undesirable microorganisms (Table 2). The chloroform extract displayed the best activity (MIC 0.039 µg/ml) against *B.subtilis* ATCC 6633 and *B.cereus* CCM 99 and *C.albicans* ATCC 10239. Similarly, the chloroform extract displayed antibacterial activity (MIC 0.078 µg/ml) against *C. luteola* TEM 05 compared to used antibiotics. In contrast, the chloroform extract showed the least activity (MIC 0.625 µg/ml) against *E. coli* ATCC 35218. The methanol/water, hexane and chloroform extracts of *E.camaldulensis* leaf showed 1.25-5.0< µg/ml, 0.625- 5.0< µg/ml and 0.039-0.625 µg/ml MIC value, respectively. The results of MIC value showed that chloroform extracts were the best activity compared to methanol/water and hexane like disc diffusion.

Type of used extract have been affected the soluble type of antimicrobial compounds. Egwaikhide *et al.*[16] reported that the water and ethyl acetate extrcats were found to have alkaloids. It was only in hexane extracts that triterpenes was observed. In the water extract, flavonoids and tannins were present which were absent in the organic extracts. The chlorofor m extract were found to saponins, volatile oils, faty acids and steroid. The ethanol chloroform extracts of Eucalyptus leaves were also evaluated for their chemical composition by GC/MS in this study. The GC/MS analyses allowed 11 compounds to be determined; the main constituents of the Eucalyptus leaves extract were chloroform (68.5%), hexane (10.85%), ethylacetate (9.12%), ethanol (8.25%), γ-terpiene (0.78%), bromochloromethane (0.59%), 1,8-cineole (0.52 %) (Table 3). Chloroform, hexane ethylacetate and ethanol are our solvent as shown in Table 3.

Table 3. Volatile components of the chloroform extracts of Eucalyptus leaves (GC-MS analysis)

Component[a]	Area (%)	Rt[b]
Hexane	10.85	3.60
Ethyl acetate	9.12	4.81
Etanol	8.22	5.25
Cis-dichloroethylene	0.29	6.29
Octamethylcyclotetrasiloxane	0.23	6.35
Chloroform	68.5	6.71
Bromochloromethane	0.59	7.70
Isocineol	0.02	8.37
d-limonene	0.11	14.16
1,8-cineole	0.52	14.43
α -pinene	0.01	15.32
γ -terpiene	0.78	16.47
Total	99.27	-

[a] Components listed in order of elution from a HP-1 capillary column
[b] Retention time (as min)

Similary, Bosniae *et al.*[23] reported that eucalyptus essential oil contained about 80v/v % 1.8-cineole, but the antimicrobial activity of eucalyptus oil was greater than the antimicrobial activity of 1.8-cineole. Other components contributed significantly to the antibacterial activity of eucalyptus leaves. The major component of crude eucalyptus oil is 1,8-cineole (eucalyptol), which ranges in percentage composition from 44.3% to 84.4%13,15 and is known to possess antimicrobial properties[24-26]. Previous studies have showed that one of main components of essential oil of *Ocimum gratissimum* L. (21.9%) and essential oil of *Elettaria cardamomum* leaves (2.675%) is γ-terpiene due to its pleasant spicy aroma and taste, is used in flavorings, fragrances, and cosmetics[27-29]. γ-terpiene was also identified as major component of essential oil of sea fennel (*Crithmum maritimum* L.) collected from Turkey[30].

CONCLUSIONS

The present study confirmed antimicrobial properties of chloroform extracts from *E. camaldulensis* that showed growth inhibition for *B. subtilis* ATCC 6633, *B. cereus* CCM 99 and *C. albicans* ATCC 10239. In addition, these results form a good basis for selection of the plant for further phytochemical and pharmacological investigation. The antibacterial activity exhibited by the chloroform extract of leaves may be due to soluble compounds in chloroform. We believe that the present investigation together with previous studies provide support to the antimicrobial properties of *E. camaldulensis*. It can be used as

antimicrobial supplement in the developing countries towards the development of new antimicrobial agents.

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