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ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF ESSENTIAL OILS FROM LEAVES OF SEVEN *EUCALYPTUS* SPECIES GROWN IN PAKISTAN

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ABSTRACT

The chemical composition of essential oils from leaves of seven *Eucalyptus* species, viz., *Eucalyptus crebra; Eucalyptus kitsoniana*; *Eucalyptus melanophloia*; *Eucalyptus microtheca*; *Eucalyptus pruinosa*; *Eucalyptus rudis* and *Eucalyptus tereticornis* (Pakistan) was analysed by GC-FID and GC-MS. The main component of *E. crebra, E. microtheca* and *E. rudis* essential oils was 1,8-cineole (31.6–49.7 %). *Eucalyptus melanophloia* and *E. tereticornis* contained *p*-cymene (41.8–58.1 %) as a major component while *E. kitsoniana* and *E. pruinosa* essential oils were dominated by α-pinene (25.8–31.4 %). *In vitro* antimicrobial activity of the essential oils was studied by agar well diffusion method at concentration range 8-250 µg/ml. The *Eucalyptus* oils inhibited the growth of Gram positive bacteria (*Bacillus spizizenii*, *Staphylococcus aureus*) significantly with zones of inhibition (IZ) ranging from 15.3-46.0 mm. The studied oil demonstrated moderate inhibitory effect against Gram negative bacteria (*Enterobacter aerogenes, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) with IZ (0.0−22.2 mm). The oils exhibited bactericidal effects at 8 µg/ml in agreement with minimum inhibitory concentration against all tested foodborne pathogens except *Pseudomonas aeruginosa* (8−250 µg/ml). Time kill assay based on four weeks studies authenticated the potency of oils as food preservatives. Antioxidant assays (free radical scavenging activity and ferric reducing power test) demonstrated good activity of the oils. The evaluated essential oils exhibited strong antioxidant activity by 45.3−75.1 % inhibition of 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH) and ferric reducing power (1.04 ± 0.05−1.95 ± 0.04 %) at 100 µg/ml. The good antioxidant and antibacterial activities of *Eucalyptus* essential oils validate their potential use as food preservatives.

Key words: 1,8-cineole, *E. melanophloia*, foodborne pathogens, GC-MS, ferric reducing power, bactericidal, time kill assay.

INTRODUCTION

The safety of food products is an important concern due to their vulnerability towards the microbes (bacteria and fungi) and atmospheric oxidation. Microbial growth is the major cause of food-borne ailments whereas the enzymatic oxidation of lipids affects the quality of food (Kanner and Rosenthal, 1992). Synthetic preservatives are preferred in food industry due to their effectiveness and low price to impede discoloration, spoilage and microbial contamination (Bajpai *et al*., 2008). However, apprehending their adverse environmental impacts, potential health hazards, development of microorganisms' resistance, the conventional synthetic chemicals are being substituted by natural products. Among the emerging natural preservatives, the interest in essential oils has been boosted up owing to their diverse biological (antimicrobial, antioxidants, anticarcinogenic) properties (Gutierrez *et al.,* 2009).

Eucalyptus is one of the important genera of *Myrtaceae* and comprises about 800 species and subspecies (Gil *et al*., 2010). Most of the species are native to Australia. However, they have been cultivated throughout the tropics and subtropics including Africa, America, China, Europe, India, Mediterranean Basin and Middle East, (Guenther, 1952). *Eucalyptus* species have also been widely planted in many parts of NWFP and Punjab (Pakistan). *Eucalyptus* species are a rich resource of essential oil of medicinal and commercial importance. *Eucalyptus* essential oils also exhibit antibacterial (Elaissi *et al*., 2012), antioxidant, anti-inflammatory (Silva *et al*., 2003), antifungal (Somda *et al*., 2007), antiviral (Schnitzler *et al*., 2001) and insecticidal activities (Jemâa *et al*., 2014). Keeping in view the recent trend of use of natural preservatives, seven common Pakistani *Eucalyptus* species, namely *E. crebra, E. kitsoniana*, *E. melanophloia*, *E. microtheca*, *E. pruinosa, E. rudis* and *E. tereticornis* have been selected to evaluate their antioxidant potential and antibacterial activity against foodborne pathogens.

MATERIALS AND METHODS

Collection of Materials: Fresh leaves of *E. crebra, E. kitsoniana*, *E. melanophloia*, *E. microtheca*, *E. pruinosa, E. rudis* and *E. tereticornis* were collected from botanical garden, Pakistan Forest Research Institute (PFI), Faisalabad, Pakistan in October, 2014. Plant herbaria were authenticated by Dr. Nasir (Professor, Department of Botany, University of Punjab, Lahore, Pakistan) and voucher specimens have been deposited at the Department of Botany, University of the Punjab, Lahore, Pakistan under the following references: BDSS # 4023; BDSS # 4024; BDSS # 4025; BDSS # 4026; BDSS # 4027; BDSS # 4028 and BDSS # 4029.

Isolation of Essential Oils: Fresh leaves of *Eucalyptus* species were subjected to hydrodistillation for 3h using Clavenger-type apparatus according to the method recommended in the European Pharmacopoeia (EDQM, 2005. Essential oils distillates were dried over anhydrous sodium sulfate, filtered and kept at -4°C till analysis. The oil yields are listed in **Table 1**.

Chemical Analysis of Essential Oils: Gas chromatography analysis of the essential oils was carried out on Shimadzu GC 2010 using DB-5 MS (30 m × 0.25 mm id, 0.25 µm film thickness) capillary column. The column oven temperature was programmed initially at 40−90°C at the rate of 2°C/min and then 90−240°C at the rate of 3°C/min. The final temperature was held constant for 5 min. Injector and detector temperatures were maintained at 240 and 280°C respectively. A sample of pure essential oil (0.5µl) was injected in a split mode ratio of 1:5. Helium was used as a carrier gas at the flow rate of 1 ml/min. GC-MS analysis was carried out on GCMS-QP 2010 Plus, Shimadzu, Japan operating in electron ionization mode at 70 eV. Column conditions were same as in GC analysis. The mass spectrometer was capable of scanning from 35 to 500AMU every second or less.

The data acquisition system continuously acquires and stores all data analyses. The components were identified by comparing their mass spectra with those of NIST mass spectral library (Mass spectral library 2001) and Adams (2001) as well as by comparing their retention indices either with those of authentic compounds or with literature values.

Evaluation of Antimicrobial Activities of Essential Oil:

Test Microorganisms

Seven bacterial strains from American Type Culture Collection (ATCC, Rockville) were selected for *in vitro* antibacterial activity of the essential oil. Gram positive bacteria comprised of *B. spizizenii* (ATCC 6633) and *S. aureus* (ATCC 25923) while Gram negative strains were *E. aerogenes* (ATCC 13048), *E. coli* (ATCC 8739), *S. enterica* (ATCC 14028), *K. pneumoniae* (ATCC 13882) and *P. aeruginosa* (ATCC 27853). All the bacterial strains were sub-cultured at 35°C for 24 h on nutrient agar slants prior to being grown in nutrient broth overnight.

Antibacterial Activity: Antibacterial activity of *Eucalyptus* essential oils was checked by agar well diffusion method (Zaika, 1988). Molten agar medium (20 ml) was inoculated with bacterial suspension containing indicator strain at 10^6 cfu/ml .The inoculated medium was poured into a petri plate and allowed to solidify. Wells were made in triplicate on solidified agar and 90 µl of the tested oil was added to each. The plates with bacterial strains were incubated at 35ºC for 24 h. The diameters of inhibition zones were measured in millimeters and results were recorded in triplicate.

Minimum Inhibitory Concentration (MIC) Assay: Serial dilutions of 8 µg/ml, 15 µg/ml, 65 µg/ml and 250 µg/ml were used in triplicate to determine MIC levels by agar well method (Zaika, 1988). The lowest concentration of oil inhibiting visible growth of each microbe after incubation was taken as the MIC.

Minimal Bactericidal Concentration (MBC): Minimum Bactericidal Concentration (MBC) was determined by the method of Rabe, *et al.,* (2002). Bacterial spore load (10^6 cfu/ml) was poured into the tubes containing respective culture broth and oil with concentration of MIC. Broth tubes with and without bacterial load were used as controls. The tubes were incubated for 24 h at 35°C. After incubation, 100 µl from tubes having no visible growth was removed and poured in plates along with agar to enumerate total viable counts. The lowest concentration with no visible growth after 24 h of incubation at 35°C was defined as the MBC, indicating 99.5 % killing of the original inoculum.

Time Kill Assay: A time kill study was carried out with the MIC values found previously agar well method to discern whether the *Eucalyptus* oils have bacteriostatic or bactericidal effect over a period of time to be used as food preservatives (White, 1996). Bacterial suspension with 10^6 cfu/ml and oil having concentration equal to MIC were added respectively in the tube of corresponding culture medium. Broth tubes with and without microbial suspension were used as controls. The cultures were incubated for one month at 35°C. An inoculant of 100 µl, removed after 2, 5, 8, 11, 14 and 30 d was poured in agar plates in triplicate to determine the total reduction in viable counts. The mean number of the colonies (cfu/ml) was counted and compared with that found in the control culture at the end of the incubation period. The test tubes with turbidity after a certain time period of incubation depict bacteriostatic effect of the evaluated essential oils at the applied concentration. To determine the bactericidal concentration of *Eucalyptus*

essential oils against that particular strain, the higher concentrations (15 µg/ml, 65 µg/ml and 250µg/ml) were applied and lethal effect of essential oils were observed as mentioned above.

ANTIOXIDANT ACTIVITY

DPPH Assay: The antioxidant activity of the essential oils from *Eucalyptus* species was assessed by measuring its ability to scavenge 2,2′-diphenyl-1-picrylhydrazyl (DPPH) stable radical. The assay was carried out spectrophotometrically as described by Shimada *et al.,* (1992) with some modifications.

Various concentrations of oils samples were prepared in methanol. To the 0.1ml of each test concentration, 3ml of methanolic solution of DPPH (0.004 %) were added. Resulting mixtures were incubated in dark for 30 min at 25°C. The decrease in absorbance was measured at 517 nm using a spectrophotometer (Cecil CE7200). Scavenging (%) exhibited by essential oils was calculated as follows:

Scavenging (%) = (A_{blank} - A_{sample}/A_{blank}) x 100

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test oil. All determinations were performed in tripilcate.

Total Reduction Ability by Fe^{3+}- Fe^{2+} Transformation: The capacity of essential oils to reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) was determined by measuring absorbance at 700 nm (Oyaizu *et al*., 1986.). Different concentrations of the essential oils were added to 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min and 2.5 ml of trichloroacetic acid (10 %) were added thereafter. The mixtures were revolved at 3000 rpm for 10 minutes. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride 0.5 ml. The resultant mixture was allowed to stand for 30min and absorbance was measured on UV spectrophotometer at 700 nm. Higher absorbance of reaction mixture indicated greater reducing power. The comparison of ferrous reducing antioxidant potential was made with butylated hydroxytoluene (BHT).

Statistical Analysis: The mean values, ± standard deviations were calculated using MS Excel 2007.

RESULTS AND DISCUSSION

The yield of essential oils ranged from 0.1−1.9 % for different *Eucalyptus* species [Table 1]**.** The highest yield was obtained from *E. rudis* (1.8 %) while *E. tereticornis* gave the lowest yield (0.1 %). The oil yields of *E. rudis* and *E. crebra* were in accordance to the previous reports (Iqbal *et al*., 2003; Ahmad *et al*., 2005; Haq *et al*., 2007; Elaissi *et al*., 2011; Jemâa *et al*., 2012; Sliti *et al*., 2015). *Eucalyptus microtheca*, *E. melanophloia, E. kitsoniana* and *E. tereticornis* oil yields were found lower than the former reports (Bachheti *et al*., 2011; Elaissi *et al*., 2011; Iqbal *et al*., 2003; Ghaffar *et al*., 2015). No data was available to compare oil yield of *E. pruinosa* in literature.

Essential oils of seven *Eucalyptus* species were found rich in monoterpene hydrocarbons and oxygenated monoterpenes. α-Phellandrene (0.0−2.0 %), α-pinene (0.7−39.4 %), *p*-cymene (0.7−58.1 %), 1,8-cineole (1.0−49.7 %) and terpinolene (1.3−7.2 %) were found as common components in volatile oils of all *Eucalyptus* species in varying concentrations

Table 1. Chemical Composition of Essential Oils from *Eucalyptus* Species.

Compounds	RI	Content (%)						
		E. cre	***E.kit***	***E. mel***	***E. mic***	***E. pru***	***E.rud***	***E. ter***
α-Pinene	930	16.9	25.8	24.1	31.0	29.5	32.5	0.7
Camphene	944	0.2	0.2	0.3	0.6	0.4	0.1	-
β-Pinene	974	-	-	-	-	0.3	-	-
4-Carene	1001	0.1	0.2	0.3	-	-	tr	0.2
α- Phellandrene	1002	0.2	2.0	1.0	tr	0.1	tr	0.3
Limonene	1011	4.1	4.0	6.2	5.7	4.6	8.4	-
p-Cymene	1026	16.1	24.8	41.8	2.4	8.2	1.6	58.1
1,8-Cineole	1029	49.7	3.3	3.3	31.6	24.2	48.5	6.5
β-*cis*-Ocimene	1043	Tr	tr	tr	-	-	0.1	-
γ-Terpinene	1055	0.2	1.2	0.8	0.1	-	0.2	0.5
Terpinolene	1083	0.1	0.6	0.6	0.1	tr	0.1	0.2
Terpin-4-ol	1089	4.1	7.2	2.9	4.8	3.9	2.4	2.1
β-Linalool	1095	-	tr	0.8	-	0.1	-	0.5
α –Campholenal	1127	0.1	-	0.1	0.1	0.1	-	-
trans-Pinocarveol	1135	2.0	0.2	-	2.2	1.3	0.9	0.1
Camphene Hydrate	1145	0.1	0.1	0.1	0.2	0.2	tr	-

p-Cymen-3-ol	1183	0.1	0.1	0.1	tr	0.1	0.4	0.4
p -Cymen-8-ol	1196	0.5	0.3	0.8	0.3	0.4	0.2	3.6
Compounds	**RI**	**Contents %**						
		E. cre	***E.kit***	***E. mel***	***E. mic***	***E. pru***	***E.rud***	***E. ter***
Nerol acetate	1365	-	-	-	-	-	0.1	0.2
α-Copaene	1374	-	-	-	tr	-	-	0.1
Fenchol	-	-	-	-	-	-	tr	-
Eugenol methyl ether	1402	0.1	0.2	0.5	-	0.1	0.8	0.7
α-Caryophyllene	1444	Tr	0.1	0.4	0.2	0.2	0.1	-
Humulen- (IV)	1452	-	-	-	0.1	-	-	-
Germacrene D	1484	Tr	-	0.1	tr	tr	0.5	0.2
Monoterpenes		37.9	58.8	75.1	39.9	43.1	43.0	60.0
Oxygenated monoterpenes		56.6	11.2	8.1	39.2	30.3	52.5	13.4
Sesquiterpenes		Tr	0.1	3.3	0.3	0.2	0.6	0.5
Oxygenated sesquiterpenes		2.5	0.5	6.9	2.5	9.7	1.0	-
Phenolic compounds		0.1	0.2	0.5	-	0.1	0.8	0.7
Others		-	tr	-	-	-	-	-
Unidentified		2.9	29.2	6.1	18.1	16.6	2.1	25.4
Oil Yield (%)		0.5	0.2	0.4	0.4	0.9	1.9	0.1

Plant abbreviations: *Eucalyptus kitsoniana: E. kit; Eucalyptus crebra; E. cre; Eucalyptus melanphloia: E.mel; Eucalyptus microtheca: E. mic; Eucalyptus pruinosa: E.pru; Eucalyptus rudis: E. rud; Eucalyptus tereticornis: E.ter*; RI: Retention Index; - : not detected; tr; traces< 0.05 %

The richness of *E. crebra*, *E. rudis*, *E. microtheca* and *E. pruinosa* essential oils in 1,8-cineole and α-pinene was in conformity with the previous reports (Isiaka *et al.* 2003; Sefidkon *et al.,* 2007; Joseph *et al*.,2008). *Eucalyptus rudis* oil contained higher concentration of 1,8-cineole (48.5 %) and α-pinene (32.5 %) as compared to Tunisian variety of *E. rudis* (19.9 % and 3.9−14.5 %, respectively). Limonene which has been identified in *E. rudis* oil was absent in the Tunisian variety of *E. rudis* (Haouel *et al* 2010; Elaissa *et al*.2012; Sliti *et al.,* 2015). *p*-Cymene was the major component in the studied species of *E. tereticornis* contrary to 1,8-cineole and α-pinene as reported previously (Pino *et al.,* 2001; Kaur *et al.,* 2010). α-Pinene was found as a major component in *E. kitsoniana* oil whereas, 1,8-cineole was reported as principal constituent in Tunisian variety of *E. kitsoniana* (Elaissi *et al.,* 2011).

The antimicrobial activity of *Eucalyptus* essential oils was evaluated by measuring the inhibition zones against the common food borne pathogens (*B. spizizenii, S. aureus, E. aerogenes, E. coli, S. enterica, K. pneumoniae, P. aeruginosa*).. Preliminary screening of antibacterial activity of *Eucalyptus* essential oils was done through agar well diffusion assay. The degree of antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) of the oils while the bactericidal activity was assessed by broth dilution method. *Eucalyptus* species manifested good antibacterial properties against all the tested microbes. However, the level of bacterial growth inhibition was dependent on the oils concentration and the bacterial strain.

The results of antibacterial activity and MIC and MBC values have been summarized in Table 3 and 4. The selected gram positive strains (*B. spizizenii* and *S. aureus*) showed high sensitivity towards *Eucalyptus* oils at concentrations of 8-250 µg/ml (Table 2). *Bacillus spizizenii* was most sensitive towards *E. microtheca* essential oil with zones of inhibition (ZI = 14.5−46.0 mm) followed by *E. rudis* (13.8−37.0mm), *E. microtheca* (ZI = 12.0−33.0 mm), *E. melanophloia* (ZI = 15.7−22.0 mm), *E. pruinosa* (ZI = 13.2−20.0 mm), *E. kitsoniana* (ZI = 12.0−17.0 mm) and *E. tereticornis* (ZI = 15.0 mm). *Eucalyptus rudis* and *E. kitsoniana* (ZI = 11.3−21.2 mm) showed good activity against *S. aureus* followed by *E. microtheca* (11.2−19.2 mm), *E. crebra* (12.3−18.7 mm), *E. tereticornis* (13.0−17.0 mm) and *E. melanophloia* (13.2-16.8 mm). Among gram negative strains, the *Eucalyptus* oils demonstrated significant inhibitory effect against *K. pneumoniae* and *E. coli* (11.5−22.2 mm) followed by *E. aerogenes*, *S. enterica* (11.0−16.8 mm) and *P. aeruginosa* (0.0−15.7 mm). Elaissa *et al*., 2011 reported smaller zones of inhibition (7.7−10.3 mm) against *S. aureus*, *E. coli* and *P. aeruginosa*. Our results on antibacterial activity are in accordance with those described by Fawad *et al*., 2011 and Ghaffar *et al*., 2015.The larger inhibitory zones induced by studied *Eucalyptus* oils when compared to Elaissa *et al*., 2011 showed their effectiveness as antibacterial agents.

Table 2. Antibacterial activity of *Eucalyptus* Essential Oils by Agar well diffusion method.

Essential Oil	Conc. µg/ml	*Tested Microbial Strains*						
		B. spizi	***S. aure***	***E. aerog***	***E. coli***	***K. pneum***	***P. aerug***	***S. enter***
E. cre	**250**	46.0 ±0.0	18.7 ±0.4	13.7 ±0.4	15.7 ±0.6	18.7 ±0.4	15.7 ±0.4	13.3 ±0.2
	65	17.7 ±0.5	16.0 ±0.0	12.3 ±0.4	13.0 ±0.0	15.3 ±0.4	14.7 ±0.4	12.3 ±0.4
	15	15.7 ±0.4	13.0 ±0.0	11.7 ±0.4	11.8 ±0.5	14.0 ±0.0	12.0 ±0.0	11.7 ±0.4
	8	14.5 ±0.0	12.3 ±0.4	11.0 ±0.0	11.7 ±0.4	12.0 ±0.0	10.3 ±0.4	11.3 ±0.4
E. kit	**250**	17.0 ±0.0	20.5 ±0.3	15.8 ±0.2	22.2 ±0.5	22.2 ±0.5	15.7 ±0.2	16.2 ±0.5
	65	13.8 ±0.2	13.2 ±0.2	13.5 ±0.3	15.7 ±0.2	15.7 ±0.2	12.3 ±0.2	13.5 ±0.0
	15	13.0 ±0.0	12.0 ±0.0	12.5 ±0.0	14.2 ±0.2	13.5 ±0.3	12.0 ±0.0	12.3 ±0.2
	8	12.0 ±0.2	11.3 ±0.2	12.0 ±0.0	13.3 ±0.2	13.0 ±0.0	11.3 ±0.2	11.3 ±0.2
E. mel	**250**	21.8 ±0.2	16.8 ±0.2	11.8 ±0.2	13.5 ±0.3	16.2 ±0.5	0.0 ±0.0	12.7 ±0.2
	65	19.5 ±0.5	14.7 ±0.4	11.3 ±0.2	12.5 ±0.3	14.2 ±0.2	0.0 ±0.0	12.3 ±0.4
	15	17.0 ±0.3	13.5 ±0.0	11.2 ±0.2	12.2 ±0.2	13.0 ±0.0	0.0 ±0.0	11.7 ±0.2
	8	15.7 ±0.2	13.2 ±0.2	11.0 ±0.0	11.5 ±0.0	12.2 ±0.2	0.0 ±0.0	11.3 ±0.4
E. mic	**250**	33.3 ±0.5	19.2±0.5	16.7 ±0.4	15.8 ±0.5	16.7 ±0.2	15.5 ±0.5	15.7 ±0.6
	65	19.3 ±0.4	13.0 ±0.3	15.8 ±0.5	15.2 ±0.5	14.8 ±0.4	13.8 ±0.2	14.8 ±0.2
	15	14.5 ±0.5	12.0 ±0.3	14.8 ±0.2	13.5 ±0.3	14.5 ±0.0	11.8 ±0.2	13.2 ±0.2
	8	12.0 ±0.0	11.2 ±0.4	14.0 ±0.0	12.8 ±0.2	13.8 ±0.2	11.3 ±0.4	12.5 ±0.0
E. pru	**250**	20.0 ±0.0	19.0 ±0.6	16.8 ±0.5	19.2 ±0.2	21.8 ±0.5	13.7 ±0.4	16.3 ±0.4
	65	16.5 ±0.3	15.0 ±0.0	13.3 ±0.5	17.3 ±0.5	16.8 ±0.5	12.2 ±0.2	14.5 ±0.3
	15	13.5 ±0.0	13.0 ±0.3	12.5 ±0.0	15.3 ±0.4	14.2 ±0.2	11.5 ±0.5	13.8 ±0.2
	8	13.2 ±0.2	12.3 ±0.2	11.5 ±0.3	13.7 ±0.4	13.8 ±0.2	10.3 ±0.4	12.8 ±0.2
E. rud	**250**	36.8 ±0.2	21.2 ±0.2	16.2 ±0.2	17.8 ±0.2	18.0 ±0.0	14.8 ±0.5	16.7 ±0.3
	65	17.8 ±0.7	18.2 ±0.2	15.0 ±0.0	14.5 ±0.3	16.8 ±0.2	12.2 ±0.2	14.0 ±0.0
	15	14.7 ±0.5	13.5 ±0.3	13.8 ±0.2	13.0 ±0.3	14.7 ±0.2	11.8 ±0.6	13.0 ±0.0
	8	13.8 ±0.2	12.2 ±0.2	13.0 ±0.0	12.3 ±0.4	13.7 ±0.4	10.0 ±0.0	11.8 ±0.3
E. ter	**250**	15.3 ±0.5	17.0 ±0.5	15.5 ±0.5	13.2 ±0.2	18.3 ±0.5	14.5 ±0.3	13.0 ±0.0
	65	14.3 ±0.4	15.3 ±0.4	14.0 ±0.5	12.5 ±0.3	15.0 ±0.0	12.7 ±0.4	12.5 ±0.3
	15	13.5 ±0.2	13.7 ±0.5	12.8 ±0.5	12.3 ±0.4	13.5 ±0.3	12.3 ±0.4	12.0 ±0.0
	8	13.0 ±0.2	13.0 ±0.0	12.0 ±0.0	11.8 ±0.2	13.3 ±0.2	11.3 ±0.4	11.7 ±0.4

*The diameter of the inhibition zones (mm), including the well diameter (6mm), are given as mean ± SD of triplicate experiments.
*Microorganisms Abbreviations: *B. spizizenii*; *B. spizi*: *E. aerogenes*; *E. aerog*: *K. pneumoniae*; *K. pneum*: *P. aeruginosa*; *P. aerug*: *S. enterica*; *S. enter*: *S. aureus; S. aure*

The high degree of antibacterial activity was further confirmed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). All *Eucalyptus* oils except *E. tereticornis* and *E. melanophloia* exhibited bacteriostic and bactericidal effect at concentration of 8µl/ml against tested strains. The MIC and MBC values of *E. tereticornis* and *E. melanophloia* were found 15 µg/ml and 250 µg/ml respectively (Table 3). Sliti *et al*., 2015 reported MIC (0.075-0.5 %) and MBC values (0.5-3.5 %) of *E. rudis* against different pathogens. They have been found higher than Pakistan grown variety of *E. rudis*. Based on the MIC results, the oil or plant extract is considered strong inhibitor with MIC below 0.5 mg/ml;moderate inhibitor with MIC 0.6–1.5 mg/ml and weak inhibitor with MIC above 1.6 mg/ml (Duarte *et al.* 2005). Hereby, considering Duarte *et al.* 2005 plants classifications for potential antimicrobial activity, the *Eucalyptus* oils with powerful antibacterial activity at 8-250 µg/ml concentrations against the evaluated bacterial strains fall within the group of strong inhibitors.

The time kill assay was carried out to check the potency of *Eucalyptus* essential oils to be used as a food preservative. The study was carried out to evaluate the bacteriostatic or bactericidal effects of tested oils for four weeks. All the tested oils showed bactericidal effect against *B. spizizenii* at 15-250 µg/ml while it ranged from 8-250 µg/ml for *S. aureus. K. pneumoniae* was found sensitive towards all tested oil with lowest MBC i-e. 8 µl/g among gram negative strains followed by *E. coli* with MBC values (8-15 µg/ml), *E. aerogenes* and *S. enterica* (15-250µg/ml). *P. aeruginosa* was found most resistant among the tested strains with MBC values ranging from 65-250 µg/ml (**Table 4**).

Table 3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) µg/ml of *Eucalyptus* essential oils.

Tested Microbial Strains	*E. cre*		*E. kit*		*E. mel*		*E. mic*		*E. pru*		*E. rud*		*E. ter*	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
B. spizizenii	8	8	8	8	8	8	8	8	8	8	8	8	8	8
S. aureus	8	8	8	8	8	8	8	8	8	8	8	8	8	8
E. aerogenes	8	8	8	8	8	8	8	8	8	8	8	8	8	8
E. coli	8	8	8	8	8	8	8	8	8	8	8	8	8	8
K. pneumoniae	8	8	8	8	8	8	8	8	8	8	8	8	8	8
P. aeruginosa	8	8	8	8	250	250	8	8	8	8	15	15	15	15
S. enterica	8	8	8	8	8	8	8	8	8	8	8	8	8	8

Table 4. Time kill Assay; bactericidal Effects (MBC) µg/ml of *Eucalyptus* essential oils for 30 days.

Tested Microbial Strains	*E. cre*	*E. kit*	*E. mel*	*E. mic*	*E. pru*	*E. rud*	*E. ter*
B. spizizenii	65	65	250	65	15	15	250
S. aureus	8	8	250	8	15	15	8
E. aerogenes	65	250	250	250	15	15	65
E. coli	8	15	15	8	15	8	8
K. pneumoniae	8	8	8	8	8	8	8
P. aeruginosa	250	250	250	65	65	65	250
S. enteric	15	15	250	15	15	15	15

The bioactivity of the essential oils depends upon their major volatile component (Cimanga *et al.,* 2002). α-Pinene, *p*-cymene and 1,8-cineole; the principal constituents in the *Eucalyptus* oils have been reported to exhibit antibacterial activities (Khamis *et al.,* 2005; Sonbolia *et al.,* 2006; Jiang *et al.,* 2011). The minor components (linalool, α-terpineol; *cis*-ocimene, limonene, terpinolene and α-phellandrene) in the *Eucalyptus* oils also possess antibacterial properties (Khamis *et al.,* 2005; Magwa *et al.,* 2006; Donsì *et al.,* 2011). Therefore, the antimicrobial activity of tested essential oils could be due to synergistic effects of major and minor components.

DPPH assay and reducing power assay were used to assess antioxidant potential of essential oils. The synthetic antioxidant BHT was used as an equivalence parameter for the antioxidant activity of the essential oils. The *Eucalyptus* essential oils showed DPPH scavenging activity (45.34-75.11 %). The DPPH scavenging activity of tested oils was found lower than synthetic antioxidant; butylated hydroxytoluene (BHT) (**Table 5**). Highest antioxidant activity was displayed by *E. microtheca* essential oil with IC_{50} value of 56.8 ± 0.9 µg/ml while *E. pruinosa* demonstrated lowest activity with IC_{50} value 113.0 ± 2.2 µg/ml among the *Eucalyptus* oils.

Table 5. DPPH Redical Scavenging Activity of *Eucalyptus* Essential Oils.

	DPPH Redical Scavenging (%)					
	20 µg/ml	**40 µg/ml**	**60 µg/ml**	**80 µg/ml**	**100 µg/ml**	**IC_{50}µg/ml**
BHT	30.8 ± 0.6	52.4 ± 1.0	66.5 ± 0.7	76.8 ± 0.6	84.9 ± 0.3	44.5 ± 0.4
E. crebra	25.9 ± 0.5	30.8 ± 0.7	38.0 ± 0.4	45.2 ± 0.6	50.7 ± 0.7	97.3 ± 2.3
E. kitsoniana	28.7 ± 0.4	35.5 ± 0.7	42.8 ± 0.6	51.4 ± 1.1	59.3 ± 1.0	76.7 ± 1.0
E. melanophloia	29.8 ± 1.0	37.3 ± 0.7	46.6 ± 1.4	58.3 ± 1.1	64.9 ± 1.7	66.8 ± 1.8
E. microtheca	29.3 ± 0.7	38.6 ± 0.8	52.6 ± 1.7	64.3 ± 0.7	74.4 ± 0.7	56.8 ± 0.9
E. pruinosa	21.5 ± 1.3	25.7 ± 0.8	35.2 ± 0.8	39.9 ± 0.8	45.5 ± 0.7	113.0 ± 2.2
E. rudis	25.0 ± 0.8	28.6 ± 1.3	37.3 ± 0.9	49.4 ± 0.7	55.2 ± 0.6	86.9 ± 1.1
E. tereticornis	27.5 ± 0.7	35.3 ± 0.9	45.6 ± 0.8	55.2 ± 1.2	63.6 ± 0.9	69.9 ± 1.0

In the reducing power assay, the *Eucalyptus* essential oils showed lower ferric reducing power than BHT. *Eucalyptus microtheca* essential oil showed highest absorbance at tested concentrations (20-100 µg/ml)

corresponding to good antioxidant activity (1.77 ± 0.02 µg/ml) while *E. pruinosa* demonstrated lowest activity with percentage absorbance of 1.04 ± 0.05 µg/ml among the *Eucalyptus* oils at 100 µg/ml (**Table 6**).

Table 6. Total Ferric Reducing Abilityof Butylatedhydroxytoluene and *Eucalyptus* Essential oils.

	Absorbance (%) at 700nm				
	20 µg/ml	**40 µg/ml**	**60 µg/ml**	**80µg/ml**	**100 µg/ml**
BHT	1.16 ± 0.18	1.36 ± 0.03	1.57 ± 0.05	1.82 ± 0.07	1.95 ± 0.04
E.crebra	0.51 ± 0.02	0.68 ± 0.03	0.81 ± 0.02	0.98 ± 0.02	1.17 ± 0.03
E.kitsoniana	0.60 ± 0.01	0.80 ± 0.02	0.95 ± 0.02	1.17 ± 0.01	1.31 ± 0.01
E. melanophloia	0.66 ± 0.02	0.84 ± 0.04	1.06 ± 0.04	1.25 ± 0.06	1.50 ± 0.10
E.microtheca	0.75 ± 0.02	1.01 ± 0.02	1.25 ± 0.02	1.46 ± 0.02	1.77 ± 0.02
E.pruinosa	0.45 ± 0.01	0.61 ± 0.02	0.75 ± 0.02	0.90 ± 0.03	1.04 ± 0.05
E.rudis	0.54 ± 0.02	0.70 ± 0.01	0.87 ± 0.02	1.02 ± 0.03	1.23 ± 0.06
E. tereticornis	0.61 ± 0.02	0.82 ± 0.03	1.05 ± 0.03	1.25 ± 0.05	1.44 ± 0.00

Antioxidant activity of monoterpene and monoterpenoids present in studied oils has been reported by many researchers. Wei and Shibamoto 2007 related antioxidant activity to the α-pinene in *Citharexylum caudatum* L. Limonene, terpinolene, and γ-terpinene have also been reported to show considerable activity (Ruberto and Baratta, 2000; Song *et al*., 2001; Elmastas *et al.* 2006). Among other monoterpenoids, 1,8-cineole and terpen-4-ol has demonstrated antioxidant and anti-inflammatory activities (Kim *et al*., 2004; Porres *et al*., 2014). Thus, antioxidant activity of *Eucalyptus* essential oils can be attributed to the synergy among the different oil constituents.

Conclusion: The *Eucalyptus* species have remarkable antibacterial activity against common food-borne pathogens and antioxidant potential so these may be suggested as new potential source of natural antimicrobial and antioxidant agents.

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