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Bioactivity of *Eucalyptus citriodora* leaves essential oil

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Introduction. – The essential oils and extracts from plants have gained popularity and scientific interests during last few years (Tepe *et al.,* 2005). Diets rich in natural antioxidants are considered to reduce risk of incidence of cardiovascular, chronic diseases and certain types of cancer (Hussain *et al.,* 2008). Foods that combine nutritional and medicinal benefits are preferred as their use have shown synergistic effects, and most natural compounds extracted from plants have established biological activities. Among these various kinds of natural substances, essential oils from aromatic and medicinal plants gain particular attention as potential natural agents for food preservation (Mimica-Dukic *et al.,* 2004). Essential oils are proved to have various pharmacological effects such as spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic effects (Mimica-Dukic *et al.,* 2003; Bozin *et al.,* 2006). Recently, essential oils have been qualified as natural antioxidants (Ruberto and Baratta, 2000) and are proposed as potential substitutes of synthetic antioxidants in various sectors of food industry. Oxidation of lipids which occurs during raw material storage, processing, heat treatment and further storage of final products is one of the basic reasons for the rancidity of food products and resultantly lead to food deterioration. Due to undesirable influences of oxidized lipids on the human organs, it seems to be essential to decrease contact with products of lipid oxidation in food (Karpinska *et al.,* 2001). According to some toxicologists and nutritionists, the carcinogenicity of some synthetic antioxidants used in food processing such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented. The governmental authorities and consumers had shown high concern about

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the safety of their food and the potential side effects of synthetic additives on health (Reische *et al.,* 1998; Tepe *et al.,* 2005).

The antimicrobial and antioxidant activities of essential oils have revealed a number of applications in fresh and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Bozin *et al.,* 2006; Celiktas *et al.,* 2007). *Eucalyptus* spp. are endemic to Australia, Tasmania and Indonesia, but are also widely introduced to India, Africa, Brazil, California, Hawaii and Portugal (Paine, 2011). *E. citriodora* is frequently grown in the North-Eastern states of India (Kharwar *et al.,* 2010) and Pakistan (Rukhsana and Iffat, 2005). The bluish green leaves carry the medicinal properties; they contain a fragrant volatile oil which have antiseptic, expectorant, antibacterial, anti-inflammatory, deodorant, diuretic, and antispasmodic properties. The leaves of *E. citriodora* also contain eucalyptol that is used in cough syrup, aromatherapy and dentistry as well as for the treatment of bronchitis, sinusitis, chronic rhinitis and asthma (Kharwar *et al.,* 2010).

Biologically active natural compounds are of interest to the pharmaceutical industry for the control of human diseases of microbial origin and for the prevention of lipid peroxidative damage (Smith *et al.,* 2002). The antioxidant and antimicrobial activities along with the identification of the essential oil components of *E. citriodora* native of sub-continental regions have not yet been reported, and the present work was undertaken with the objective to appraise the physico-chemical composition of the essential oil isolated from the leaves of *E. citriodora* native of Pakistan.

Materials and Methods. – *Collection of plant material.* – The fresh leaves of *E. citriodora* were collected from the Punjab Forest Research Institute, Gutwala, Faisalabad, Pakistan in summer season of 2010. All the leaves were washed with cold water to remove dirt particles and these specimens were air dried to constant weight.

Chemicals. – The chemicals and reagents like plaster of Paris, diethyl ether, beef infusion (30%), casein hydrolysate (1.75%), starch (0.15%), agar (1.7%), mycological peptone, glucose, agar-agar, butylated hydroxytoluene (BHT), linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, ethanol, sodium phosphate buffer, ammoniun thiocyanate and ferrous chloride were of analytical grade.

Extraction and recovery of essential oil. – Weighed amount (733 g) of crushed plant material was taken in distillation assembly, and steam produced in a separate chamber circulated through plant material. On evaporation, the vapours of the pure essential oil along with steam were condensed while passing through a water condenser, and collected in a receiver flask which was kept in ice water bath in order to prevent the evaporation of low boiling constituents of oil. The upper oily layer of condensed material was dissolved in diethyl ether (40 mL) and separated from the distilled water component with the help of a separating funnel. The volume of essential oil recovered from a known

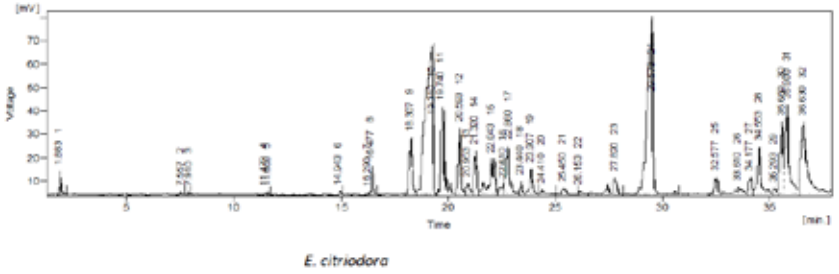

Fig. 1. – Gas chromatogram of *E. citriodora* essential oil.

quantity of plant material (leaves) was calculated and the average percentage yield of essential oil was determined using the relation (%Y = wt. of oil extracted/wt. of plant material) x 100. After extraction the essential oil was stored in a clean, dry and sterile vial and stored at 4°C until further analysis.

Essential oil analyses. Physical analysis. – The refractive index and density of *E. citriodora* essential oil were determined following standard methods adopted by Hussain *et al.* (2008). A digital refractometer RX-7000α (Atago Co. Ltd., Tokyo, Japan) was used for the determination of the refractive index of oil.

Gas chromatographic analysis. – GC analysis of *E. citriodora* essential oil was performed using a GC-17A Modal (Shimadzu) gas chromatograph equipped with a DB-wax (30 m × 0.25 mm) column with flame ionization detector (FID). Injector and detector temperatures were set at 250°C and 260°C, respectively. Column temperature was kept at 90°C for 2 min and increased to 180°C at 2°C/min and to 240°C at 3ºC/min. Helium was used as a carrier gas at a flow rate of 1.5 mL/min at 150 psi. A sample of 1.0 µL was injected, using the split mode (split ratio 1:100). All quantifications were carried out using a built-in data-handling program provided by the manufacturer of the gas chromatograph. The composition was reported as a relative percentage of the total peak area.

DPPH radical-scavenging assay – The evaluation of the antiradicalic activity of *E. citriodora* essential oil was determined by measuring its scavenging ability against 2,2'-diphenyl-1-picrylhydazyl (DPPH) stable radicals (Hussain *et al.,* 2008). The sample (0.5 µg/mL) was mixed with 1 mL of 90 µM DPPH solution and filled up with 95% methanol and the final volume was made up to 4 mL. The absorbance of the resulting solutions and the blank were recorded after 1 h at room temperature. BHT was used as a positive control, and for each sample three replicates were recorded. The disappearance of DPPH was measured spectrophotometrically at 515 nm (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Inhibition of DPPH free radicals (%) was calculated by the relation [I% = 100 (A_{blank}-A_{sample}/A_{blank})], where A_{blank} is the absorbance of the control reaction mixture excluding the test compounds and A_{sample} is the absorbance of the test compounds.

Percent inhibition in linoleic acid system. – The antioxidant activity of *E. citriodora* essential oil was also determined using inhibition of linoleic acid oxidation (Hussain *et al.,* 2008). The test samples (50 µL) were dissolved in 1 mL of ethanol, mixed with linoleic acid (52 µL), 4 mL ethanol and 4 mL of 0.05 M sodium phosphate buffer (pH 7). The solution was incubated at 40°C for 175 h. The extent of oxidation was measured

by the peroxide value obtained by a colorimetric method. To 0.2 mL sample solution, 10 mL of ethanol (75%), 0.2 mL of an aqueous solution of ammonium thiocyanate (30%) and 0.2 mL of ferrous chloride solution (20 mM in 3.5% HCl) were added sequentially. After 3 min of stirring, the absorbance was measured at 500 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). A control was performed with linoleic acid without the essential oil; BHT was used as a positive control. Inhibition of linoleic acid oxidation was expressed as percentage, and was measured with the following relation: % inhibition of linoleic acid oxidation = 100 - [(Abs. increase of sample at 175 h/Abs. increase of control) x 100].

Antimicrobial activity. Microbial and fungal strains. – The essential oil extracted from *E. citriodora* was checked for antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* and two fungal strains, namely *Aspergillus niger* and *Rhizopus solani.* The pure cultures of both fungal and bacterial strains were obtained from the Department of Veterinary Microbiology, University of Agriculture, Faisalabad. Bacterial strains were cultured overnight at 37°C in nutrient agar (NA, Oxoid), while fungal strains were cultured overnight at 30°C using potato dextrose agar (PDA, Oxoid).

Disc diffusion method. – The antimicrobial activity was determined by the agar disc diffusion method as described by Tepe *et al.* (2005). Sterilized medium was poured in Petri plates (0.15 mL) and left for solidification. Agar formed a firm gel of 2-3 mm thickness in each plate and sub-culturing was also essential for obtaining pure, isolated and single species of the test bacteria and fungi in the medium. Discs of 6 mm were fixed on the polystyrene foam with the help of common pins and 20 µL of essential oil were dropped on discs by a micropipette. Discs of test essential oil along with standard microbial strain were applied on the solid agar medium under the laminar flow chamber. These Petri plates were incubated at 37°C for 24 h and zones of inhibition (mm) were measured with a transparent ruler.

Determination of minimum inhibitory concentration (MIC). – For the determination of MIC, a micro-dilution broth susceptibility assay was adopted (Hussain *et al.,* 2008). All tests were performed in nutrient broth and sabouraud dextrose broth supplemented with Tween 80 detergent to a final concentration of 0.5% (v/v) for bacteria and fungi, respectively. Bacterial strains were cultured overnight at 37°C in nutrient broth, and the fungi were cultured overnight at 30°C in sabouraud dextrose broth. A 160 µL of nutrient broth for bacteria and sabouraud dextrose broth for fungi were added onto microplates together with 20 µL of tested solution. Finally, 20 µL of 5 × 105 CFU/mL of standard microorganism suspension were inoculated onto microplates. Plates were incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for fungi. The same test was performed simultaneously for the growth control (nutrient broth + Tween 80) and sterility control (nutrient broth + Tween 80 + test oil). Neomycin and erythrosine were used as reference compounds for antibacterial and Amoxil and Nizoral for antifungal activities.

Results and Discussion. – *Essential oil yield and physical analysis* – Table 1 shows the average percentage yield and physical properties of *E. citriodora* essential oil. The yield of fresh leaves of *E. citriodora* extracted through steam distillation showed a percentage yield of 1.82%. The percentage yield of the essential oil was found to be comparable with the reported values of 0.5-2% by Dutta *et al.* (2007). The

TABLE 1. – *Physical properties and antioxidant potential of* E. citriodora *essential oil.*

Physical properties	
Colour	Pale yellow
Odour	Citronellal odour
Solubility	Soluble in alcohols
Insolubility	Insoluble in water
Density (g cm^{-3}), 25°C	0.85
Weight of plant material (g)	733
Weight of oil (g)	13.4
Average percentage yield	1.82%
Refractive index (25°C)	1.49
Boiling point	177ºC
Antioxidant activity	
DPPH % inhibition	92.10%
Inhibition in linoleic acid system (%)	93.80%
BHT (Control)	92%

essential oil extracted from *E. citriodora* was soluble in 80% ethanol, but insoluble in water and a similar solubility behaviour has also been reported previously (ELLIOT and DAVID, 1983). The specific gravity of *E. citriodora* essential oil was found to be 0.85 which was observed to be close to the standard values of 0.95 presented by DIONGLAY *et al.* (2010). The boiling point was recorded to be 177°C, which was almost similar to the authenticated value of 179°C by CAB international (2000). The value of refractive index of *E. citriodora* essential oil extracted from leaves was 1.49 and is similar to that reported by KIM *et al.* (2005). The analysed physical values and percentage yield were compared with standard values which specify the purity of essential oil and efficiency of the extraction method used. Similar studies regarding essential oil percentage yield have also been reported previously by ZRIRA *et al.* (1992), DETHIER *et al.* (1994) and MOUDACHIROU *et al.* (1999).

Antioxidant activity. – The essential oil extracted from *E. citriodora* leaves was subjected to screening for its antioxidant activity through DPPH free radical scavenging and linoleic acid systems. Free radical scavenging capacities of the extracts measured by DPPH assay is given in Table 1. The reduction of DPPH into DPPH-H in response to the tested agent showed good antioxidant activity, and the

examined essential oil was able to reduce the stable, purple-coloured radical (DPPH) into the yellow-coloured ones (DPPH-H). The antiradicalic activity of *E. citriodora* essential oil was found to be higher than control (BHT). The DPPH radical scavenging activity of the oil and BHT were found to be 92.1 and 91.5%, respectively. Table 1 shows the percent inhibition of linoleic acid by the essential oil extracted from *E. citriodora.* The percentage inhibition of linoleic acid oxidation depends on the concentration of peroxides produced during the reaction, and a higher absorbance indicates lower antioxidant activity. The antioxidant activity of *Eucalyptus* essential oil might be attributed to the presence of some phenolic compounds (Lu and Foo, 2001). The essential oil of *E. citriodora* inhibited the oxidation of linoleic acid (93.8%) to a greater extent than the positive control BHT (92%). Few authors have reported similar results for other species of *Eucalyptus*, and the antioxidant activity observed for *E. citriodora* was comparable with that of other species (Singh *et al.,* 2009; Fadel *et al.*, 1999). Fadel *et al.* (1999) correlated the antioxidant activity of *Eucalyptus* to the essential oil components *p*-cymen-7-ol and thymol, because a significant higher antioxidant activity was observed in extracts having higher contents of these components.

Chemical composition. – The chemical composition of *E. citriodora* essential oil showed total 31 major components like citronellal (22.3%), citronellol (20%), patchoulene (9.4%), germacrene D (7.5%), α-terpinol (6.3%), sabinene (4.2%), γ-phellandrene (3.2%), eugenol (3.9%), α-pinene (3.6%), β-citronellal (3.2%), citrinyl acetate (2.8%), geranial oxime (1.9%) and paraldehyde nitrile (1.9%), while remaining 18 component percentages were lower than 1% (Table 2, Fig. 1). Batish *et al.* (2006) also reported total 17 components out of which citronellal (21.2%), citronellol (12.2%) and isopulegol (11.9%) were the major components. Fadel *et al.* (1999) also performed the GLC-MS analysis of *Eucalyptus camaldulensis* var. *brevirostris* leaves volatile oil obtained by hydrodistillation and supercritical fluid extraction methods, and there was a light difference in yield and chemical composition of oil extracted by both methods. In both extracts the main constituents were found to be β-phellandrene (8.94 and 4.09%), *p*-cymene (24.01 and 10.61%), cryptone (12.71 and 9.82%) and spathulenol (14.43 and 13.14%). The yield of the monoterpene hydrocarbons in the hydrodistillation oil (0.288 g/100 g fresh leaves) was slightly higher compared to that in the supercritical fluid extraction (0.242 g/100 g fresh leaves). The supercritical fluid extraction possessed higher concentrations of the

TABLE 2. – *Chemical composition of* E. citriodora *essential oil.*

No.	Component	Retention time	%
1	*cis*-β-ocimene	7.557	0.1
2	Limonene	7.910	0.1
3	Citral	11.473	0.1
4	Eucalyptol	11.600	0.1
5	Geranial	14.943	0.1
6	Isopulegol	16.290	0.1
7	Terpinene	16.477	1.0
8	γ-phellandrene	18.307	3.2
9	Citronellal	19.310	22.3
10	α-terpinol	19.740	6.3
11	γ-terpinene	20.593	0.7
12	β-citronellal	20.953	3.2
13	Paraldehyde nitrile	21.320	1.9
14	Eugenol	22.043	3.9
15	Geranial nitrile	22.610	0.7
16	α-pinene	22.860	3.6
17	Linalool	23.440	0.5
18	Citronellal oxime	23.907	1.4
19	Terpinene-4-ol	24.410	0.3
20	Cyclopentanone	25.450	0.6
21	Cymen-8-ol	26.153	0.3
22	Geranial oxime	27.820	1.9
23	Citronellol	29.573	20.0
24	*trans*-pinocarveol	32.577	1.1
25	β-phellandrene	33.610	0.9
26	Ylangene	34.177	0.9
27	Citrinyl acetate	34.553	2.8
28	Solanone	35.293	0.3
29	Sabinene	35.650	4.2
30	Germacrene D	35.900	7.5
31	Patchoulene	36.630	9.4

TABLE 3. – *Antimicrobial activities of* E. citriodora *essential oils and antibiotics, and minimum inhibitory concentration (MIC).*

Microorganism	Essential oil	Neomycin	Erythrosine	Amoxil	Nizoral	MIC (mg/mL)
S. aureus	31[a]	14	-	-	-	0.48
B. subtilis	28	17	-	-	-	0.55
E. coli	15	-	11	-	-	0.90
A. niger	29	-	-	17	-	1.45
R. solani	18	-	-	-	19	1.85

[a]Antimicrobial activity is expressed as width of inhibition zones (mm).

sesquiterpene compounds than the hydrodistillation oil. Our findings are also comparable with BATISH *et al.* (2006) regarding the chemical compositions of essential oil. MULYANINGSIH *et al.* (2011) also reported aromadendrene (31.17%), citronellal (90.07%) and citronellol (4.32%) as the major compounds. Our study was also in line with SARTORELLI *et al.* (2007) who observed that the leaf-essential oils from *E. robusta* and *E. saligna* contained monoterpene α-pinene (73.0%), *p*-cymene (54.2%) and γ-terpinene (43.8%).

Antimicrobial activity. – The antimicrobial activity of *E. citriodora* essential oil against *S. aureus, B. subtilis, E. coli, A. niger* and *R. solani* is shown in Table 3. The oil extracted from *E. citriodora* leaves showed significantly higher antimicrobial activity versus the standards neomycin and erythrosine (antibacterial) as well as Amoxil and Nizoral (antifungal). The zone of inhibition of *E. citriodora* oil were 31, 28, 15, 29 and 18 mm against *S. aureus, B. subtilis, E. coli, A. niger* and *R. solani,* respectively. The zones of inhibition for neomycin, erythrosine, Amoxil and Nizoral were 14-17, 11, 17 and 19 mm, respectively, while the MIC values were found to be 0.48, 0.55, 0.9, 1.45 and 1.85 mg/L for *S. aureus, B. subtilis, E. coli, A. niger* and *R. solani,* respectively. It is very clear from the data that *E. citriodora* essential oil has significant higher antibacterial and antifungal activities, and previous studies have also reported similar results (RUBERTO *et al.,* 2000; CIMANGA *et al.,* 2002) for different species of *Eucalyptus*. DELAQUIS *et al.* (2002) reported the antimicrobial activity of *E. dives* against *S. cerevisiae, P. fragi, E. coli, S. typhimurium, L. monocytogenes and S. aureus*. While studying the antimicrobial activity of essential oils from *Eucalyptus* against multidrug-resistant bacteria pathogens, MULYANINGSIH *et al.* (2011)

reported the presence of aromadendrene (31.17%), citronellal (90.07%) and citronellol (4.32%) as the major compounds in *Eucalyptus* which were active against gram positive and gram negative bacterial strains. Similarly, GHALEM and MOHAMED (2008) revealed the antibacterial activities of essential oils from leaves of two *Eucalyptus* spp. (*E. globulus and E. camaldulensis*) against *S. aureus* and *E. coli* and indicated the usefulness of the *Eucalyptus* species as a microbiostatic, antiseptic or as disinfectant agent. The results of the present investigation were also in accordance with SARTORELLI *et al.* (2007) indicating that the antimicrobial activity of the essential oil against *S. aureus, E. coli* and *C. albicans* was comparable with standards and that essential oil of this species could be used practically.

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Summary. – Chemical composition, antioxidant and antimicrobial activities of the essential oil from *Eucalyptus citriodora* leaves were investigated. The higher steam-distilled essential oil content was recorded up to 1.82% which consisted of citronellal (22.3%), citronellol (20%), patchoulene (9.4%), germacrene D (7.5%), α-terpinol (6.3%), sabinene (4.2%), γ-phellandrene (3.2%), eugenol (3.9%), α-pinene (3.6%), β-citronellal (3.2%), citrinyl acetate (2.8%), geranial oxime (1.9%), paraldehyde nitrile (1.9%), and the remaining 18 minor components were lower than 1% each. This investigated essential oil exhibited a good antioxidant activity measured by DPPH free radical-scavenging ability and inhibition of linoleic acid oxidation. Evaluation of antimicrobial activity of the essential oil indicated that the essential oil from *E. citriodora* leaves was active against bacterial strains like *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and pathogenic fungi as *Aspergillus niger and Rhizopus solani*. The results of antimicrobial assays indicated that all the tested microorganisms were affected by potential ingredients of the essential oil from *E. citriodora* leaves that could be used as potential antioxidant and antimicrobial agent.