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Assessment of *in vitro* antioxidant activity of essential oil of *Eucalyptus citriodora* (lemon-scented Eucalypt; Myrtaceae) and its major constituents

Harminder Pal Singh [a,*], Shalinder Kaur [a], Kirti Negi [a], Savita Kumari [b], Varinder Saini [a], Daizy R. Batish [b], Ravinder Kumar Kohli [b]

[a] Department of Environment Studies, Panjab University, Chandigarh 160014, India
[b] Department of Botany, Panjab University, Chandigarh 160014, India

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ABSTRACT

We investigated the chemical composition and antioxidant activity of *Eucalyptus citriodora* (lemon-scented eucalyptus) leaf oil in terms of total antioxidant activity, ferric reducing antioxidant power (FRAP) assay, ferrous ion chelating activity, and scavenging of hydrogen peroxide (H_2O_2) and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radicals and inhibition of lipid peroxidation. GC–MS analysis of essential oil revealed the presence of 43 components constituting 99.2% of oil. The oil was monoterpenoid (94.35% of oil) with citronellal (60.66%), β-citronellol (12.58%) and isopulegol (8.19%) as the major monoterpenoids. Oil and its major monoterpenes exhibited moderate to strong antioxidant activity in terms of TAA, FRAP and Fe^{+2} chelating, DPPH$^{\bullet}$ and H_2O_2 scavenging, and lipid peroxidation inhibition. The study concludes that *E. citriodora* leaves contain monoterpenoid rich oil exhibiting antioxidant activity.

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1. Introduction

Eucalyptus is a large genus of family Myrtaceae comprised of about 900 species and subspecies (Brooker & Kleinig, 2004). Though it is a native of Australia, it is found in almost all parts of the world due to human introduction. It is now cultivated in many tropical, sub-tropical and even sub-temperate countries (Batish, Singh, Kohli, & Kaur, 2008). *Eucalyptus* is generally grown for its wood, which is primarily used in making cellulose pulp and secondly for boards and panels (Brooker & Kleinig, 2004). *Eucalyptus* species are well-known for their essential oils that are extracted through steam or hydrodistillation. These have been widely used in the perfumery and fragrance industries, and in fact, these are among the world's top-traded oils (Batish et al., 2008). Essential oil from *Eucalyptus* species has been used as an antiseptic, antipyretic and analgesic since ancient times (Brooker & Kleinig, 2004). It has been reported to possess a wide range of biological activities including antimicrobial, fungicidal, insect-repellant, fumigant, pesticidal and acaricidal activity (Batish et al., 2008).

Free radicals have deleterious effects on the human body and cause oxidative damage to proteins, carbohydrates, lipids, enzymes, and DNA (Halliwell & Gutteridge, 1989). Living cells possess an excellent scavenging mechanism to avoid free radical-induced injury; however, under the influence of external stresses these mechanisms become inefficient. Therefore, food stuffs are supplemented with synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). However, due to toxicological concerns linked to their use over a long period and increasing awareness about natural foods, there has been a renewed interest in the use of natural substances as antioxidants (Babich, 1982). In this context, essential oils and their components due to their relatively non-toxic nature, acceptance with consumers, and a wide spectrum of biological activity, have gained much attention as a potential source of antioxidants (Batish et al., 2008). Among essential oils, eucalyptus oils hold a good promise. These have been placed under the GRAS (Generally Regarded as Safe) category by the Food and Drug Authority (FDA) of the USA and classified as non-toxic (USEPA, 1993) and approved as flavoring agent in food ($\leq$5 mg/kg) and confectionery items ($\leq$15 mg/kg) in Europe (Council of Europe, 1992). It is therefore worthwhile to evaluate the essential oils from the eucalyptus species for their antioxidant activity with a view of their pharmaceutical application.

Eucalyptus citriodora is one species of eucalyptus widely used in perfumery, for cleaning air, and as an important ingredient of cosmetics and room fresheners. Earlier studies have demonstrated that essential oil from *E. citriodora* possesses antibacterial, antifungal, anticandidal, insecticidal, acaricidal, antitrypanosomal and

* Corresponding author. Tel.: +91 172 253 4095.
E-mail addresses: hpsingh_01@yahoo.com, hpsingh_01@pu.ac.in (H.P. Singh).

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herbicidal activity (Batish et al., 2008; Habila et al., 2010; Ramezani, Singh, Batish, & Kohli, 2002; Singh, Batish, Setia, & Kohli, 2005). Burning of the leaves of *E. citriodora* has been reported to provide a cost-effective method of household protection against mosquitoes in Africa (Seyoum, Killeen, Kabiru, Knols, & Hassanali, 2003). Additionally, the oil has been reported to exhibit analgesic and anti-inflammatory effects during cold, flu and sinus congestion (Silva et al., 2003). However, to the best of our knowledge, no study has been conducted to evaluate the essential oil from *E. citriodora* for antioxidant activity. Thus, the aim of the present study was (a) to investigate the chemical composition and (b) to determine the antioxidant activity of essential oil extracted from foliage of *E. citriodora,* and its three major constituents, *in vitro*. We evaluated antioxidant activity in terms of ferric reducing antioxidant power (FRAP) assay, ferrous ion chelating activity, scavenging of hydrogen peroxide (H_2O_2) and DPPH• radicals, total antioxidant activity and inhibition of lipid peroxidation.

2. Materials and methods

2.1. Plant material

In September 2010, fresh leaves were plucked from trees of *E. citriodora* growing on the campus of Panjab University, Chandigarh, India. These were brought to the laboratory, cleaned and used for extraction of oil. The leaves were identified and authenticated by the herbarium, Department of Botany, Panjab University, where the voucher specimen was deposited. The pure reference compounds used for identification and antioxidant assays were purchased from Sigma–Aldrich Co., St. Louis, MO, USA; Lancaster, Morecambe, England; AlfaAesar, UK; Acros Organics, Geel, Belgium; and TCI, Tokyo, Japan. All other chemicals and reagents used were of analytical grade and purchased from Sigma–Aldrich Co., St. Louis, MO, USA; Merck Ltd., Mumbai, India; Sisco Research Laboratory Pvt. Ltd., Mumbai, India; or Loba Chemie Pvt. Ltd., Mumbai, India.

2.2. Extraction of volatile oil

Fresh leaves were picked in the morning. The essential oil was isolated by the process of hydrodistillation using Clevenger's apparatus for 3 h. The obtained oil was dried over anhydrous sodium sulfate, measured, and stored at 4 °C for further identification by gas chromatography–mass spectroscopy (GC–MS) and antioxidant analysis.

2.3. GC–MS

The essential oil was analyzed by GC–MS, as described by Singh, Mittal, Kaur, Batish, and Kohli (2009). GC–MS analyses were performed on a Shimadzu QP 2010 mass spectrophotometer (Shimadzu, Kyoto, Japan) fitted with a AOC 20*i* auto sampler and BP-1 MS capillary column (30 m × 0.25 mm, i.d.: 0.25 μm film thickness). Helium (He) at a split ratio of 1:50 and a linear velocity of 40.8 cm/s was used as carrier gas. Initially, the oven temperature was 40 °C, which was held isothermally for 4 min, followed by an increase in temperature to 220 °C at 4 °C/min and this was held isothermally for 15 min. Mass spectral range was recorded from *m/z* 50–600 amu. The mass spectrometer source temperature was 200 °C, and interface temperature and injector temperature was 220 °C.

2.4. Identification of the compounds

Different constituents of the oil were identified on the basis of (i) co-elution and comparison of retention times with those of pure reference samples, (ii) comparison of their retention indices (RI) relative to homologous series of *n*-alkanes (C_{10}–C_{40}, Sigma–Aldrich Co., St. Louis, MO, USA), and (iii) comparison of their mass spectra and RI with that of pure reference samples consulting the libraries of Wiley 275 and NBS 75K, NIST 98, and the compilation by Adams (Singh et al., 2009).

2.5. Antioxidant activity

The antioxidant activities of the essential oil and its three major components (citronellal, β-citronellol and isopulegol) were determined in terms of total antioxidant activity (TAA), ferric reducing antioxidant power (FRAP) assay, Fe^{+2} chelating activity, and H_2O_2 and DPPH• scavenging.

2.5.1. Total antioxidant activity (TAA)

It was measured as per the method of Preito, Pineda, and Aguilar (1999) with slight modification. To different concentrations of the sample (50–400 μg/ml), 1 ml of reagent solution containing 0.6 M H_2SO_4, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate was added. Eppendorf vials were then heated at 95 °C for 90 min in a hot water bath. The mixture was then cooled and the absorbance was read at 695 nm. Increased absorbance indicates increased TAA. Ascorbic acid was used as a positive control, and the TAA values have been expressed as μM ascorbic acid equivalents (AAE).

2.5.2. Free radical scavenging capacity (RSC)

RSC was evaluated by measuring scavenging activity against DPPH (2,2′-diphenyl-1-picrylhydrazyl) as per Singh et al. (2009). Briefly, samples (50–400 μg/ml) were mixed with 1 ml of a 90 μM DPPH (Fluka, Buchs, Switzerland) solution in methanol, and the final volume was made 3 ml with methanol. A parallel blank (without sample) and positive control (BHT) were also maintained. The solutions were incubated for 30 min in the dark at 25 °C, and the absorbance was read at 515 nm. A decrease in the absorbance of the DPPH solution indicates an increased RSC.

2.5.3. Ferric reducing antioxidant power (FRAP) assay

It was carried out as per the method of Yildirim, Mavi, and Kara (2001). Samples (50–400 μg/ml) were mixed with 1.25 ml of PO_4^{3-} buffer (0.2 M, pH 6.6) and 1.25 ml of potassium ferricyanide (1%, *w/v*). The mixture was incubated at 50 °C for 30 min, followed by addition of 1.25 ml of trichloroacetic acid (10%, *w/v*), and then centrifuged at 4000× *g* for 10 min. After centrifugation, the supernatant was extracted and the residue was discarded. To 1.25 ml of the supernatant, 1.25 ml of distilled water and 0.25 ml of ferric chloride (0.1%, *w/v*) was added. The mixture was kept for 10 min and the absorbance was read at 700 nm against a blank containing ferric chloride and distilled water. Increased absorbance of the reaction mixture indicates increased reducing activity. BHT was used as a positive control.

2.5.4. Ferrous ion (Fe^{+2}) chelating activity

Fe^{+2} ion chelating ability was determined as per the method of Decker and Welch (1990). Briefly, to 500 μl of sample (50–400 μg/ml), 50 μl of 2 mmol/L $FeCl_2$ and 100 μl of 5 mmol/L ferrozine was added. The mixture was diluted by addition of 2 ml methanol and then shaken vigorously. After 10 min, the absorbance of resulting solutions was recorded at 562 nm. Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions (Halliwell & Gutteridge, 1989). Ferrozine forms a complex with Fe^{2+} ions to give color and Fe^{2+}/ferrozine complex has a strong absorbance at 562 nm. The higher the Fe^{2+} chelating ability, the lower the absorbance will be. EDTA is a strong metal chelator; hence, it was used as a positive control for this test.

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