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ORIGINAL ARTICLE

Chemical composition, antioxidant activity and inhibitory effects of essential oil of *Eucalyptus teretecornis* grown in north-western Himalaya against *Alternaria alternata*

Sanjay Guleria · A. K. Tiku · Sahil Gupta · Gurjinder Singh · Apurva Koul · V. K. Razdan

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Abstract The essential oil obtained from fresh leaves of *Eucalyptus teretecornis* (family Myrtaceae) was analysed by gas chromatography/mass spectrometry (GC/MS). Twenty eight compounds were identified and β-pinene (22.55%), α-pinene (22.50%), 1,8-cineole (19.84%), limonene (5.62%), β-fenchol (3.10%), α-phellandrene (2.90%), α-eudesmol (2.66%) and 4-(2-methylcyclohex-1-enyl)-but-2-enal (2.34%) were the main components. The antifungal activity of the essential oil was assayed against *Alternaria alternata* using bioautography. Two main bioactive components namely a_1 (R_f=0.27) and a_2 (R_f=0.33) were observed that produced inhibition zone of 4 mm and 8 mm in diameter respectively. The minimum inhibitory amount (MIA) of a_1 and a_2 against *A. alternata* was determined as 28 μg and 10 μg, respectively using bioautography assay. Components corresponding to a_1 and a_2 were determined as β-fenchol (oxygenated monoterpene) and α-eudesmol (oxygenated sesquiterpene) respectively using GC/MS analysis. The antioxidant activity of the essential oil and its bioactive fraction was evaluated by DPPH radical scavenging assay, β-carotene/linoleic acid bleaching assay, reducing power assay and metal chelating assay. In addition fraction of the essential oil that showed antioxidant activity was analyzed using GC/MS and α-fenchol, 4-terpineol and carvacrol were the main components.

S. Guleria (✉) · A. K. Tiku · S. Gupta · G. Singh · A. Koul
Natural Product Laboratory,
Division of Biochemistry and Plant Physiology,
SK University of Agricultural Sciences and Technology,
Chatha 180 009 Jammu, India
e-mail: guleria71@rediffmail.com

V. K. Razdan
Division of Plant Pathology,
SK University of Agricultural Sciences and Technology,
Chatha 180 009 Jammu, India

Keywords *Eucalyptus teretecornis* · Antioxidant activity · β-fenchol, α-eudesmol · Antifungal activity · Monoterpenoids

Abbreviations

BHT	Butylated hydroxyl toluene
BHA	Butylated hydroxyl anisole
GC/MS	Gas chromatography/mass spectrometry
TLC	Thin layer chromatography
PTLC	Preparative thin layer chromatography
MIA	Minimum inhibitory amount
DPPH	1,1-diphenyl-2-picrylhydrazyl
ISM	Indian system of medicine

Introduction

The interest in medicinal plants and their biologically active secondary metabolites has increased in recent years, in relation to the possible development of novel antioxidant molecules and potential biocides (Hedberg 1993). The antimicrobial properties of volatile oils have been recognized since antiquity. The use of essential oils is important not only in preservation of foods but also in control of human and plant diseases of microbial origin (Baratta et al. 1998).

Fungal diseases are responsible for heavy toll of field crops every year and the micro-organisms have developed resistance to many synthetic fungicides due to their indiscriminate use. This has forced the researchers to search for new antimicrobial substances from various sources including medicinal plants (De Leo et al. 2004).

The role of free radicals and active oxygen species is becoming increasingly recognized in the pathogenesis of many human diseases including cancer, aging, and athero-

sclerosis (Perry et al. 2000). Free radicals can also cause lipid peroxidation in foods that leads to their deterioration. Although there are some synthetic antioxidants such as BHT and BHA but, these compounds are suspected to cause side effects (Ito et al. 1983). Therefore, the investigations on determination of natural sources of antioxidants and biocides from plants are important.

The genus *Eucalyptus* (family: Myrtaceae) comprises plants of 800 species (Louppe et al. 2008). Although most of the plants are native to Australia, numerous species have been introduced to other parts of the world including north-western Himalaya (India). Its leaves contain oil glands which produce oils of different composition (Grieve 1992), which mainly contain 1,8-cineole, monoterpenes, sesquiterpenes, aldehydes and ketones (Newall et al. 1996). *Eucalyptus* oil is used for industrial and medicinal purposes around the world for food, flavouring, nasal and cough drops. Also essential oils containing cineole had shown antimicrobial and nematicidal activity (Gundidza et al. 1993).

Eucalyptus teretecornis is the most commonly planted species in India (Singh et al. 2009). It is planted for its heartwood which is used for timber and railway sleepers (Boland et al. 1991). There are reports in literature on the antioxidant and antimicrobial activities of crude essential oil from *E. teretecornis* (Singh et al. 2009; Batish et al. 2008). However, to the best of our knowledge there is no information on isolation and identification of bioactive molecules from *E. teretecornis* essential oil having free radical scavenging and antifungal activity.

The level of interest in antioxidant and antimicrobial properties of volatile oils is just one aspect of the practical potential such oils have in various protective roles. There also appears to be a revival of traditional approaches to livestock welfare and food preservation in which essential oils play a part (Thomann et al. 1997). It is intended here to examine the antioxidant and antimicrobial activity of *E. teretecornis* essential oil to assess its potential application to human health care, food preservation and plant protection.

Materials and methods

Plant material and isolation of essential oil

Fresh leaves from *E. teretecornis* were collected locally in November, 2009. They were identified at Herbal Garden and Herbarium Research Institute in ISM, Joginder Nagar, District Mandi (HP), India and a voucher specimen was deposited at the herbarium of the institute. They were subjected to hydro-distillation for 2 h using a Clevenger apparatus, followed by exhaustive extraction of the distillate with petroleum ether. The resulting extract was dried over anhydrous sodium sulphate.

GC/MS analysis

Analysis of the oil was carried out at Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India. A GC-MS 4000 (Varian, USA) system with a varian CP-SIL 8CB column (30 m×0.32 mm i.d., 1 μm film thickness). Injector temperature was 230°C. Oven temperature programme used was holding at 60°C for 5 min, heating to 250°C at 3°C/min and keeping the temperature constant at 250°C for 10 min. Helium was used as a carrier gas at a constant flow of 1.0 ml/min and an injection volume of 0.20 μl was employed. The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40–500 m/z. The identification of components of the essential oil was based on comparison of their mass spectra with those of NIST05 (version 2.0) library.

Bioautography

This technique was used to determine the active constituents of the essential oils (Marston et al. 1997). Aliquotes of 25–50 ml of inoculum spray solution (ca. 3×10^5 conidia/ml) were prepared for test fungus (*A. alternata*) with liquid potato dextrose (potato 200 g, dextrose 20 g, and water to make total volume of 1 l). Using a 100 ml chromatographic sprayer plate was sprayed lightly (to a damp appearance) three times with spore suspension and incubated for 4 d in a dark moist chamber at 25°C. Fungal growth inhibition appeared as clear zones against a dark background. The experiment was repeated thrice.

Isolation of antifungal constituents

After identification of the inhibition zones on the TLC plate, PTLC was performed by loading the essential oil onto a pre-activated silica gel 60 F_{254} coated glass plate (20×20 cm, 500 μm thickness) which was developed in *n*-hexane/ethyl acetate (9:1, v/v) solvent system (Sridhar et al. 2003). The separated compounds were visualized under UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid spray reagent. The isolation was carried out by scrapping off the detected zones corresponding to the antifungal constituents a_1 (R_f=0.27) and a_2 (R_f=0.33) and transferring them into percolator. The substances were then set free from silica gel by elution with dichloromethane.

Antifungal activity of bioactive molecules isolated from *E. teretecornis*

Different amounts of a_1 and a_2 were loaded onto the TLC plate and bioautography was performed as described earlier using *A. alternata* as test pathogen. Antifungal activity was

determined as MIA of active compounds required for the inhibition of fungal growth on TLC plate.

Free radical scavenging activity on TLC

Hydrogen or electron donating ability of the essential oil was evaluated from the bleaching of the purple coloured solution of stable free radical DPPH. Five microlitres of 1:10 dilution of essential oil in dichloromethane were applied to the TLC plate (aluminium sheets covered with silica gel 60 F_{254}, Merck) and hexane-ethyl acetate (9:1) mixture was used as mobile phase. The plate was sprayed with a 0.2% DPPH$^{\bullet}$ reagent in methanol and left at room temperature for 20 min. Yellow spots formed from the bleaching of DPPH$^{\bullet}$ were evaluated as positive free radical scavenging activity (Mimica-Dukic et al. 2003).

Isolation of antioxidant fraction

For the isolation of the antioxidant compounds, 100 mg of the crude oil were subjected to preparative TLC (silica gel 60 G_{254}, Merck) in *n*-hexane/ethyl acetate (9:1,v/v) solvent system (Sridhar et al. 2003). The antioxidant fraction (F_1) was detected by spraying the TLC plate with 0.2% DPPH$^{\bullet}$ reagent in methanol (Mimica-Dukic et al. 2003). The silica gel corresponding to F_1 was scratched and washed with dichloromethane to remove the bioactive compounds (3.7 mg).

DPPH radical scavenging assay

The radical scavenging activity was determined using DPPH$^{\bullet}$ method (Bozin et al. 2006). 1 ml of different concentrations of the essential oil or bioactive fraction were mixed with 1 ml of a 90 μM DPPH$^{\bullet}$ solution in methanol, and final volume was made to 4 ml with methanol. The mixtures were well shaken and kept at 25°C in the dark for 1 h. The absorbance was measured at 517 nm. Inhibition of DPPH radical in percent was calculated as:

$$\mathrm{RSC}(\%) = \left(1 - \mathrm{A_{sample}}/\mathrm{A_{blank}}\right) \times 100$$

Where A_{blank} is the absorbance of the control (containing all the reagents except the test compound), and A_{sample} is the absorbance of the test sample. Oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition% against oil concentration. BHT was used as reference.

β-carotene bleaching inhibition assay

The antioxidant activity of essential oil/fraction was evaluated using β-carotene-linoleic acid model system (Kabouche et al. 2007). β-carotene (0.5 mg) in one ml of chloroform was added to 25 μl of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. Chloroform was evaporated at 40°C by a rotary vacuum evaporator. Then, 100 ml of distilled water saturated with oxygen were slowly added to the residue and the solution was vigorously agitated to form a stable emulsion. Four thousand microlitres of this mixture were transferred into test tubes containing 0.2 ml portion of the essential oil/fraction prepared in methanol at different concentrations. As soon as the emulsion was added to each tube, zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 120 min at 50°C. A blank, devoid of β-carotene, was used for background subtraction. Antioxidant activity was calculated as percent of inhibition (I%) relative to the control using the following equation:

$$\mathrm{I}\% = \left[1 - \left(\mathrm{A_{s(0)}} - \mathrm{A_{s(120)}}\right)/\mathrm{A_{c(0)}} - \mathrm{A_{c(120)}}\right)\right] \times 100$$

Where $A_{s(0)}$ was the initial absorbance of the sample at 0 min, $A_{s(120)}$ was the absorbance of the sample at 120 min, $A_{c(0)}$ was the initial absorbance of the negative control at 0 min, and $A_{c(120)}$ was the absorbance of the negative control at 120 min. The essential oil/fraction concentration providing 50% antioxidant activity (IC_{50}) was calculated from the graph of antioxidant activity percentage against essential oil/fraction concentration. BHT was used as standard antioxidant.

Reducing power assay

The reducing power of essential oil/fraction was determined by the method as described previously (Oyaizu 1986). Different concentrations of essential oil/fraction were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$](1%). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 ml) of 10% trichloroacetic acid were added to the mixture. The above mixture was then centrifuged for 10 min at 1,036 g. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 1% ferric chloride solution. The absorbance was measured at 700 nm in a double beam UV-VIS spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. The essential oil/fraction concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against essential oil/fraction concentration and compared with those of standard antioxidant.

Chelating capacity assay

The chelating effect on ferrous ions of *E. teretecornis* essential oil/fraction was estimated by the method fol-

lowed by Chua et al. 2008. Briefly, 200 μl of different concentrations of essential oil/fraction and 740 μl of methanol were added to 20 μl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 μl of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min before determining the absorbance of the mixture at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation: % inhibition = [(absorbance of control− absorbance of test sample)/absorbance of control)] ×100.

Identification of bioactive compounds

The isolated bioactive compounds corresponding to a_1 and a_2 and antioxidant fraction were determined using GC/MS analysis described earlier.

Statistical analysis

All experiments were carried out in triplicate. To determine whether there is a statistically significant difference among

Table 1 Percent composition of the leaf essential oil from *Eucalyptus teretecornis*

S. No.	Compound	Type of terpene	RT [min]	Percent (%)
1	α-thujene	Monoterpene	12.423	0.11
2	α-pinene	Monoterpene	12.891	22.50
3	Camphene	Monoterpene	13.800	0.65
4	β-pinene	Monoterpene	15.233	22.55
5	α-phellandrene	Monoterpene	16.635	2.90
6	α-terpinene	Monoterpene	17.173	0.05
7	Limonene	Monoterpene	17.839	5.62
8	1,8-cineole	Oxygenated monoterpene	18.055	19.84
9	δ- Terpinene	Monoterpene	19.308	0.60
10	Terpinolene	Monoterpene	20.741	0.26
11	α-fenchol	Oxygenated monoterpene	22.584	0.75
12	α-campholenal	Oxygenated monoterpene	22.911	0.18
13	Sabinyl acetate	Oxygenated monoterpene	23.744	2.17
14	4-(2-methylcyclohex-1-enyl)-but-2-enal	Monoterpene	25.296	2.34
15	4-terpineol	Oxygenated monoterpene	25.626	0.49
16	β-fenchol	Oxygenated monoterpene	26.303	3.10
17	Myrtenol	Oxygenated monoterpene	26.407	1.45
18	Fenchyl acetate	Oxygenated monoterpene	27.334	0.17
19	Piperitone	Oxygenated monoterpene	29.177	0.07
20	Carvacrol	Oxygenated monoterpene	31.003	0.24
21	Unknown	–	33.230	2.06
22	α-cubene	Sesquiterpene hydrocarbon	34.693	0.04
23	*allo*-aromadendrene	Sesquiterpene hydrocarbon	37.506	0.55
24	Aromadendrene	Sesquiterpene hydrocarbon	38.422	0.11
25	Sinularene	Sesquiterpene hydrocarbon	42.571	0.11
26	Viridiflorol	Sesquiterpene hydrocarbon	43.879	0.07
27	γ- eudesmol	Oxygenated sesquiterpene	45.201	1.55
28	Valencene	Sesquiterpene hydrocarbon	45.542	0.21
29	α-eudesmol	Oxygenated sesquiterpene	46.157	2.66

RT retention time

Monoterpenes=57.58%

Oxygenated monoterpenes=28.46%

Sesquiterpene hydrocarbons=1.09%

Oxygenated sesquiterpenes=4.21%

Total identified=91.34%

the results obtained for antioxidant activity assays, variance analyses were carried out using the SPSS 14.0 software package. Values of $p<0.05$ were considered to be statistically different.

Results and discussion

Hydro-distillation of the fresh leaves from *E. teretecornis* grown in the north-western Himalaya gave yellowish oil with a yield of 1.35%. The oil was analyzed by GC/MS to determine its main constituents. It was further investigated for antifungal and antioxidant activity. The GC/MS analysis led to the identification of 28 components eluted between 10 and 47 min (Table 1), accounting for 91.34% of the total components present in the crude essential oil of *E. teretecornis*. The major constituents of the volatile oil were β-pinene (22.55%), α-pinene (22.50%), 1,8-cineole (19.84%), limonene (5.62%), β-fenchol (3.10%), α-phellandrene (2.90%), α-eudesmol (2.66%) and 4-(2-methylcyclohex-1-enyl)-but-2-enal (2.34%). The oil contained 10 monoterpenes (57.58%), 28.46% oxygenated monoterpenes, 1.09% sesquiterpene hydrocarbons and 2 oxygenated sesquiterpenes (4.21%). In general, the oil was rich in monoterpenoids and the presence of 1,8-cineole and α-pinene as the major constituents in the fresh leaf oil of *E. teretecornis* was in agreement with earlier studies (Singh et al. 2009; Pino et al. 2002). However, we have also observed higher level of β-pinene in essential oil of *E. teretecornis* grown in north-western Himalaya (Table 1).

Antifungal potential of *E. teretecornis* essential oil was examined using direct bioautographic procedure and *A. alternata* as test organism. Bioautography is a simple procedure for evaluating plant extracts and essential oils against human and plant pathogens (Marston et al. 1997). This technique helps in activity guided tracking of bioactive compounds on the TLC plate. Application of *E. teretecornis* essential oil in the bioautography system mentioned above showed the presence of two distinct inhibition zones (Fig. 1b) on the TLC plate with diameters of 4 mm (R_f=0.27) and 8 mm (R_f=0.33) corresponding to a_1 and a_2 respectively (Table 2, Fig. 1c). As the essential oil presented antifungal activity in the bioautography test, it was further subjected to PTLC for isolation of pure molecules corresponding to the inhibition zones. PTLC of the essential oil yielded 3.3 mg of a_1 and 4.8 mg of a_2 and the MIA of bioactive molecules a_1 and a_2 was determined against *A. alternata* using bioautography test. The above study revealed stronger inhibition of *A. alternata* spores by a_2 (MIA=10 μg) than a_1 (MIA=28 μg) (Table 3). Furthermore, the antifungal compounds corresponding to a_1 and a_2 were determined as oxygenated terpenoids β-fenchol and α-eudesmol respectively using GC/MS analysis (Fig. 2). The above results are in agreement with earlier report that fungicidal effect of the essential oils would be due to oxygenated compounds (Griffin et al. 1999). The antifungal activity of essential oils extracted from other *Eucalyptus* species such as *E. salgina* and *E. camaldulensis* has been shown on *Candida albicans* and *Phaeoramularia angolensis* (Oyedeji et al. 1999; Dongmo et al. 2008).

E. teretecornis essential oil also exhibited free radical scavenging activity in TLC based DPPH$^{\bullet}$ assay (Fig. 1a).

Table 2 Antifungal activity of essential oil isolated from *Eucalyptus teretecornis* essential oil against *Alternaria alternata* using bioautography

Compound	R_f value	Diameter of inhibition zone [mm][a]
a_1	0.27	4.00±0.30[b]
a_2	0.33	8.00±0.48

[a] 6 μl of 10 times diluted *Eucalyptus teretecornis* essential oil in dichloromethane was loaded onto the TLC plate. The plate was developed with *n*-hexane/ethyl acetate (9:1, *v/v*). Zones of fungal growth inhibition were produced after bioautography with *Alternaria alternata*

[b] Values are mean ± standard deviation of three parallel measurements

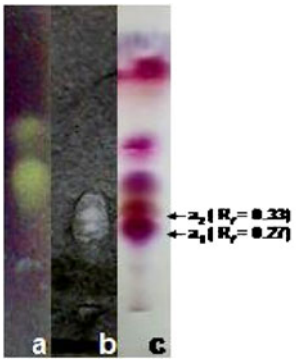

Fig. 1 TLC of essential oil from *Eucalyptus teretecornis* leaves. **a** detection of free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (0.5 mM); **b** bioautography with *Alternaria alternata* spores; **c** detection with vaniline/sulphuric acid reagent. a_1 and a_2 correspond to the components of the essential oil having antifungal activity

Table 3 Antifungal activity of compounds isolated from *Eucalyptus teretecornis* essential oil against *Alternaria alternata*

Compound	Antifungal activity [μg][a]
a_1, β-fenchol	28±1.68[b]
a_2, α-eudesmol	10±0.40

[a] Minimum inhibitory amount (MIA) of active constituents required for the inhibition of fungal growth on TLC plate in bioautography assay

[b] Values are mean ± standard deviation of three parallel measurements

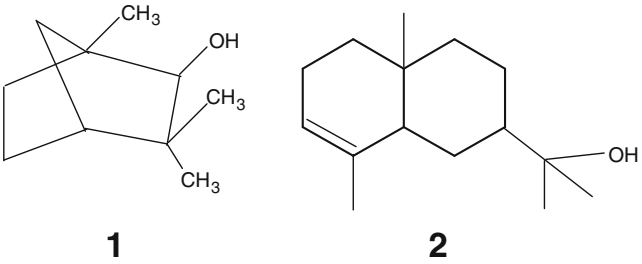

Fig. 2 Chemical structures of the compounds having antifungal activity. 1. β-fenchol; 2. α-eudesmol

The capacity of *E. teretecornis* essential oil to scavenge DPPH free radical, β-carotene bleaching inhibition, reducing power and metal ion chelating capacity was quantified using spectrophotometer based assays and the results obtained are presented in Table 4. Though the oil showed striking antioxidant activity in TLC based antioxidant assay (Fig. 1a), it exhibited low scavenging activity (IC_{50}, 5.17±0.26 mg/ml) as compared to the positive control BHT (IC_{50} value of 0.023±0.001 mg/ml) in the spectrophotometric assay (Table 4). Moderate DPPH radical scavenging activity (IC_{50}, 1.54±0.04 mg/ml) has been reported in essential oil from *Eucalyptus oleosa* leaves (Marzoug et al. 2011). Similarly, β-carotene bleaching inhibition (IC_{50} value of 6.89±0.32 mg/ml) and reducing power (IC_{50} value of 14.51±0.63 mg/ml) of *E. teretecornis* essential oil was also low as compared to BHT (IC_{50} value of 0.025±0.002 mg/ml and 0.15±0.004 mg/ml, respectively). Furthermore, the metal ion chelating activity was not detected in the essential oil at test concentration of 0.5 mg/ml.

The bioactive fraction (F_1) corresponding to the spots showing DPPH radical scavenging activity in TLC assay was eluted using PTLC and tested for antioxidant activity. F_1 exhibited significant ($p<0.05$) radical scavenging activity (IC_{50} value of 0.53±0.02 mg/ml), β-carotene bleaching inhibition (IC_{50} value of 0.81±0.034 mg/ml) and reducing power (IC_{50} value of 1.98±0.076) as compared to the essential oil (Table 4). Intriguingly, F_1 showed good (54.67±1.92%) metal ion chelating capacity at the test concentration (0.5 mg/ml). The major components of F_1 were analyzed using GC/MS analysis and determined as α-fenchol (16.84%), 4-terpineol (17.41%) and carvacrol (26.19%). Antioxidant activity of essential oil of *E. teretecornis* leaves can be attributed to the presence of these components, although the amounts of these compounds are relatively low in oil (Table 1). Phenolic compounds such as thymol and carvacrol and essential oils rich in phenolic compounds show potent antioxidant and DPPH radical scavenging activities (Alma et al. 2003). Carvacrol was detected in low amount in essential oil of *E. teretecornis* (Table 1). Antioxidant activities of volatile components from other *Eucalyptus* species such as *E. polyanthemos*, *E. globulus* and *E. perriniana* have also been reported (Lee and Shibamoto 2001).

In the present study we investigated the antifungal and antioxidant activity of *E. teretecornis* essential oil. To the best of our knowledge this is the first report on isolation and identification of the bioactive constituents responsible for antifungal and antioxidant activity from the essential oil of *E. teretecornis* grown in north-western Himalaya. The results conclusively demonstrate the antifungal potential of bioactive molecules namely β-fenchol and α-eudesmol from *E. teretecornis* essential oil against *A. alternata* a pathogen of agricultural importance. In addition, though fraction from *E. teretecornis* essential oil showed significant antioxidant activity, the antioxidant compounds were present at low level in the essential oil. Future research is warranted on studying the seasonal variation in the level of bioactive molecules from *E. teretecornis* essential oil for determining its commercial value.

Table 4 Antioxidant activity of *Eucalyptus teretecornis* essential oil and bioactive fraction in DPPH radical scavenging, β-carotene bleaching inhibition, reducing power and chelating capacity assays

Component	DPPH radical scavenging activity, IC_{50} (mg/ml)	β-carotene bleaching inhibition, IC_{50} (mg/ml)	Reducing power, IC_{50} (mg/ml)	% Chelating capacity at 0.5 mg/ml
Crude oil	5.17±0.26^{c}	6.89±0.32^{c}	14.51±0.63^{c}	n.d.
Antioxidant fraction	0.53±0.02^{b}	0.81±0.034^{b}	1.98±0.076^{b}	54.67±1.92
BHT	0.023±0.001^{a}	0.025±0.002^{a}	0.15±0.004^{a}	–

Values are mean ± standard deviation of three parallel measurements and statistical analysis of data were performed using one way analysis of variance using SPSS version 14.0 programme. Different letters (a, b and c) with in a column for a particular treatment represent significance at $P<0.05$

n.d. not detected

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