

Antioxidant Mechanism of Active Ingredients Separated from *Eucalyptus globulus*

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Abstract

The present study aimed to evaluate the antioxidant activity of petroleum ether, methanolic extracts and active ingredients separated from *Eucalyptus globulus* using three different antioxidant assays: 2,2 diphenyl picryl hydrazyl (DPPH), 2,2'- azino-bis [ethylbenzthiazoline-6-sulfonic acid] (ABTS) and β -carotene bleaching assay and identify the mode of action. The results revealed that, crude methanolic extract showed higher antioxidant activity against both DPPH and ABTS radicals than petroleum ether extract. The promising methanol soluble fraction of *Eucalyptus globulus* wood was fractionated on a silica gel column, using hexane, chloroform and ethyl acetate as the mobile phase to give three fractions (C1, C2 and C3), and both the antioxidant activity and chemical composition for raw and fractions were determined. One of the fractions isolated (C2) showed a remarkable antioxidant activity (EC_{50} of 64.4 μ g/ml, in comparison with 52.74 μ g/ml for crude extracts) against ABTS radical method, and the chemical structures of separated active ingredients were identified using different spectroscopic methods such as 17-pentatricontene (C1), N,N-diphenyllauramide (C2) and O-benzyl-N-tert-butoxycarbonyl-D-serine (C3). Also, the mode of action of the promising fraction was determined.

Keywords: *Eucalyptus globulus*; Antioxidant activity; Chemical constituents; Mode of action

Introduction

Eucalyptus globulus is one of the main forest species in Galicia, representing 27% of total wood volume. Eucalyptus wood is used mainly to produce cellulose pulp and, secondly, panels and boards. In both cases, eucalyptus bark is separated as a waste product and used as fuel. Yazaki and Hillis [1] detected ellagitannins, methyl and glycosyl derivatives of ellagic acid and free ellagic and gallic acids in methanolic extracts of the bark of various eucalyptus species. Gallotannins and catechin were found in the tannins extracts obtained after acid hydrolysis of eucalyptus barks [2]. Methanolic extracts of *E. globulus* bark were characterized by an abundance of total phenols, polymeric proanthocyanidins and ellagitannins. Increasing interest in the replacement of synthetic antioxidants has led to the research into natural sources of antioxidants, especially in plant materials. Flavonoids and other polyphenols possess anti-tumoral, anti-allergic, anti-platelet, anti-ischemic, and anti-inflammatory activities, among others, and most of these effects are believed to be due to the antioxidant capacity [3]. Phenolic compounds in edible and non-edible plants have been reported to have antioxidant capacity. Several types of plant materials, such as vegetables, fruits, seeds, hulls, wood, bark, roots and leaves, spices and herbs, etc. have been examined as potential sources of antioxidant compounds. The antioxidant compounds from natural sources could be used to increase the stability of foods by preventing lipid peroxidation and also to protect oxidative damage in living systems by scavenging oxygen radicals. Natural antioxidants have been also proposed for use in topical pharmaceutical and cosmetic compositions [3]. The yield and antioxidant activity of natural extracts is dependent on the solvent used for extraction. Several procedures have been used [4].

Eucalyptus contains many chemical compounds that play several roles in the plant. These include defense against insect and vertebrate herbivores and protection against UV radiation and against cold stress. The best-known compounds are the terpenoids, which form most of the essential oil giving *Eucalyptus* foliage its characteristic smell. However, Eucalyptus is also a rich source of phenolic constituents such

as tannins and simpler phenolics. Some of these have formed the basis of industries in the past. For example, tannins were extracted from *Eucalyptus astrigens* and rutin from *Eucalyptus astrigens* [5].

The synthetic antioxidants include butylated hydroxyanisole and butylated hydroxytoluene (BHA and BHT, respectively), propyl gallate (PG) and tertbutylhydroquinone (TBHQ). Their manufacture costs, the relative poor efficiency of natural tocopherols (also used as antioxidant agents) and the need of increased food additive safety give rise to a crescent demand on other natural and safe antioxidants sources. The search for cheap and widespread feedstock's for this purpose has led to the evaluation of residual materials, including several leaves, seeds and peels, generally considered as wastes [3,6]. The fibrous part of vegetal biomass can yield antioxidants after hydrolytic processing [7].

Extractions of phenolic compounds as antioxidants from eucalyptus (*Eucalyptus globulus*) bark were done by Vázquez et al. [8] who demonstrated the potential of eucalyptus bark as a source of antioxidant compounds.

This investigation was designed to identify the mechanism of active ingredients isolated from *Eucalyptus globulus* for their antioxidant activity, using three different antioxidant methods.

Materials and Methods

Materials

Source of plant samples: Sample of Eucalyptus (*Eucalyptus*

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Received April 03, 2012; Accepted April 26, 2012; Published May 03, 2012

Citation: El-Moein NM, Mahmoud EA, Shalaby EA (2012) Antioxidant Mechanism of Active Ingredients Separated from *Eucalyptus globulus*. Organic Chem Curr Res 1:106. doi:10.4172/2161-0401.1000106

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Globulus Family – Myrtaceae) was obtained from Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University, Giza.

Chemicals: All chemicals and reagents were obtained from Sigma chemical Co. (London, Lab. Poole), England (Cairo branch).

Preparation of extracts: Dried plant materials were pulverized using a mechanical grinder. 500 gr of powdered material was extracted with 1000 ml of petroleum ether (40-60) continuously for 6 hrs using the soxhlet apparatus. Then the residue of plant material extracted with 1000 ml of absolute methanol. Thereafter, the resulting petroleum ether and methanol extracts were reduced in rotary evaporator (40°C; N₂ stream), stored at 4°C until further use in the experiment.

Methods

Proximate analysis

Determination of moisture: The moisture content was determined according to the A.O.A.C. [9].

Determination of crude proteins: Total nitrogen (TN) was determined by the method of A.O.A.C. [9] using microkjeldahl method. Crude protein was calculated by multiplying TN by a factor of 6.25.

Determination of total hydrolysable carbohydrate: Total carbohydrate was estimated according to the method described by Dubois et al. [10].

Determination of crude lipids: Crude lipids were determined as described by A.O.A.C. [9] method using chloroform: methanol (2:1) as a solvent in soxhlet apparatus.

Separation of fatty acids and unsaponifiable matter: One gram of plant extracted lipids was saponified with methanolic KOH (30 ml, 1N) containing BHT (1 mg) at 60°C for 1 hr. under reflux. The unsaponifiable matter was extracted with ethyl ether, washed several times with distilled water and dried over anhydrous sodium sulfate and the solvent was evaporated.

Separation of fatty acids: The soap solution was acidified with sulfuric acid (5N) and the liberated fatty acids were extracted with ethyl ether, washed several times with distilled water then methylated with diazomethane ethereal solution [11].

Identification of fatty acids: Fatty Acids Methyl Esters (FAME) were analyzed by Gas Liquid Chromatography (GLC) according to Farag et al. [11] under the following conditions: column, a Thermo TR - FAME 70% cyanopropyl polysilphenylene - siloxane (30 m x 0.25 mm, film thickness 0.25 µm); detector, flame ionization; carrier gas, nitrogen; initial column temperature was 80°C and increased to 180°C at rate 4 C/min and hold at 140°C for 10 min; temperatures for injection (split ratio 1:100) and detector were 230 and 240°C, respectively. The fatty acids were identified by comparing their retention times with those of standard fatty acid methyl ester (purity 99% by GLC, sigma Co.).

Identification of unsaponifiable matter: The unsaponifiable compounds were identified by GLC using an instrument equipped with a Flame Ionization Detector (FID), a 30 m x 0.25 mm i.d. glass column. Thermo TR – 5MS (5% Phenyl Polysil Phenylene Siloxane). Initial column temperature was 70°C and increased to 280°C at 5°C/min and hold at 280°C for 20 min. injector and FID detector temperatures were 235 and 280°C, respectively and N₂ was the carrier gas. The unsaponifiable compounds (hydrocarbon and sterols) were identified by comparing their retention times those of standard hydrocarbons

from C14 to C32 and some authentic sterols (Stigmasterol and β-sitosterol).

Quantitative analysis of secondary metabolites

Determination of total Glycosides: Plant tissue was hydrolyzed with 2M HCl (Acid hydrolysis) at 100°C for 30 - 40 min. The cooled solution was extracted twice with ethyl acetate and the combined extracts were taken to dryness and the residue was taken up in a small volume of ethanol for spectrophotometer. The total sugars were determined as glucose with the phenol – sulfuric acid method according to Dubois et al. [10] using Jenway 1640 U.V/ Visible spectrophotometer for investigation.

Determination of total Terpenes: Total terpenes were estimated according to the method described by Ebrahimzadeh and Niknam [12].

One gram of dried plant sample was boiled with 15 ml of 40% ethyl alcohol for 4 hrs. A small amount of activated charcoal was added and the extract was filtered through Whatman filter paper No. 41 and the extract was completed to 50 ml (in a measuring flask) with distilled water.

10 ml of total extract was transferred to wide neck test tube, and then placed in an oven at 100°C in order to remove the water and after cooling; freshly prepared vanillin reagent (5 ml, 0.7% in 65% H₂SO₄) was added. The test tube was heated at 60 ± 1°C in a water bath for 1 hr, and then cooled in a crushed ice bath. The absorbance was determined spectrophotometrically using Jenway 1640 U.V/ Visible spectrophotometer after 1 min at 473 nm. A blank was prepared using distilled water instead of extract solution. Cholesterol was chosen as the standard

Determination of total Alkaloids: Total alkaloids contents were extracted according to Sabri et al. [13]. The alkaloid extract was dissolved in 2ml of chloroform, then 25ml of 0.02 N H₂SO₄ was added. The resulting solution was warmed to driven off the chloroform, cooled and titrated back the excess acid against 0.02 N NaOH solution, using methyl red as indicator. Each ml of H₂SO₄ (0.02N) was equivalent to 5.78 mg, of alkaloid.

Determination of total polyphenols: Total polyphenols were estimated according to the method described by Meda et al. [14]. Briefly, aliquots of 0.1 g sample extracts were dissolved in 1 ml ethanol. This solution (0.1 ml) was mixed with 2.8 ml of deionized water, 2 ml of 2% sodium carbonate and 0.1 ml of 50% folin-ciocalteu reagent. After incubation at room temperature for 30 min, the reaction mixture absorbance was measured at 750 nm against a deionized water blank using Jenway 1640 U.V/ Visible spectrophotometer. Ferulic acid was chosen as the standard.

Determination of total flavonoids

A known weight (3 g) of dried plant materials was extracted with ethanol (10 ml, 70% v/v). The ethanolic extract was concentrated under vacuum to dryness, and the residue was dissolved in distilled water (10 ml). The aqueous solution was extracted with chloroform to remove the pigment and fatty materials. The defatted aqueous extract was evaporated to dryness and weighted. A known weight of the extract was placed in 10 ml volumetric flask. Then, 5 ml distilled water and 3 ml AlCl₃ (1:10, w/v) were added. After 6 min., 2 ml CH₃-COOK (1 M) was added and the total volume was made up to 10 ml with distilled water. The solution was well mixed and the absorbance was spectrophotometrically measured against a blank at 415 nm [15].

Quercetin was served as the standard compound for the preparation of calibration curve.

Antioxidant activity

DPPH method: Quantitative measurement of radical scavenging properties was carried out according to Burits and Bucar [16]. The reaction mixture contained sample extracts 50, 100 µg/ml (or 80% MeOH as a blank) and 1 mL of a 0.002% (w/v) solution of DPPH in methanol. Butylated hydroxyl toluene (BHT) and Ascorbic acid were used for comparison or as a positive control. Discoloration was measured at 517 nm after incubation for 1, 15, 30 and 60 min. using Jenway 6305 U.V/ Visible spectrophotometer. Measurements were taken at least in triplicate. DPPH radical's concentration was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample. The actual decrease in absorption induced by the test compounds was compared with the standards.

β-Carotene-linoleic acid assay: The antioxidant activity of the all extracts, based on coupled oxidation of β-carotene and Linoleic acid emulsion, was evaluated following the method of Taga et al. [17]. Briefly, 1ml of β-carotene (0.5 mg/ml) dissolved in chloroform was pipetted into a small round bottom flask. After removing the chloroform using a rotary evaporator under reduced pressure at low temperature (less than 30°C), 10 mg of linoleic acid, 100 mg of Tween 40 and 50 ml of distilled water were added to the flask with vigorous shaking. Aliquots (1ml) of the prepared emulsion were transferred to a series of tubes each containing 50 and 100 µg/ml of extract or BHT and ascorbic acid as synthetic and natural standards. 3 ml of ethanol was finally added to them. A control sample was prepared exactly as before but without adding antioxidants. Each type of sample was prepared in triplicate. The test systems were placed in a shaking water bath at 50°C for 60 hrs. the absorbance of each sample was read spectrophotometrically at 362 nm.

ABTS radical method: This assay was based on the ability of different substances to scavenge 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS⁺) radical cation in comparison to a standard (BHT and Vit. C, 50 and 100 µg/ml). The radical cation was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4-16 h until the reaction was complete and the absorbance was stable. The ABTS⁺ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9 ml of ABTS⁺ solution and 0.1 ml of tested samples (at 50 and 100 µg/ml in MeOH solution) and mixed for 45 sec; measurements were taken at 734 nm after 1 min. The antioxidative activity of the tested samples was calculated by determined the decrease in absorbance at different concentrations by using the following equation:

$E = \frac{(A_0 - A_t)}{A_0} \times 100$, where: A_t and A_0 are the respective absorbance of tested samples and ABTS⁺ [18].

Separation of active gradient

25 gr of the *Eucalyptus globulus* crude Methanolic extract was fractionated using Liquid Chromatographic Column (50 x 1.5 cm, i.d packed with silica gel H (100 g). Gradient elution was carried out with petroleum ether, chloroform and their mixture with an increased polarity pattern (100% Petroleum ether to 100% chloroform), then with

chloroform, ethyl acetate and their mixture with an increased polarity pattern (100% chloroform to ethyl acetate 100%). Fractions (200 ml of each) were collected. Each was separated, evaporated under reduced pressure to dryness, redissolved in 5 ml of ethanol and monitored by TLC, using the solvent system Petroleum ether: ethyl acetate (70: 30, v/v and 90: 10, v/v). The TLC chromatogram was visualized under U.V. light at 365 nm and 245 nm before exposure to vaniline/sulfuric acid reagent.

All the sub-fractions were tested for their antioxidant activity by using ABTS and DPPH radical scavenging. The most potent fractions (C1, C2 and C3) were chosen for further identification using the chromatographic and spectroscopic methods as the following

Gas chromatography-mass spectrometry: GC-MS of National Research Center was used for identification the active groups in active gradients. Run time 2.02 min, low mass 49.97 m/z and high mass 700.00 m/z.

FTIR: JASCO FTIR spectra 460 plus, Japan was used for identification the active groups in active gradients.

¹H-NMR: The identification of compounds was confirmed by carried out H-NMR analysis using NMR Joel GIM, EX 270 (400 Hz).

Statistical analysis

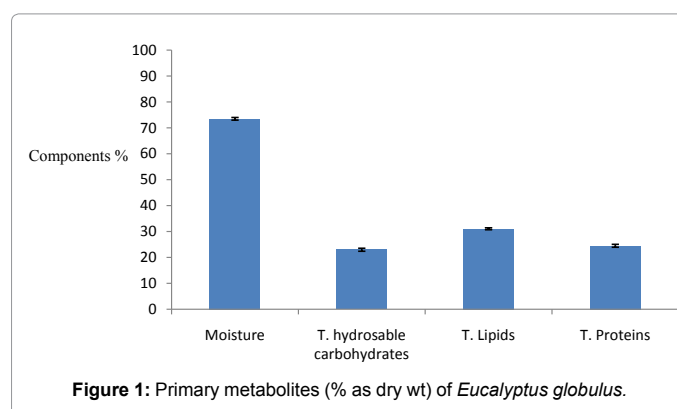
The present data was subjected to analysis of variance and the Least Significant Difference (L.S.D.) test was calculated to allow for a comparison between the average values of the studied factors.

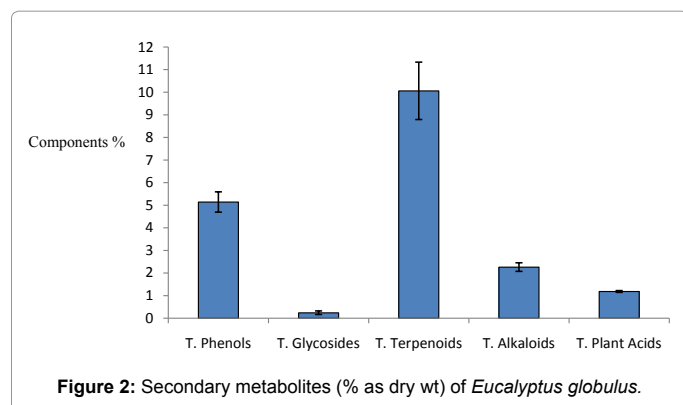
Results and Discussion

Chemical composition

Primary and secondary metabolites: The chemical composition (primary and secondary metabolites) of eucalyptus bark is given in (Figure 1 and 2). The results indicated that, *Eucalyptus globulus* have high carbohydrate percentage reached to 20% as dry matter and less than lipids and protein content (30.0 and 25.0%) as illustrated in (Figure 1). These results are in agreement with those of Emara and Shalaby [19].

The total contents of alkaloids, terpenoids, glycosides and phenolic compounds of *Eucalyptus globulus* are shown in Figure 2. It could be noticed that the terpenoids content of sample have the maximum percentage (10.2%), With regard to Phenolic compounds content, it reaches to 5.0%. However, the glycoside and flavonoids content have the lowest value (0.2 and 0.05 % respectively). These results are in agreement with those of Mishra et al. [20] who found





that, Phytochemical screening of the *Eucalyptus globulus* showed the presence of flavonoids, terpenoids, saponins and reducing sugars.

Fatty acids and hydrocarbons composition: The relative percentages of total fatty acids are presented in Table 1. The total fatty acids patterns from *Eucalyptus globulus* shows relatively high level of saturated fatty acid compared with the unsaturated components (73.16 and 26.84 % respectively). Among saturated fatty acids Capric, Lauric, and palmitic acids, Lauric acid is the most common (29.6%). However, Palmitoleic acid has the highest amount of unsaturated fatty acids (10.13%).

The relative percentages of total unsaponifiable substances are presented in (Table 1). The levels of hydrocarbons C_{20} and C_{24} occur in large quantities among all the hydrocarbons isolated (11.14 and 34.86 % respectively). In addition, The *Eucalyptus globulus* have the high amount of sterol as hydrocarbon. From these sterols, β -sitosterol and stigmasterol have a percentage of 4.11 and 11.14%, respectively. These results are in agreement with those of Gutiérrez et al. [21], who studied the chemical composition of lipophilic Extractives from *Eucalyptus globulus* Labill. Wood and reported that, the main compounds identified included sterols, sterol esters, fatty acids, steroid ketones, hydrocarbons and triglycerides. Minor compounds such as fatty alcohols, mono- and diglycerides, waxes and tocopherols were also identified among the lipids from *E. globulus* wood.

Antioxidant activity

Antioxidant efficiency of each extracts (methanol and petroleum ether) was carried out using three antioxidant methods. DPPH method was used as a principal antioxidant for fast test (H-donor method) and the other two methods were used to understand the mechanism of antioxidant activity.

The obtained results using the antioxidant bioassays (by different solvents) showed that, the antioxidant activity is concentration dependant. Tables 2 recorded the activity (using DPPH method) which increased by doubling the extract concentration (50 and 100 μ g/ml). These results are in agreement with those of Mishra et al. [20].

The antioxidant activity against DPPH, ABTS and B-carotene bleaching methods of the methanolic extract is 75.6, 81.60 and 60.40% at 50 μ g/ml respectively, and is increased to 90.24, 94.8 and 71.6% on doubling the extract concentration to 100 μ g/ml respectively. However, petroleum ether extract give an antioxidant activity lower than methanolic extract at 50 and 100 μ g/ml as shown in (Table 2) when also compared to BHT as a synthetic stander and ascorbic acid as a natural standard (ranged from 78 to 87%).

The obtained data clearly shows that the ABTS method recorded the highest antioxidant activity (94.8%), at a extract concentration of 100 μ g/ml, which exceeds that of the standard BHT (84.6%), ascorbic acid (87%) and petroleum ether extract at the same concentration (22.84%). β -carotene bleaching antioxidant methods recorded much lower activities than those of DPPH and ABTS, as illustrated in (Table 2). These results are in agreement with those of Awika et al. [22] who found that, ABTS is a better choice than DPPH and is more sensitive than DPPH. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant

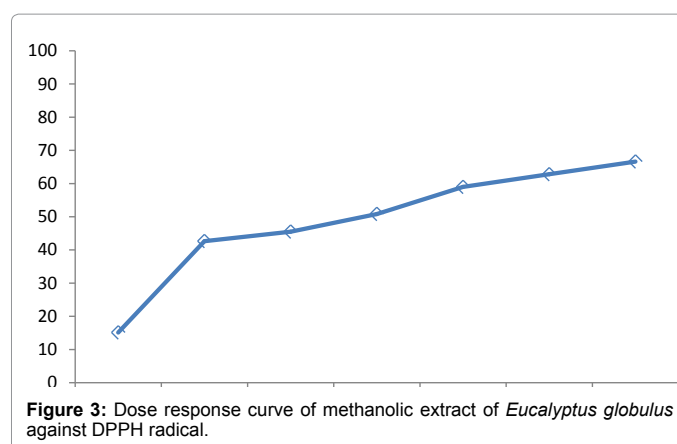


Figure 3: Dose response curve of methanolic extract of *Eucalyptus globulus* against DPPH radical.

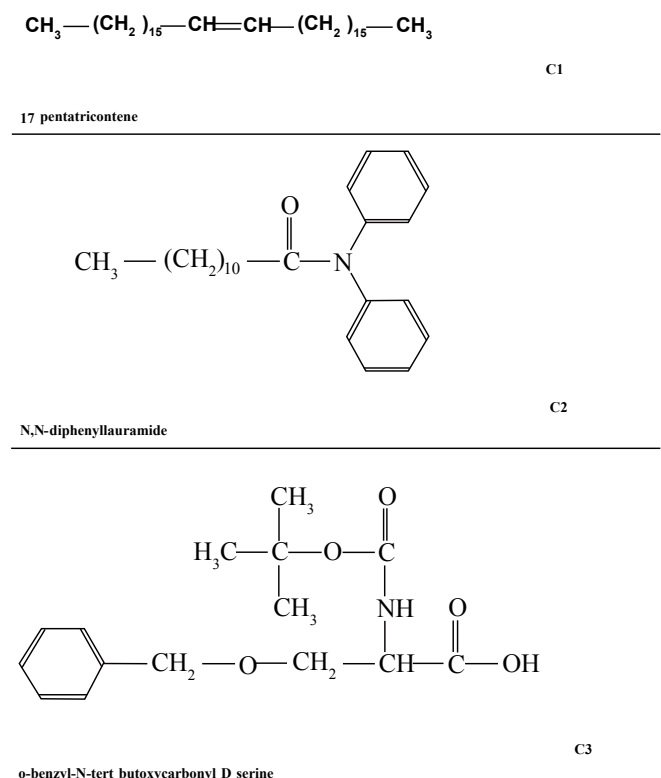


Figure 4: Suggested chemical structure of active principles separated from methanolic extract of *Eucalyptus globules*.

activity of various compounds. It is also useful for measuring antioxidant activities of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media. It is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer containing 150 mM NaCl) (PBS). Another advantage of ABTS method is that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS) reaching a steady state within 30 min. The DPPH reacted very slowly with the samples, approaching, but not reaching, the steady state after 8 hrs. This slow reaction was also observed when ABTS was reacted with samples in alcohol.

The antioxidant activity of methanol and petroleum ether extract correlated with the chemical constituents of *E. globulus* as shown in Figure 2, since phenolic compounds have been reported by Vazquez et al. [8] to bear high antioxidant activity.

The dose response curves of promising sample (Crude methanolic extract) was analyzed and the results are shown in Figure 3. It suggests that there is a positive correlation between the concentration of the sample (5-50 µg/ml) and the antioxidant activity against DPPH radical (12-70 %).

Active ingredients structure and their antioxidant activity

Chromatographic and spectroscopic analysis of active compounds separated from methanol extract of *Eucalyptus globulus* suggest that, 17-pentatricontene (C1), N,N-diphenyllauramide (C2) and O-benzyl-

N-tert-butoxycarbonyl-D-serine (C3) (Figure 4) were present with a molecular weight of 490, 351 and 295, respectively, and with a molecular formula of $C_{35}H_{70}$, $C_{24}H_{33}NO$ and $C_{15}H_{21}NO_5$, respectively. These compounds have high antioxidant activity (44.15, 77.59 and 49.00%, respectively against ABTS radical and 0.0, 22.42 and 12.18%, respectively against DPPH radical), compared to standard synthetic antioxidant BHT which has an activity of 78.85 and 84.60 % towards ABTS radical and DPPH radical, respectively.

This active compound was identified using different spectroscopic analysis methods as a terpenoid (C1) and amides (C2 and C3) (Figure 4) and was shown to exert potent antioxidant activity (Table 3) [23].

Our results also went parallelly with Lim et al. [24] who reported that dichloromethane fraction from methanol extract exhibited the strongest antioxidant activity (in red blood cell hemolysis and lipid peroxidation assays). Further fractionation by column chromatography, TLC, UV and IR showed that the separated four sub-fractions contain phenolic compounds and manifested potent antioxidant activities.

The mass spectrum of separated active ingredient (C1) indicates the presence of the following fragment ions: 490, 349, 322, 279, 202, 169, 149, 111, 97 and 71 Dalton.

These results were confirmed by IR and 1H -NMR. The IR spectrum of active compound (C1) showed absorption at 2956 (– CH aliphatic asymmetric stretch), 1630 (C=C), 1461 (Aliphatic CH_2 scissor for the methylene group).

Hydrocarbons			Fatty acids		
RR _t	No. of carbons	Relative %	RR _t	No. of carbons	Relative %
0.73	C14	8.52	0.40	C8:0	3.8
0.76	C16	0.18	0.51	C10:0	20.54
0.78	C18	0.59	0.62	C12:0	29.6
0.79	C19	3.12	1.0	C16:0	19.1
0.83	C20	11.14	1.10	C16:1	10.13
0.86	C21	1.18	1.81	C18:2	6.99
0.97	C22	2.39	2.05	C18:3α	8.74
1.0	C24	34.86	2.43	C18:3γ	0.98
1.14	C25	4.30	% SFA		73.16
1.22	C26	6.28	% MUFA		10.13
1.27	C27	3.67	%PUFA		16.71
1.28	C28	6.48			
1.30	Stigmas sterol	11.14			
1.31	B-Sitosterol	4.11			
1.33	C32	1.99			

Table 1: The hydrocarbons and Fatty acids composition (Relative percentage) of *Eucalyptus globules*.

Antioxidant methods	Pet. ether		Methanol		BHT		Ascorbic acid	
	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml
DPPH	8.78 ± 3.42	17.7 ± 6.85	75.60 ± 9.79	90.24 ± 0.18	78.85 ± 2.18	84.60 ± 3.00	80.06 ± 12	87.63 ± 4.36
ABTS	15.89 ± 2.4	22.84 ± 1.9	81.60 ± 2.64	94.80 ± 5.72	80.2 ± 4.50	88.0 ± 2.61	84.61 ± 4.63	92.48 ± 4.0
B-carotene bleaching	14.85 ± 1.84	20.54 ± 2.00	60.40 ± 0.84	71.65 ± 4.73	26.7 ± 1.05	44.85 ± 4.51	45.31 ± 2.04	67.30 ± 6.45

Table 2: Antioxidant (%) activity of Methanol and pet. ether extracts of *Eucalyptus globulus* against three different antioxidant activities.

Compounds	DPPH method		ABTS method	
	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml
C1	0.0	0.0	32.27 ± 3.48	44.15 ± 2.08
C2	9.2 ± 0.67	22.42 ± 1.53	66.39 ± 2.06	77.59 ± 1.15
C3	9.86 ± 0.68	12.18 ± 2.79	34.95 ± 2.52	49.00 ± 2.58

Table 3: Antioxidant activity (%) of pure compounds separated from methanolic extract of *Eucalyptus globules*.

The ¹H-NMR data indicated that, the compound under study (C1) had the following types of protons; A multiplex signal at δ 3.372 ppm which is characteristic for unsaturated protons and the singlet signal at δ 1.280 ppm is characteristic for methylene group $-\text{CH}_2-$ protons. Moreover, the singlet signals at δ 1.063-0.889 ppm is characteristic for methyl group- CH_3 protons.

The mass spectrum of separated active ingredient (C2) indicates the presence of the following fragment ions: 351, 305, 255, 203, 191, 149, 119, 97, 81 and 71 Dalton.

These results were confirmed by IR and ¹H-NMR. The IR spectrum of active compound (C2) showed absorption at 3423 (N-H), 2924 ($-\text{CH}$ aliphatic asymmetric stretch), 1735 ($-\text{C}=\text{O}$ absorption), 1600 (Aromatic nucleus), 1460 (Aliphatic CH_2 scissor for the methylene group).

The ¹H-NMR data indicated that, the compound under study (C2) had the following types of protons; A multiplex signal at δ 7.260 ppm which is characteristic for aromatic protons, a singlet signal at δ 2.175 ppm which is characteristic for $\text{CH}-\text{C}=\text{O}$ and a singlet signal at δ 1.640 ppm which is characteristic for methylene group $-\text{CH}_2-$ protons. Moreover, the singlet signals at δ 1.063- 0.889 ppm is characteristic for methyl group- CH_3 protons.

The mass spectrum of separated active ingredient (C3) indicates the presence of the following fragment ions: 295, 282, 246, 224, 211, 162, 149, 125, 104 and 77 Dalton.

These results were confirmed by IR and ¹H-NMR. The IR spectrum of active compound (C2) showed an absorption at 3436 is characteristic for (O-H and N-H), 2927 for ($-\text{CH}$ aliphatic asymmetric stretch), 1727 for ($-\text{C}=\text{O}$ absorption), 1600-1500 for (Aromatic nucleus), 1460 for (Aliphatic CH_2 scissor for the methylene group) and (C-O, C-C and C-N in the finger print region).

The ¹H-NMR data indicated that, the compound under study (C3) had the following types of protons: a multiplex signal at δ 7.264 ppm which is characteristic for aromatic protons, a singlet signal at δ 2.172 ppm which is characteristic for $\text{CH}-\text{C}=\text{O}$, a singlet signal at δ 2.44 ppm which is characteristic for methylene group $-\text{CH}_2-$ protons beside aromatic group, and a singlet signal at δ 2.78 ppm which is characteristic for N-H protons. Moreover, the singlet signals at δ 1.256 ppm is characteristic for methyl group- CH_3 protons.

Suggested mechanism

The antioxidant activity of the promising active compound especially C2 which was separated from *Eucalyptus globulus* against ABTS radical may be due to one of the following reasons:

First, it may be caused by the resonance phenomena of double bonds and lone pair atoms (N, O) in the chemical structure of the active compound. This structure may lead to radical formation at more than one site e.g.: benzene ring, and formation of new covalent bond with ABTS radical and non-radical products [25].

Second, the presence of different electro-negative groups in the structure may lead to a less stability of different atoms (e.g.: methylene group) because these groups can attract electrons from methylene group and convert it to a radical or a carbonium ion. So, the activity of the active compound (C2) may be due to the reaction between methylene group radicals or hydrogen proton with ABTS radical [25].

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