Int J Pharm Bio Sci 2015 April; 6(2): (B) 883 - 892

Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ANTIOXIDANT, ANTIPROLIFERATED ACTIVITIES AND GC/MS ANALYSIS OF *EUCALYPTUS CAMALDULENSIS* ESSENTIAL OIL

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ABSTRACT

The present investigation is design to determine the chemical composition of the essential oil of *Eucalyptus camaldulensis* grown in Egypt and to study its antioxidant and cytotoxic effects on different cancer and normal cell lines. The analysis of *E. camaldulensis* oil using GC/MS resulted in the identification of 43 compounds. The current results indicated that the essential oil has weak radical scavenging activity comparing with ascorbic acid and Butylated hydroxyanisole (BHA), while it has high potent ferrous ions chelating and total antioxidant activities comparing to ascorbic acid and BHT. Regarding cytotoxic effect, the essential oil has high potency cytotoxic effect on colon, prostate and breast cancer cell lines as well as moderate potency against liver and lung cell lines with IC_{50} 19.8, 31.5, 34.9, 51.7 and 64.0µg/ml respectively. In the same pattern, the oil has high cytotoxic effect on normal epithelial retina cell line and moderate effect on normal skin fibroblast cell with IC_{50} 41.3 and 60.6µg/ml respectively. This inhibition may be relevant to antioxidant and /or apoptotic effects. Therefore, the essential oil from *E. camaldulensis* is active candidates which could be used as antioxidant and antiproliferated agents against colon, breast and prostate cancer.

KEYWORDS: ***Eucalyptus camaldulensis*; essential oils; Fe2+ chelating; GC/MS; antioxidant; antiproliferated activity.**

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INTRODUCTION

Essential oils are concentrated natural products with strong smells that are produced as secondary metabolites by aromatic plants. They are extracted from various aromatic plants that are generally found in temperate or warm countries, where they often represent an important part of the traditional pharmacopoeia [1]. Essential oils are present as variable mixtures of primarily terpenoids, especially monoterpenes (C10) and sesquiterpenes (C15), although diterpenes (C20) may also be present [1]. These oils have been used for centuries in medicine, perfumery, cosmetic, and have been added to foods as part of spices or herbs and have been shown to possess antibacterial, antifungal, antiviral and insecticidal properties [2,3]. Antioxidants are molecules that are capable of neutralizing the harmful effects of the Radical oxygen species (ROS) through the endogenous enzymatic defense system such as the superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in human system. Interestingly, overproduction of ROS arising from either the mitochondrial electron transport chain or excessive stimulation of NAD (P) H or from exposure to environmental pollutants such as cigarette, smoke, UV-rays, radiation and toxic chemicals results in a weakened body defense system [4]. Hence, this creates the need to provide the body with a constant supply of plant chemicals (antioxidants) through dietary supplementation [5]. In recent years, antioxidants derived from natural sources mainly plants have been intensively used to prevent oxidative damage because of its advantages over synthetic ones; as they are easily obtained, economical and have slight or negligible side effects [6]. Thus, antioxidants presented in herbs and spices may offer resistance against oxidative stress by scavenging free radicals [7]. In recent years the screening of medicinal plants with potential antioxidant properties has received attention due to increasing concern for safe and non-toxic alternative antioxidants. Recent studies on medicinal plants and herbal constituents have shows their beneficial therapeutic potentials [8]. Eucalyptus is a large genus of family Myrtaceae comprised of about 900 species and subspecies and are well-known for their essential oils [9,10]. In addition, Eucalyptus has been used in folk medicine as anti-inflammatory, analgesic and antipyretic remedies for the symptoms of respiratory infections, such as cold, flu and sinus congestion [11,12]. In addition, the essential oils of Eucalyptus species possesses important biological activities including diaphoretic, disinfectant, antimalarial, antiseptic, analgesic, antipyretic, antiinflammatory and antibacterial properties since ancient times [9,13,14]. Therefore, the aim of the present study is to evaluate the antioxidant and proliferative activities of *E. camaldulensis* essential oil. The chemical composition of *E. camaldulensis* essential oil was also carried out.

MATERIALS AND METHODS

(i) Plant materials

The leaves of *E. camaldulensis* were collected in March 2014 from one farm in Qalyubia Governorate, Egypt. The plant was botanically identified by the staff at the herbarium of the botanical garden of the Orman, Giza, Egypt. Voucher specimen was deposited at the herbarium of Orman garden, Cairo, Egypt (ASU-ECM2007).

(ii) Extraction of essential oils [15]

Fresh adult leaves (200 g) were washed and submitted to hydrodistillation for 5 h using a Clevenger-type apparatus. Two replicate extractions were done. The essential oil was dried over anhydrous sodium sulfate and was stored at 4^{o}C until further analysis.

(iii) Gas Chromatography/Mass Spectrometry (GC/MS) analysis of the essential oil

The *E. camaldulensis* essential oil analysis was performed on a Thermo Scientific capillary gas chromatography (model Trace GC ULTRA) directly coupled to ISQ Single Quadruple MS and equipped with TG-5MS non polar 5% Phenyl Methylpolysiloxane capillary column (30 m x 0.25 mm ID x 0.25 µm). The operating condition of GC oven temperature was maintained as: initial

temperature 40°C for 3 min, programmed rate 5°C/min up to final temperature 280°C with isotherm for 5 min. For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. One microliter of diluted essential oil (1:1, v/v, in diethyl ether) was injected automatically in the splitless mode. Detection was performed in the full scan mode from 40 to 500 m/z. The identification of the chemical components was carried out based on the retention time of each component (R_t) compared with those of the Wiley9 and NIST08 mass spectra libraries (NIST, 2010).

(iv) Antioxidant activities

Evaluation of free radical scavenging activity using the following methods

1. DPPH method [16]

The determination of the radical scavenging activity of *E. camaldulensis* essential oil was carried out using the DPPH (2, 2-diphenyl-1 picrylhydrazyl) assay with a slight modification. One milliliter of DPPH methanol solution (0.1 mM) was added to 3 ml of the essential oil at various concentrations (500, 1000, 1500 and 2000 µg/ml). Discoloration of the mixture was measured at 517 nm after 30 min incubation in dark at room temperature. BHT was used as positive control. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [A_C - A_S / A_C] \times 100$$

Where, A_C is the absorbance of the control reaction (containing all reagents except the sample) and A_S is the absorbance when the sample extract is added. The extract concentration providing 50% inhibition of radical scavenging activity (IC_{50}) was calculated and expressed as mg/ml.

2. Ferrous ions chelating activity [17]

The ferrous ion-chelating activity of essential oil was estimated. Three milliliters of the essential oil at various concentrations (500, 1000, 1500 and 2000 µg/ml), were added to 60 µl of $FeSO_4$ (2 mM). The reaction was started by adding 100 µl of ferrozine (5mM). The mixture was shaken vigorously and kept back to stand at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. EDTA was used as positive control. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated according to the following equation:

$$\text{Ferrous ion-chelating activity (\%)} = (1\text{-}A_1/A_0) \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in presence of samples.

3. Total antioxidant activity [18]

The total antioxidant activity of the essential oil was carried out. One ml of the essential oil at different concentrations (25, 50, 75 and 100 µg/ml) was mixed with 3 ml reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min, after cooling at room temperature; the absorbance was measured at 695 nm using spectrophotometer against blank (The reaction mixture without the sample). Gallic acid was used as positive control. The antioxidant activity was expressed as absorbance of the sample.

(v) Antiproliferated activity of essential oil [19-22]

The *in vitro* antitumor screening was performed adopting previously reported procedures. Cells were suspended in RPMI 1640 medium for HepG2, MCF7 and HCT116 and DMEM for A549, 1% antibiotic-antimycotic mixture (10,000 u/ml potassium penicillin, 10,000 mg/ml streptomycin sulfate and 25 mg/ml amphotericin B) and 1% L-glutamine at 37° C, fewer than 5% CO2 and 95% humidity. Cells were seeded at concentration of 10 x 103 cells/well in fresh complete growth medium in 96-well microtiter plates for 24 h. Media was aspirated, fresh medium (without serum) was added and cells were incubated

with different concentrations of sample to give a final concentration of (100, 50, 25, 12.5, 6.25, 3.125, 0.78 and 1.56 Ug/ml). 0.5% DMSO was used as negative control and 100 mg/ml of doxorubicin was used as positive control. MTT assay was used for assessment of cytotoxicity. After 48 h of incubation, medium was aspirated, 40 ml MTT salt (2.5 mg/ml) were added to each well and incubated for further 4 h. To stop the reaction and dissolving the formed crystals, 200 ml of 10% sodium dodecyl sulfate (SDS) in deionized water were added to each well and incubated overnight at 37 °C. The absorbance was then measured at 595 nm and a reference wave length of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The % cytotoxicity was calculated according to the formula: [1-(OD compound/OD negative control)] x100]. A probit analysis was carried for LC50 determination using SPSS 11 program.

RESULTS AND DISCUSSION

1. *Chemical composition of E. camaldulensis essential oil*

The chemical composition of *E. camaldulensis* essential oils were analyzed by GC-MS. The GC/MS analysis revealed the presence of 43 compounds eluted between 10 min and 47 min and consisting 99.94% of the oil (Table 1). The major constituents of the oil were Eucalyptol (20.81%) followed by O-Cymene (19.11%), α- Phellandrene (9.21%) and Crypton (9.4%). The presence of O-Cymene, M-Cymene and Crypton as the major compounds in the leaf oil of *E. camaldulensis* is in agreement with earlier reports of Batish et al [23]. However, it is in contrast with the recent study of Habila et al [24] who found 6-octenal (77.11%) is the major constituent in the leaf oil of *E. camaldulensis*. Recently, Mital and Ali [25] demonstrated that, α-pinene (38.6%), B-pinene (25.6%) and α-thujene (11.9%) [24] are the major components. In the present study, these three constituents are present in small amounts, where 6-octenal is absent. This is not surprising since the composition of the essential oil varies with season, location, climate, soil type, age of the leaves, fertility regime and the method used for drying the plant material and oil extraction [26]. In addition the present results reveal the presence of M-Cymene, Terpinenol and Spathulenol represented 6.16%, 4.57% and 3.80%, respectively. Moreover, other compounds ranged from 0.02% to 2.9% were also detected (Table 1).

2. *Antioxidant activity*

To assess the antioxidant capability of *Eucalyptus camaldulensis* essential oil, three different methods were used in this study because it is generally accepted that a mix of methods should be used for assessing *in vitro* antioxidant activities so, all aspects of antioxidant efficacy are covered [27]. The antioxidant activity of medicinal plant may be due to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts [28]. The methods chosen are the most commonly used for the determination of antioxidant activities of plant extracts and/or essential oils.

2.1. DPPH free radical scavenging activity

The results in Fig (1) indicated that the essential oil has weak DPPH radical scavenging activity, with an IC_{50} value of 32 mg/ml. The results of the radical scavenging activity of essential oil was lower than of synthetic antioxidants vitamin C and BHT, with IC_{50} values of 12± 3.5 µg/ml and 53 ±3.1 µg/ml, respectively. Also, essential oil DPPH scavenging activity exhibited dose-dependent relationship. The reduction ability of DPPH radicals formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [29].

Table 1
GC/MS chemical profile of E. camaldulensis essential oil.

No.	[a]R_t	Compounds name	Area (%)[b]
1	10.022	α- Thujene	1.21
2	10.340	α- Pinene	2.55
3	10.818	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	0.23
4	12.360	β- Pinene	2.10
5	12.978	β- myrcene	0.62
6	13.819	α- Phellandrene	9.21
7	14.264	(+) -4- Carene	0.46
8	14.800	O- Cymene	19.11
9	14.866	M- Cymene	6.16
10	15.191	Eucalyptol	20.81
11	16.284	α- Terpinen	0.83
12	16.910	Cis – Linaloolonide	0.19
13	17.574	α-Terpinolene	0.27
14	17.843	α- P-Dimethylstyrene	0.23
15	18.394	β-Linabol	0.44
16	18.670	Isovaleric acid	0.22
17	19.173	α-Thujone	0.22
18	19.565	2-Cyclohexene-1-ol, 1-methyl-4-(1-methylethyl)-, trans-	0.68
19	20.451	1.3-Isopropenyl-5- methyl cyclohexene	0.52
20	20.707	Cyclopentene, 4,4-dimethyl-	0.95
21	21.176	4-Isopropylcyclohexanone	0.20
22	21.300	2 (10)-Pinen-3-one	0.16
23	21.688	5,5-Dimethyl -1, 3 –hexadiene	0.33
24	21.885	Biisobutenyl	0.35
25	22.306	Terpinenol	4.57
26	22.710	Crypton	9.40
27	23.015	Terpenol	1.66
28	25.187	Cuminal	2.57
29	25.434	Carvotanacetone	0.36
30	25.513	Tetrahydropyridine	0.20
31	25.661	Piperitone	0.56
32	26.795	Phellandral	2.46
33	27.833	P-Thymol	0.77
34	29.491	Bezenemethanol, 3-hydroxy-	0.16
35	33-695	(+) –Aromadendrene	0.13
36	34.593	Albaromadendrene	0.26
37	35.793	Benzene, 1-(2-butenyl)-2,3-dimethyl-	0.02
38	38.843	Nerolidol	2.90
39	39.441	Spathulenol	3.80
40	39.548	Caryophyllene oxide	0.91
41	39.738	Globulol	0.54
42	40.665	3-Buten -2- one, 4-(6,6- dimethyl-1-cyclohexen-1-yl)-	0.43
43	47.322	2-Pyridylacetamide	0.21

[a]Retention time (as minutes).
[b]The percentage composition was computed from the gas chromatography peak areas.

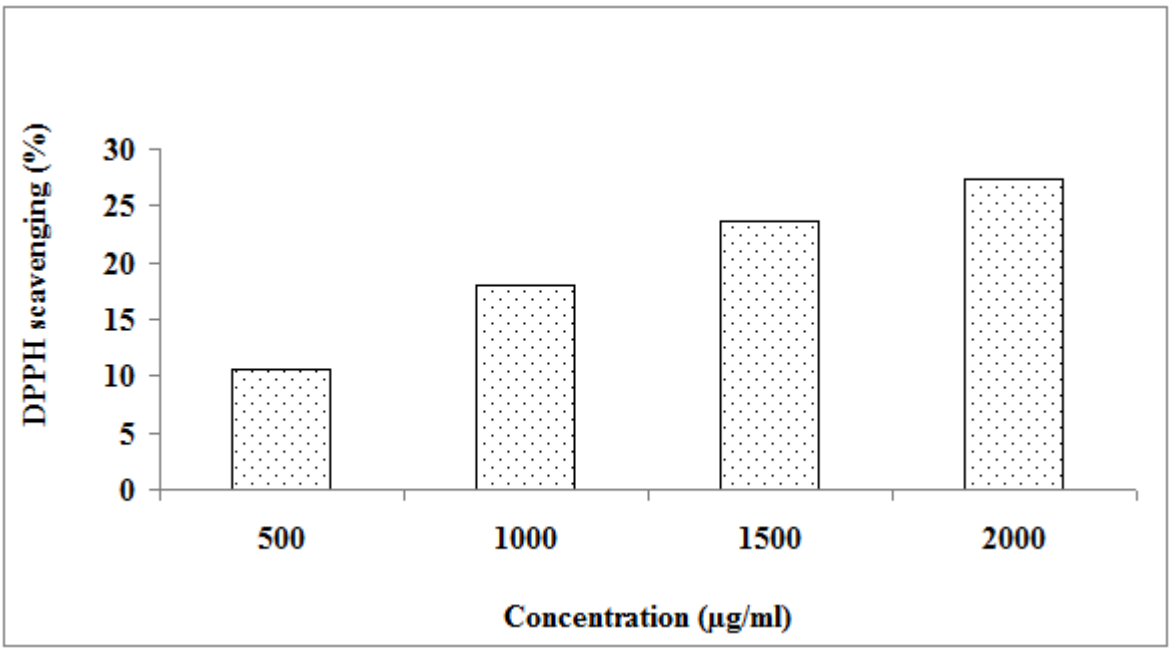

Figure 1
DPPH scavenging activity (%) of essential oil of E. camaldulensis.

2.2. Ferrous ions chelating activity (FICA)

In the present study, IC_{50} value for standard ascorbic acid ferrous ion chelating activity is observed at 1.5 mg/ ml concentration. However, essential oil FICA is detected at 1.23 ± 0.27 mg/ml. (Fig. 2). The presence of chelating agents in different plant extracts disrupts the complex (Ferrozine with Fe^{2+}) formation, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the coexisting chelator [30]. Concisely metal ion chelating activity of an antioxidant molecule can inactivate, catalyse and inhibit the harmful transition metal ions responsible for the generation of oxygen free radicals in living organisms [31,32].

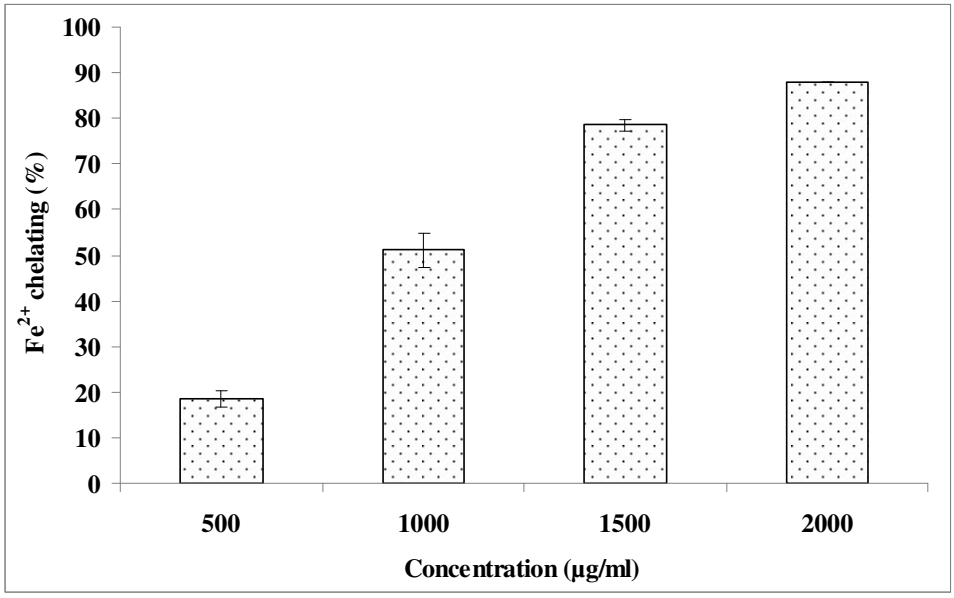

Figure 2
Ferrous ion chelating activity (%) of essential oil of E. camaldulensis.

2.3. Total antioxidant activity

Total antioxidant capacity was determined by phosphomolybdenum method assay which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the equivalents number of gallic acid. The total phenolic contents in the examined essential oil using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: y = 0.014x + 0.139, r2 = 0.970). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract and recorded 48 ug /ml GA/g (Fig.3), for essential oil.

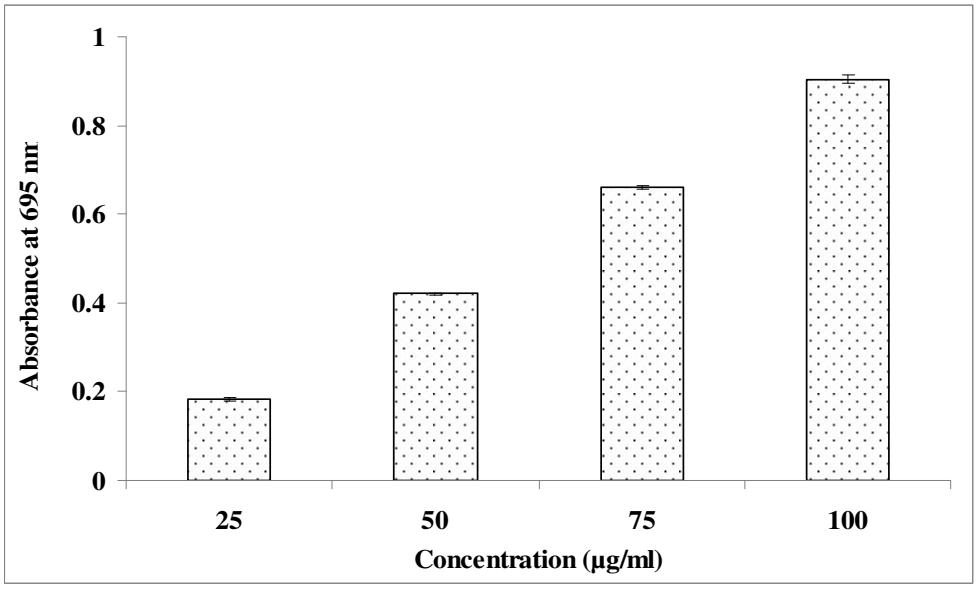

Figure 3
Total antioxidant activity of essential oil of E. camaldulensis.

3. *Antiproliferated activity of essential oil*

Essential oil of eucalyptus was subjected to *in vitro* antitumor screening against five human cancer cell lines namely: hepatocellular carcinoma HepG2, caucasian breast adenocarcinoma MCF7, colon carcinoma HCT116, prostate cell line (PC3) and lung carcinoma A549. Beside one immortalized normal cell line namely retina epithelial cells immortalized with hTER (RPE1) and normal skin fibroblast (BJ1) to evaluate their antitumor potency by using MTT method (Table 2). In regard to the antitumor selectivity among the used tumor cell lines, essential oil proved to be selective toward the colon carcinoma HCT116 cell line, prostate carcinoma cell line PC3 and caucasian breast adenocarcinoma MCF7 cell line with LC50 values of 19.8 ug/ml, 31.5 ug/ml and 34.9, respectively; while the cytotoxicity effect of essential oil decreased on the HEPG2 and A549 cell line with LC50 51.7 ug/ml and 64.0 ug/ml, respectively. While, the cytotoxicity activity of essential oil on BJ1and RPE1 normal cell lines the activity on BJ1 fibroblast cell line was less than on epithelium retina cell line with LC50 values 60.6 ug/ml and 41.3ug/ml. These results clearly indicated that, the essential oil has selectivity effect on colon cancer followed by hormonal dependant cancer cell lines MCF7and PC3. Despite the results on liver and lung cancer cell lines were matched to that obtained on normal cell line, the oil exhibited antitumor potency against colon, prostate and breast cell lines comparable to the standard drug doxorubicin.

Table 2
Antiproliferative activity of E. camaldulensis essential oil and Doxorubicin on different cell lines

Cell line	IC_{50} 0f Eucalyptus essential oil (µg/ml)	IC_{50} 0f doxorubicin
HCT116	19.8	37.6
PC3	31.5	23.8
MCF7	34.9	26.1
A549	64.0	28.3
HepG2	51.7	21.5
BJ1	60.6	74.2
RPE1	41.3	41.9

CONCLUSION

It could be concluded that, the essential oil of *E. camaldulensis* is a very good source of monoterpenes. The oil and its selected components exhibited moderate to strong antioxidant activity thereby implying its potential in providing protection against oxidative diseases and its use as a natural antioxidant food supplement and in confectionery industries. In addition, the essential oil had selectivity effect on colon cancer followed by hormonal dependant cancer cell lines MCF7, PC3 as well as it declared antitumor potency against colon, prostate and breast cell lines comparable to the standard drug doxorubicin.

Conflict of Interest: Conflict of Interest declared none.

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