



Full Length Article

Essential Oil Compositions and Cytotoxicity from Various Organs of *Eucalyptus camaldulensis*

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Abstract

In the present study essential oil compositions from leaves, stems and immature flowers of *Eucalyptus camaldulensis* Dehnh. from Malaysia and their cytotoxicity effects were investigated. The essential oils were obtained by hydrodistillation and the composition was determined by gas chromatography-mass spectrometry (GC-MS). The cytotoxicity of the essential oils was evaluated by MTT assay. The oil yields of leaves, stems and immature flowers of the plant represented 1.40, 0.57 and 0.46 %, respectively, based on dry weight. Fifteen major compounds were identified represented 98.8, 97.5 and 99.5% of the compounds in the leaves, stems and immature flowers, respectively. Monoterpenes hydrocarbons were predominant in the three oil samples. The flower oil was the highest in oxygenated monoterpenes content (19.6%). The most abundant compounds in the three essential oils were γ -terpinene, as a principal oil component (57.4–72.5%) followed by *o*-cymene (14.6–26.3%) and terpinen-4-ol (6.6–16.2%). *E. camaldulensis* leaves essential oil demonstrated cytotoxic effects in three tested cancer cell lines; WEHI-3, HT-29 and HL-60. WEHI-3 was the most sensitive with IC₅₀ 16.1. The essential oil exhibited less cytotoxic effects in HT-29 and HL-60 cells (IC₅₀=50.5 and 42.1, respectively). Also, it exhibited a weak cytotoxic effect in RAW 264.7 cells. The main component of the oil, γ -terpinene also, showed a very weak cytotoxic effect in the tested cell lines without reaching IC₅₀ values within the studied concentration ranges. These findings add significant information to the pharmacological effects of *E. camaldulensis* essential oil and to its cytotoxic properties, thus justifying and reinforcing the pharmaceutical use of this plant oil. © 2015 Friends Science Publishers

Keywords: *Eucalyptus camaldulensis*; Malaysia; Plant organs; Essential oil; GC-MS; Cytotoxicity

Introduction

The genus *Eucalyptus* belongs to Myrtaceae family, the member of which are shrubs and forest trees native to Australia and Tasmania. The genus was successfully introduced in many other regions of the world for various purposes. *Eucalyptus* species for the time being occur in most tropical and subtropical regions became an important and world widely planted genus containing more than 700 species (Ozel *et al.*, 2008; Dibax *et al.*, 2010). However, the genus members are not common occurrence in Peninsular Malaysia.

Eucalyptus spp. are a rich resource of essential oils of medicinal and commercial importance. The chemical composition of a specific plant species essential oil varies from species to species and subspecies, plant chemotype and genotype and specific environmental conditions (Pearson, 1993). Thus, the two main components detected in *E. camaldulensis* from Morocco and Jerusalem were spathulenol and *p*-cymene (Zrira and Benjilali, 1996;

Chalchat *et al.*, 2001), 1,8-cineole and pinene from Mozambique (Pagula *et al.*, 2000) and 1,8-cineole and limonene from Burundi (Dethier *et al.*, 1994). Some tropical *E. camaldulensis* leaf oil are rich in 1,8-cineole and they are potential commercial sources of medicinal-grade *Eucalyptus* oil (Doran and Brophy, 1990).

Eucalyptus spp. essential oils are widely used in medicine, pharmaceuticals, cosmetics and food industries. Many species of the *Eucalyptus* have been used widely in folk medicine for a variety of medicinal applications (Silva *et al.*, 2003; Marzoug *et al.*, 2011). Moreover, essential oil from *E. camaldulensis* has been reported to have a variety of beneficial efficacies and contains different bioactive ingredients capable to display antibacterial activity (Ghalem and Mohamed, 2008), antifungal activity (Falahati *et al.*, 2005), larvicidal activity (Cheng *et al.*, 2009), antioxidative and antiradical activities (Siramon and Ohtani, 2007). In addition there are many reports on the cytotoxic effects of essential oils belong to Myrtaceae plants as described by Ashour (2008) and Schnitzler *et al.* (2008).

Cancer diseases have been treated with a number of bioactive agents mostly being chemicals, but the naturally occurring and derived anticancer agents have increased recently. Since these plant-derived agents have shown lesser adverse effects than synthetic drugs (Kinghorn *et al.*, 2003; Newman and Cragg, 2007).

In the present work we have investigated the essential oil compositions obtained from *E. camaldulensis*. We have also investigated the cytotoxicity activity of the leaf essential oil in four different cell lines; WEHI-3, HT-29 and HL-60 and RAW 264.7. Nearly, all parts of the plant have a fragrance. Neither the effect of different parts of the plant nor the effect of the predominant environment have been reported in the literature. Moreover, some essential oils have been reported recently to have anticancer effects as reported by Ashour (2008). Therefore, the purposes of this study were to determine the contents and components of essential oil of *E. camaldulensis* different parts; leaves, stems and flowers grown in Malaysia to be compared with previous studies from other countries, to evaluate the cytotoxicity activity in different cell lines, and to contribute for future studies in the essential oil of the species.

Materials and Methods

Plant Material Collection

Fresh plant materials such as mature leaves, green stems and immature flowers of the tree *E. camaldulensis* were collected from mature tree in the University of Malaya Main Campus, Kuala Lumpur, Malaysia during November 2012, identified by the Taxonomist Assoc. Prof. Dr. Noorma Wati Haron from Institute of Biological Sciences. Voucher specimen was deposited in The University of Malaya Herbarium under the code KLU 47786.

Isolation of Essential Oils

Crushed clean fresh plant materials were separately subjected to 4 h hydrodistillation using a clevenger-type apparatus. The obtained essential oils were pale yellow. The experiment was repeated thrice. Oils floating on the water was collected in separate glass vials by discharging the water, dried by Sodium sulfate and saved in a freezer at -20°C until used. The yield percentage of the essential oil was calculated based on the dry weight (Table 1).

Analysis of Essential Oils

The analysis of essential oils was performed using GC equipped with FID and GC-MS provided with an HP- 5MS column of 30 m X 0.25 mm i.d. and 0.25 µm film thickness (Agilent J and W Scientific Inc, USA). The GC settings were done as described by Cheng *et al.* (2009) as follows: the initial temperature of the oven was set at 40°C for 2 min and increased to 140 °C at 3°C/min, then increased from 140 to 250°C at a rate of 10°C/min. Helium was the carrier

gas at 1 mL/min flow rate. The temperature of sample injection was maintained at 250°C. Diluted samples (1.0% in hexane) were injected (1 µL) separately, with 1:10 split ratio. Spectra were obtained over the mass range 40-450 amu. The electron ionization energy was 70 eV and 230°C ion source temperature. The retention index calculation was performed using a homologous n-alkane series of C₈-C₂₀ (Sigma-Aldrich, USA).

Identification of Essential Oil Components

The identification of the oil constituents was performed using their mass spectra, relative retention indices and by using authentic reference compounds. The data were compared to the NIST MS library, Wiley/NBS registry and reported mass spectra and retention indices (Adams, 2007, Hognadottir and Rouseff, 2003, Pino *et al.*, 2005; Qiao *et al.*, 2008).

Cytotoxicity Assay

Cell cultures: Murine leukemic cell line; WEHI-3, human colon carcinoma cells; HT-29, Human promyelocytic leukemia cells; HL-60 and murine macrophage cell Lines; RAW 264.7 were kindly provided by The Department of Pharmacy, University of Malaya, Kuala Lumpur.

Evaluation of cytotoxic activity: The cytotoxicity profiles of the oil were determined using MTT assay. Cell cultures were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with fetal calf serum (10%) and maintained in a humidified atmosphere in CO₂ (5%) at 37°C. Ninety six well plates (5×10³ Cells/mL) were used for plating of the cells. Plant essential oil was dissolved in Dimethyl sulfoxide (DMSO). The samples; essential oil and γ -terpinene (from Sigma-Aldrich, USA) were prepared in serial dilutions using Dimethyl sulfoxide. Dimethyl sulfoxide was applied as control treatment. Experiments were done in triplicates and each plate contained control (untreated cells). After 24 h of incubation, MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) (5 mg/mL) was added, and 96 plates were further incubated for 4 h. After that the medium was discarded and 100 µL DMSO was added to each well to dissolve formazan crystals. Absorbance (OD) was measured at a wavelength of 570 nm with ELISA microplate reader. The viability rate of the treated cells was expressed as percentage of viable cells compared to untreated cells (control) using the formula:

$$\text{Cell viability (\%)} = [\text{OD treated cells} / \text{OD negative control}] \times 100.$$

The half maximal inhibitory concentrations (IC₅₀) was calculated for each cell line.

Experimental Design and Statistical Analysis

Experiments were designed based on a complete randomized design (CRD). Data were subjected to statistical

analysis using Duncan's multiple range test ($P \leq 0.05$). The values are represented as the mean \pm SE. of three replicates for each experiment. Dose response curves and the half maximal inhibitory concentration (IC_{50}) were obtained using GraphPad Prism and SPSS version 20.

Results

Determination of Essential Oil Yield and Chemical Composition

The yields of isolated essential oils from adult fresh leaves, stems, and immature flowers of *E. camaldulensis* were 1.4, 0.57 and 0.46% (w/w), respectively (Table 1). The results demonstrated that plant leaves were the highest in the oil content whereas, there was no significant difference in oil yields percentages in stems and immature flowers.

The results of GC-MS analysing of the different investigated plant organs essential oils are presented in Table 2. A total of 15 compounds were identified in the three oil samples, representing 98.8, 97.6 and 99.5% in the essential oils of the leaves, stems and immature flowers, respectively. Comparing the three oil samples, plant leaf was shown to be the highest in monoterpenes (90.2%), followed by stems (88.8%) and the immature flowers (78.3%). The main producers of oxygenated monoterpenes were shown to be plant immature flowers (19.6%). Generally, the results showed that this species oil was low in sesquiterpene contents (0.3–1.6%). The monoterpene hydrocarbon γ -terpinene was shown to be the most abundant component in the three plant parts. Representing 72.5% of the leaf oil, 61.0% of the stem oil and 57.4% of the immature flower oil followed by *o*-cymene showing the of 14.6, 26.3 and 15.7% oil content, respectively (Table 2). Among oxygenated monoterpenes, terpinen-4-ol was detected in higher percentages in the oils, particularly, high percentage of this compound was shown in the essential oil of the flowers (16.2%).

Table 2 shows the main identified *E. camaldulensis* essential oil compounds composed from mono- and sesquiterpenes and their composition percentages, their retention times (RT) and their retention indices (RI). The most common compounds in the *E. camaldulensis* essential oil three parts were α -pinene, *o*-cymene, γ -terpinene, terpinolene, 1,8-cineole, terpinen-4-ol and globulol (Fig. 1). Their chemical structures are shown in Fig. 2 (Source: NIST MS Library).

In Vitro Cytotoxic Assay

Experiments were conducted to assess the toxicity of *E. camaldulensis* leaf essential oil using four cell lines. These cell lines were the murine leukemic cell line WEHI-3, human colon carcinoma cells; HT-29, Human promyelocytic leukemia cells; HL-60 and murine macrophage cell Lines; RAW 264.7. The cell toxicity was assessed using MTT assay by incubating the cells with

increasing concentrations of the samples ranging from 0.0 μ g/mL to 100 μ g/mL followed by 24 h incubation period. The results revealed a promising cytotoxic behavior in the cancer cell cultures. As seen in Fig. 3, the essential oil caused significant cytotoxic effect against WEHI-3, HL-60 and HT-29 cell lines, inhibiting the growth of the cells in a dose-dependent manner. The maximum cytotoxic effect of the oil (IC_{50}) was obtained against the leukemia cell line WEHI-3 ($IC_{50} = 16.1$) and less cytotoxic effects of the plant essential oil were recorded in both cell lines HT-29 and HL-60 ($IC_{50} = 50.5$ and 42.1, respectively) (Table 3; Fig. 3). In both leukemia cell lines, WEHI-3 and HL-60 the viability decreased rapidly with concentrations (until essential oil concentration 25 μ g/mL) after which the decrement remained stable or very slow (Fig. 3a, c). In colon cancer cell lines HT-29, the viability decreased sharply in low concentrations until 3 μ g/mL and remained slow decrement until 25 μ g/mL. After which it gradually decreased with the increasing of concentrations (Fig. 3b). At the same time, *E. camaldulensis* oil exhibited less cytotoxic effect in Macrophage cells; RAW 264.7, in a dose-depend manner without affording IC_{50} within the studied concentration ranges (Fig. 3d). The main compound in the oil, γ -terpinene showed low cytotoxic effects in the tested cells; WEHI-3 and Macrophage cells, without reaching IC_{50} values and the treated cells showed high survival rates even at higher doses such as 100 μ g/mL (Fig. 4). Macrophage cells seemed to be more sensitive to γ -terpinene treatment than WEHI-3 cells showing survivability rate remained lower than 80% at high concentrations (Fig. 4).

Table 3 shows the half maximal inhibitory concentration (IC_{50}) evaluating the effectiveness of the essential oil in inhibiting the growth of the tested cell lines. Fig. 3 and Fig. 4 illustrate the effectiveness of the leaf essential oil and its main component γ -terpinene on the viability of the cell lines cultured in DMEM for 24 h and measured spectrometrically by ELISA reader. Data are mean \pm S.D. from a representative experiment done in triplicate.

Discussion

Essential oils contain a large number of biochemical constituents include terpenes, alcohols, esters, coumarins, ketones, aldehydes, phenols etc. A wide range of pharmaceutical products contain essential oils or its constituents are available in the market such as soaps, antiseptics, deodorants and flavors. This encourages more researches in essential oil characterization and its potential applications. The results presented in Table 1, showed that out of the studied plant parts, leaves were the best for essential oil production showing 1.4% oil yield. In the literature; essential oil yields from *E. camaldulensis* leaves grown in Taiwan and Pakistan (Cheng *et al.*, 2009; Ashraf *et al.*, 2010), were found to be lower than those obtained in this research (0.57% and 0.98%, respectively).

Table 1: Essential oil yields of leaves, stems and immature flowers of *E. Camaldulensis*

Plant part	Colour	Oil yield (% w/w)
Leaf	Light yellow	1.40 ± 0.10a
Stem	Light yellow	0.57 ± 0.04b
Immature flower	Light yellow	0.46 ± 0.03b

The yield of essential oils was calculated on the basis of dry weight (units: % w/w). Oil yields were the means of three replicates. (Values with different superscripts are significantly different at P < 0.05)

Table 2: Major compounds in the Essential oils of different organs of *E. Camaldulensis*

Chemical compound	RT	RI	Relative percentage (%)			Method of identification
			Leaves	Stems	Immature flowers	
α -Pinene	10.2	931	0.7	0.4	0.7	MS, RI, ST
α -Phellandrene	13.4	1003	0.2	0.0	0.6	MS, RI
α -Terpinene	13.9	1015	0.4	0.1	1.0	MS, RI
<i>o</i> -Cymene	14.4	1024	14.6	26.3	15.7	MS, RI
Limonene	14.5	1027	0.8	0.5	0.9	MS, RI
1,8-Cineole	14.6	1029	0.9	0.5	2.0	MS, RI, ST
γ -Terpinene	16.2	1062	72.5	61.0	57.4	MS, RI, ST
Terpinolene	17.4	1087	1.0	0.5	2.0	MS, RI
Linalool	18.1	1101	0.3	0.1	0.4	MS, RI
Terpinen-4-ol	21.7	1178	6.7	6.6	16.2	MS, RI
α -Terpineol	22.3	1191	0.2	0.1	0.5	MS, RI
Piperitone	25.2	1253	0.2	0.1	0.5	MS, RI
Globulol	39.1	1587	0.1	1.3	1.1	MS, RI
γ -Eudesmol	40.4	1634	0.1	0.0	0.5	MS, RI
δ -Cadinol	40.7	1645	0.1	0.1	0.0	MS, RI
Total Identified Compounds						
Total monoterpene hydrocarbons			90.2	88.8	78.3	
Total oxygenated monoterpenes			8.3	7.4	19.6	
Total oxygenated sesquiterpenes			0.3	1.4	1.6	

RT: Retention Time, RI: Retention Index, MS: Mass Spectra, ST: Standard compound

Table 3: Half maximal inhibitory concentration (IC₅₀) evaluation of the essential oil and γ -terpinene in the cell lines using MTT assay

Cell line	IC ₅₀	
	Essential oil	γ -Terpinene
WEHI-3	16.1	-
HT-29	50.5	NT
HL-60	42.1	NT
RAW 264.7	-	-

-: No IC₅₀ reached within the tested concentrations (0.0 -100.0 μ g/mL)
NT: Did not tested

On the other hand, Ozel *et al.* (2008) reported 1.18% plant leaf essential oil content obtained from plant grown in Turkey. Also, Siramon *et al.* (2013) reported essential oil content in ranging from 1.07 to 2.23% in the same plant grown in Thailand. It can be argued that the variability of the yield of entire essential oil can be assigned to many factors including soil, age of the plants, climate and time of harvesting as reported previously (Doran and Bell, 1994; Boira and Blanquer, 1998).

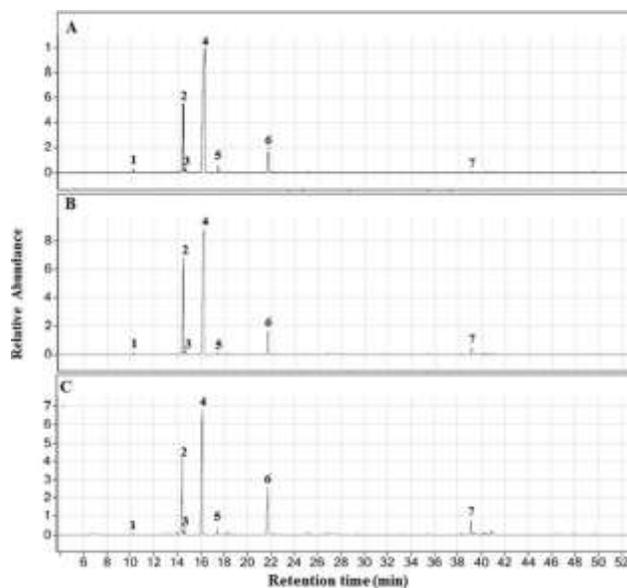


Fig. 1: GC-MS chromatograms of essential oils separated from leaves, stems and immature flowers of *E. camaldulensis*

A, B and C. *E. camaldulensis* represent essential oil chromatograms of leaves, stems and immature flowers, respectively. 1- 7 represented the main identified compounds. 1. α -pinene, 2. *o*-cymene, 3. 1,8-cineole, 4. γ -terpinene, 5. terpinolene, 6. terpinen-4-ol and 7. globulol. The chromatograms were fulfilled by Agilent GC-MS equipped with an HP-5MS column

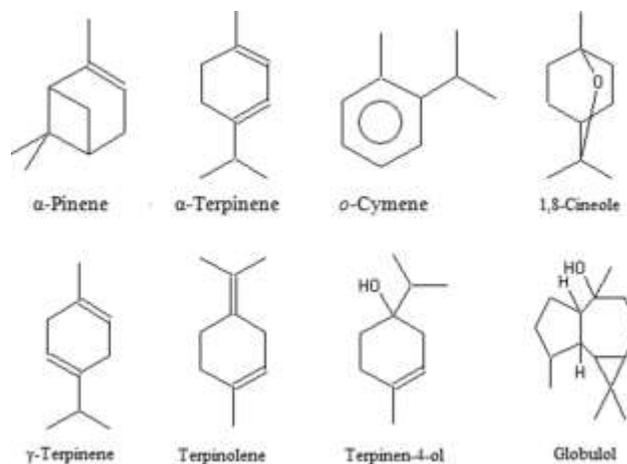


Fig. 2: Structures of the most abundant compounds identified in *E. camaldulensis* essential oils

As indicated in Table 2, analysis of the essential oils using GC-MS indicated that highest monoterpene hydrocarbon content was shown in the leaves (90.2%). On the other hand, the main producer of oxygenated monoterpenes was the immature flowers (19.6%). The results showed that the species with low sesquiterpene contents (0.3–1.6%) using the same protocol previously reported in the species by many authors (Su *et al.*, 2006;

Cheng *et al.*, 2009). Although, the chemical composition of the essential oil of *E. camaldulensis* especially, from leaves has been reported before (Ozel *et al.*, 2008; Siramon *et al.*, 2009; Cheng *et al.*, 2009; Ashraf *et al.*, 2010), The present study showed differences from those who reported 1,8-cineole as a main component in *E. camaldulensis* essential oil (Shieh, 1996; Tsiri *et al.*, 2003; Su *et al.*, 2006). Beside the environmental factors, climatic effect on the plant, geographical location, age of the plant part, nature of the soil, the status of the used plant material (dried or fresh) and time of collection etc. (Marzoug *et al.*, 2011), we assume that such variations might be attributed to the differences in the genotype and the chemotype of the plant species as reported previously by Brophy and Boland (1990) and Zouari *et al.* (2012), since no report is available on the composition of *E. camaldulensis* essential oils grown in Malaysia. The present study

indicated that the percentage of the main chemical volatiles varied among the three organs of the plant. As it is shown plant leaves and stems were characterized by higher percentages of monoterpenes respectively, while the plant flowers were characterized by higher percentages of oxygenated monoterpene (Marzoug *et al.*, 2011; Azam *et al.*, 2013).

A widespread use of plant essential oils in pharmacy, food and industry necessitates research on their cytotoxicity. Myrtaceous essential oils are rich in active phytochemicals such as 1,8-cineole, α -pinene and eugenol, which display various biological activities (Reynertson *et al.*, 2008; Harkenthal *et al.*, 1999). In the present study the maximum cytotoxic concentration of the essential oil was obtained against the leukemic cell line WEHI-3 showing IC₅₀ equal to 16.1 μ g/mL. In literature many plant essential oils have been reported for their cytotoxic effects on cancer cell lines.

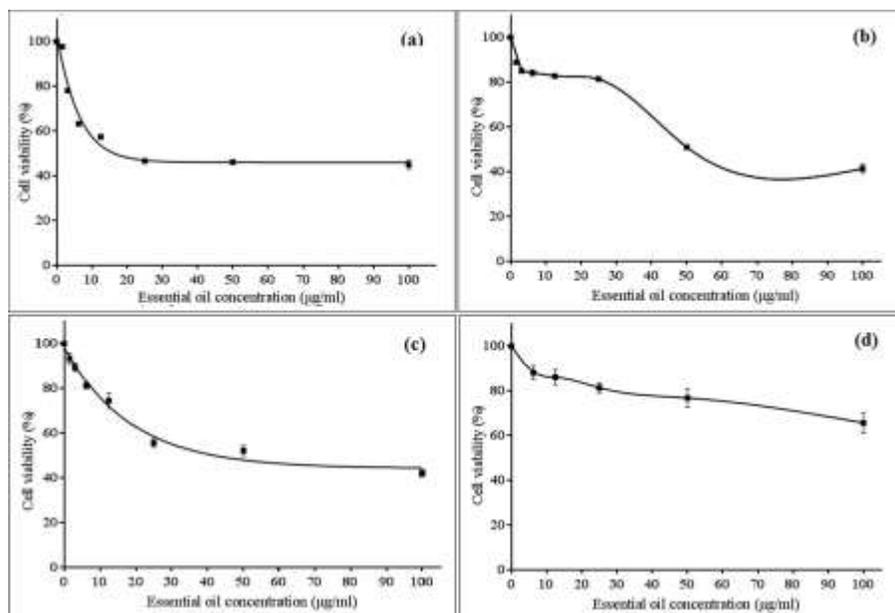


Fig. 3: Cytotoxic effects of *E. camaldulensis* essential oil in the cell lines. The cytotoxicity effect was screened using MTT assay. The values are mean \pm SE of independent triplicate treatments. (a): WEHI-3 cell line (b): HT-29 cell line. (c): HL-60 cell line. (d): RAW 264.7 cell line

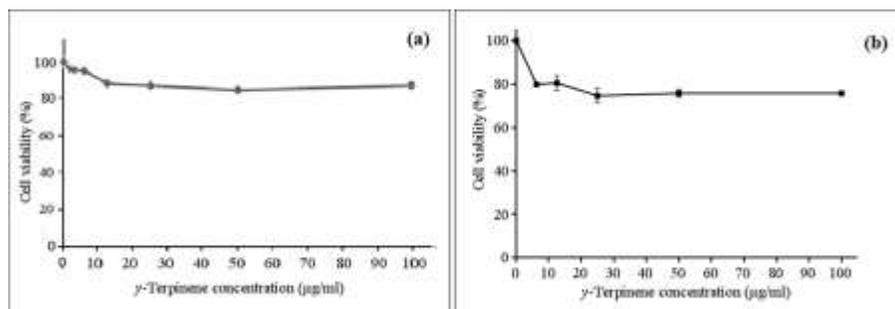


Fig. 4: Cytotoxic effects of γ -terpinene in the cell lines. The cytotoxicity effect was screened using MTT assay. The values are mean \pm SE of independent triplicate treatments. (a): WEHI-3 cell line. (b): RAW 264.7 cell line

For instance, essential oil obtained from *Hibiscus cannabinus* seeds was reported to induce apoptosis effect in WEHI-3 cell line by Foo *et al.* (2012). Another *in vitro* study showed that the active compound in essential oil, α -phellandrene reduced cell viability and induced chromatin condensation in WEHI-3 cells as reported by Lin (2012). However, in this study, α -phellandrene was found to be in a low concentrations in leaves of *E. camaldulensis* essential oil (0.02%) (Table 2). According to the previous reports by Greay *et al.* (2010) and Doll-Boscardin *et al.* (2012), terpinen-4-ol exhibited a cytotoxic efficiency in Jurkat cell line (IC₅₀ = 50.2 μ g/mL), while α -pinene and γ -terpinene exhibited very low inhibitory effects against the cell line (IC₅₀ 192.4 and 136.6 μ g/mL, respectively). Our results showed that *E. camaldulensis* essential oil efficiently inhibited the growth of tumor cells with a promising effect against WEHI-3 cells, while it exhibited low cytotoxic effect on the non-cancerous cells, Macrophage cells; RAW 264.7 without giving an IC₅₀ within the concentration range 0 – 100 μ g/mL (Fig. 3). It can be suggested that the cytotoxic effect of this essential oil might be attributed to the presence of terpinen-4-ol (6.7%) and/or the presence of other oil constituents. Nevertheless, further research is substantial to isolate and identify the bioactive compounds found in *E. camaldulensis* essential oils.

Conclusion

The essential oils of *E. camaldulensis* leaves, stems and immature flowers have shown variations mainly in the relative percentages of the major components (i.e. γ -terpinene, *o*-cymene and terpinen-4-ol). The leaves were the main producers of monoterpene hydrocarbons (e.g. γ -terpinene, 72.5%), whilst immature flowers had higher oxygenated monoterpenes (e.g. terpinen-4-ol, 16.2%). Plant essential oil shown to have promising anticancer effects and further could be a potential source of pharmaceuticals.

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