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Chemical composition, antioxidant and anticancer activities of the essential oil from *Eucalyptus citriodora* (Hook.) leaves

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ABSTRACT

The essential oil from the fresh leaves of Eucalyptus citriodora Hook. (Myrtaceae) was isolated via hydro-distillation technique and was analyzed by gas chromatography-mass spectrometry (GC-MS), yield (1.0% v/w). Thirteen compounds were identified in the essential oil representing 88.76% of the total oil composition. Citronellol (33.52%), pulegol (25.20%) and citronellyl acetate (14.70%) were the major components. The antioxidant activity was carried out quantitatively via 1,1'-diphenyl-2-picraylhydrazyl free radical and qualitatively via dot-blot and DPPH staining methods. The anticancer activity was carried out via MTT assay using three human tumor cell lines namely; hepatocellular carcinoma (liver) HePG-2, mammary gland (breast) MCF-7 and colorectal carcinoma (colon) HCT-116. The antioxidant results revealed that Eucalyptus citriodora oil showed antioxidant activity against DPPH radical (SC_{50}= 32.58 µg/ml) in comparison with ascorbic acid as standard (SC_{50}= 7.0 µg/ml), also qualitatively acts as a potent radical scavenger via showing a wide white zone on the purple background in comparison with ascorbic acid and quercetin. Furthermore, the essential oil exhibited high anticancer activity against HCT-116 (IC_{50}= 4.75 µg/ml) followed by MCF-7 (IC_{50}= 8.8 µg/ml) and HepG-2 (IC_{50}= 11.8 µg/ml). In conclusion, Eucalyptus citriodora oil may be a good source of antioxidant and anticancer agents. Citronellol and pulegol as a major components play vital role in antioxidant and anticancer activities of the essential oil.

Keywords: *Eucalyptus citriodora,* essential oil, GC-MS, antioxidant, anticancer.

INTRODUCTION

Reactive oxygen species (ROS) are highly unstable compounds containing an odd electron has the ability to attack cells and tissues in the human body followed by destructive effects leading to initiation of cancer **[1-3]**. Moreover, cancer defined as a rapid abnormal uncoordinated proliferation of aberrant cells in tissue or organ of the human body which may mass together to form tumor. Otherwise, cancer is considered as one of the most fearsome causes of morbidity and mortality in all over the world after heart disease, and must be fight via surgery or chemotherapy **[4, 5]**. Essential oils (EOs) are the volatile constituents that occur in plants and responsible for their characteristic odor. The chemical profile of the volatile oils is so complicated because in most cases they contain a complex mixture of ingredients like; monoterpenes, sesquiterpenes and diterpenes as well as their oxygenated derivatives. Furth more, they possess a wide spectrum of biological and pharmalogical activities such as; antioxidant, anticancer, antimicrobial and insecticidal **[6, 7]**. The genus *Eucalyptus*, a native of Australia and commonly known as gum tree. It is composed of a variety of volatile monoterpenes such as; cineole, citronellol, citronellal, limonene, linalool, and α-terpinene **[6, 8]**. *Eucalyptus citriodora* (Myrtaceae); commonly known as "Lemon-Scented Eucalyptus", is widely distributed in certain countries around the world like; Taiwan and Australia. The twigs and leaves of this tree have been used to produce an essential oil, a source of citronellal, which is used in soap making as a soap perfume, soap flake, detergent, and spray **[9, 10]**. Previous studies on the essential oil of *Eucalyptus citriodora* revealed that it possess vital biological activities such as; antituberculosis **[11]**, antiviral **[12]**, anti-inflammatory and analgesic **[13, 14]**, antifungal **[15, 16]**, hypoglycemic **[17]**, phytotoxicity **[18, 19]**, insecticidal **[20]**, antimicrobial **[21- 26]**,

acricidal **[27]** and larvicidal **[28, 29]**. Moreover, prior studies revealed that the essential oil isolated from *Eucalyptus citriodora* leaves, in different regions contains major constituents such as; citronellal, citonellol, geranyl acetate, limonene and terpene-4-ol **[30-32]**. The aim of the present study is to isolate and identify the essential oil of the *Eucalyptus citriodora* leaves as well as to evaluate its antioxidant and anti-cancer activities.

MATERIALS AND METHODS

1. Plant Materials

Fresh leaves of *Eucalyptus citriodora* Hook. (Myrtaceae) were collected from Zoo Garden, Giza, Egypt in May 2015. The identity of the plant was established by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy-Faculty of Science- Cairo University- Giza- Egypt, also by Eng. Tereez Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman Botanical Garden, Giza, Egypt. Voucher specimen (given number EC_1 for the plant) was kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI).

2. Isolation of the Essential Oil

Fresh leaves of *E. citriodora* Hook. (850 g) were subjected to hydro-distillation using Clavenger-type glass apparatus for 3 hours, after the mixture had reached boiling point (90°C). The obtained light yellow oil was separated, and the yield was determined. It was stored in a freezer in air-tight container at -20°C until analyzed by GC-MS **[33]**.

3. GC/MS Analysis

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS and TGSMS Fused Silica Capillary Column (30m, 0.251 mm, 0.1 mm Film thickness), National Research Center, Giza, Egypt. For GC/MS detection, an electron ionization system with ionization energy for 70 ev was used as the carrier gas at a constant flow rate of 1ml/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min) to 280°C was a final temperature at an increasing rate of 5°C /min (hold 5 min). The identified components were investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY Library data of the GC/MS system **[34]**.

4. Antioxidant assays

4.1. Rapid screening of antioxidant by dot-blot and DPPH staining

An aliquot of diluted sample of the *E. citriodora* essential oil (5 µl) was carefully loaded onto a 20 cm×20 cm TLC layer (silica gel 60 F254; Merck) against ascorbic acid in order of decreasing concentration (2, 1, 0.5, 0.25 and 0.125 mg/ml), along the row and allowed to dry (3 min). The staining of the silica plate was based on the reported procedures **[35]**. The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH$^\cdot$ solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair-dryer blowing cold air. Stained silica layer revealed a purple background with white spots at the location where radical-scavenger capacity presented. The intensity of the white color depends upon the amount and nature of radical scavenger present in the tested sample **[36, 37]**.

4.2. DPPH free radical-scavenging activity

The DPPH radical scavenging activity of essential oil was determined using the method proposed by Hung et al. (2005) with simple modification. The essential oil at concentrations of 10 to 100 µg/ml was mixed with an equal volume of 0.2 mM methanol solution of DPPH$^\cdot$. The disappearance of the DPPH after 20 min of incubation at room temperature was determined via spectrophotometry at 517 nm in a spectrophotometer zeroed with methanol. The absorbance of the DPPH radical without antioxidant served as the control and was measured daily. The amount of sample necessary to decrease the absorbance of DPPH by 50% (SC_{50}) was calculated graphically and the percentage inhibition was calculated according to the equation:

Inhibition % = (Ab – As/Ab) × 100

Where, Ab is the absorbance of the control reaction and As is the absorbance of the tested oil sample. The sample concentration providing 50% inhibition (SC_{50}) was calculated by plotting inhibition percentages against concentration of the sample **[38]**.

5. Antitumor activity assay

The tested human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were

subcultured two to three times a week. For antitumor assays, the tumor cell lines were suspended in medium at concentration $5x10^4$ cell/well in Corning® 96-well tissue culture plates, then incubated for 24 hr. The tested compounds were then added into 96-well plates (six replicates) to achieve eight concentrations for each compound. Six vehicle controls with media or 0.5% DMSO were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO_2 for 4 hours. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) **[39, 40]**.

6. Identification of the essential oil components

Qualitative analysis of volatile compounds was carried out by identification of compounds from mass spectra with the aid of mass spectral data book. The spectrum of each analyzed volatile compound agreed with that present in the mass spectrum library. Identification of the components was performed by comparing their retention times and mass spectral fragmentations with those of the available database libraries [Wiley (Wiley Int.), USA and NIST (Nat. Inst. St. Technol., USA)] and Xcalibur library and/or published data **[34]**.

RESULTS AND DISCUSSION

1. Oil yield (%)

The hydro-distillation of fresh leaves part of *E. citriodora* afforded a light yellow color essential oil. The percentage of the volatile oil was 1.0% (v/w% relative to fresh weight of leaves).

2. Identified Compounds

The GC-MS analysis of the essential oil revealed the presence 13 volatile components **(Figure 1 and Table 1)**, from which 3 components were identified represent (73.42%) of the total oil composition, in addition monoterpene hydrocarbon represented the most abundant constituents of the oil, citronellol (33.52%), pulegol (25.20%) and citronellyl acetate (14.70%) **(Figure 2)**. Previous study by Yu-Chang *et al.*, 2006 confirmed the high content of monoterpenes in the leaves oil; citrionellal (49.5 %), citronellol (11.9%), *iso*-isopulegol (10.4%) and citronellyl acetate (5.2%) which agreed with our results to some extent **[41]**. Moreover, Vahirua *et al.*, 2007 reported that the French leaves oil was mainly constituted of citronellal (31.15%), citronellol (24.28%), isopulegol (19.24%) and citronellyl acetate (1.34%) **[42]**.

On the other hand our results were not in line with the results were obtained from other regions *i.e.,* leaves oil isolated from Bangladesh sources constituted mainly of citronellal (76.98%), neoisopulegol (7.28%) citronellol (5.94%) and isopulegol (4.13%) **[8]**. Also, leaves oil isolated from India sources revealed the presence of main constituents; α-pinene (38.6%), β-pinene (25.7%), sabinene (19.6%) and α-thujene (11.9%) **[10]**. Furthermore, Bedi *et al.*, 2009 reported that leaves oil isolated from Côte d'Ivoire was mainly constituted of citronellal (68.9%), citronellol (5.3%), isopulegol (9.0%) and linalool (4.0%) **[43]**. Abd El Mageed *et al.*, 2011 reported that the essential oil isolated from the air dried leaves of the *E. citriodora* growing in Egypt, was rich in 3-hexen-1-ol (31.26%), cis-geraniol (19.66%), citronellol acetate (13.65%), δ-hepten-1-ol,3,6-dimethyl (13.14%) and citronellal (9.36%), and the difference may be due to the fact that we used the fresh leaves in our study **[44]**. It was observed by some authors that the variations in the chemical composition of the oil (qualitative and/or quantitative), may be attributed to the difference in certain conditions like; geographical location, climatic conditions, used part (leaves or seeds), type of sample (fresh or dried), time of harvest and length of vegetation period **[45-47]**.

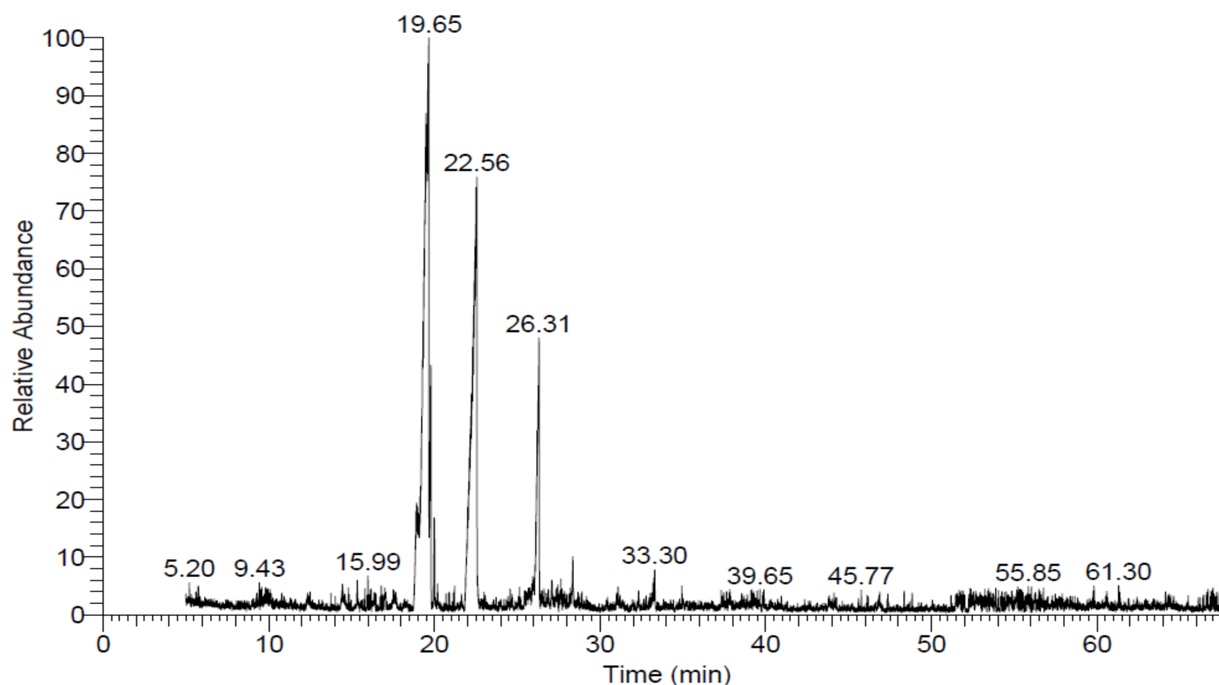
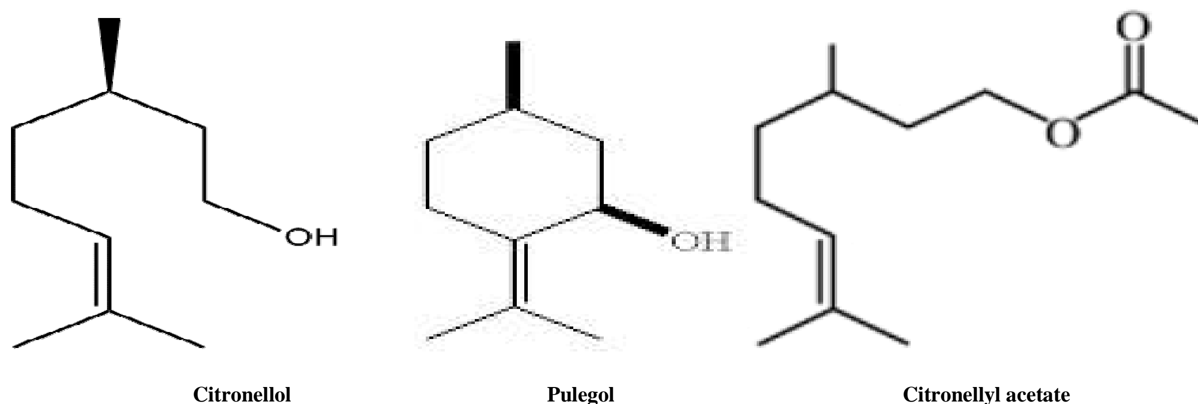


Fig. 1: Gas chromatography-mass spectrometry (GC-MS) chromatogram of the essential oil of *E. citriodora* fresh leaves

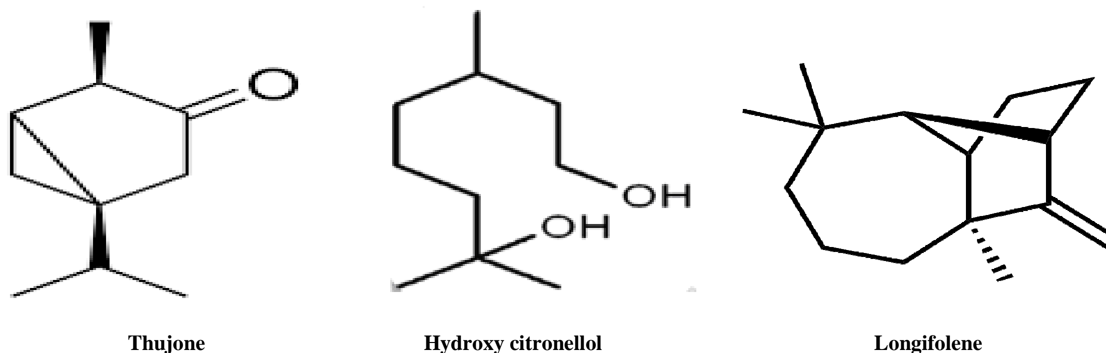
Table 1: Chemical compositions of the essential oil of *E. citriodora* fresh leaves

PeakNo.	Compound[a]	M.F.	Area %	M^+	Main Fragments	R_t
1	Thujone	$C_{10}H_{16}O$	2.26	152	(41,55,67,81,95,109,121,136, 152)	11.12
2	Isopulegol	$C_{10}H_{18}O$	1.26	154	(41,55,67,81,95,111,121,137, 154)	12.33
3	Citronellal	$C_{10}H_{18}O$	0.98	154	(41,55,69,81,95,111,121,136,154)	12.63
4	Menthone	$C_{10}H_{18}O$	0.52	154	(41,55,69,84,97,112,139,154)	12.68
5	Pulegol	$C_{10}H_{18}O$	25.20	154	(41,55, 69 ,83, 95, 111, 121, 154)	12.76
6	Cis-3-Pinanone	$C_{10}H_{16}O$	3.71	154	(41, 55, 69, 83, 91, 109, 121, 152)	12.92
7	Isomenthone	$C_{10}H_{18}O$	1.52	154	(41,55,69,84,97,112,121, 139,154)	13.08
8	Neomenthol	$C_{10}H_{20}O$	0.29	156	(41,55,67,71,81,95,123,138 156)	13.15
9	Citronellol	$C_{10}H_{20}O$	33.52	156	(41,55,69,81,95,123,138, 156)	15.83
10	*Trans*- Myrtanol	$C_{10}H_{18}O$	0.83	154	(41,55,67,81,93,123, 154)	16.82
11	Hydroxy citronellol	$C_{10}H_{22}O_2$	1.52	174	(43,55,59,69,83,123,157, 174)	21.58
12	Citronellyl acetate	$C_{12}H_{22}O_2$	14.70	198	(43, 55, 67, 81, 95, 123, 198)	26.30
13	Longifolene	$C_{15}H_{24}$	2.45	204	(41, 55, 79 91, 105, 161, 204)	28.31
Total			**88.76 %**			

[a] *Compounds identified via comparison its mass spectrum with NIST library, Adams, 2001 and literature.*
MF: Molecular formula; M^+: Molecular weight; R_t: Retention time



Citronellol **Pulegol** **Citronellyl acetate**



Thujone **Hydroxy citronellol** **Longifolene**

Fig. 2: Major compounds identified in the essential oil of *E. citriodora* fresh leaves

3. Antioxidant activity

The previous reported studies revealed that most of essential oils isolated from medicinal plants exhibited numerous health benefits and therapeutic applications among them antioxidant activity and such activity could be attributed to their hydrogen donating ability. Furthermore, due to the complex chemical profile of the essential oil the antioxidant activity should be carried out via more than one antioxidant assay **[48, 49]**. Therefore, in our current study antioxidant activity of *E. citriodora* essential oil was quantitatively evaluated via 1,1'-diphenyl-2-picraylhydrazyl free radical and qualitatively via dot-blot DPPH staining assays. In, DPPH assay the scavenging concentration (SC_{50}), which led to 50% inhibition for the essential oil is 32.58 µg/ml against ascorbic acid as standard (SC_{50} = 7.0 µg/ml) **(Table 2 and Figure 3)**, indicating to the H-donating ability of the oil components. Many prior reported studies showed that the essential oils which contain oxygenated monoterpenes (e.g. citronellol, pulegol, citronellyl acetate, .ect) exhibited high antioxidant activities which are in agreement with our results **[50]**. Also, Zhuang *et al.*, 2009 reported that the antioxidant and anticancer activities of geranium essential oil may be attributed to the major contents of citronellol and *trans*-geraniol **[51]**. On the other hand, some authors reported that; the antioxidant properties of essential oils can be attributed to the co-activity (synergistic activity) between their main components and minor components, and minor components can be strongly sharing and play a vital role in scavenging of the free radicals **[52]**. Moreover, our oil showed a potent antioxidant activity using dot-blot qualitative antioxidant assay compared to two known free radical scavengers ascorbic acid and quercetin. The white wider zone on the purple background reflected the strong antioxidant activity of the oil with respect to the standards **(Figure 4) [36, 37]**.

Table 2: Free radical scavenging activity of the essential oil of *E. citriodora* fresh leaves

Oil Sample Conc. µg/ml	Mean DPPH scavenging effect (%)[a]
10	24.36 ± 2.27
20	37.99 ± 1.83
40	60.67 ± 0.29
60	75.46 ± 3.69
80	89.13 ± 0.33
100	99.94 ± 0.90
Mean value of (SC_{50} µg/ml)[b]	**32.58**
Ascorbic acid (AA)	**7.0 ± 1.20**

[a] *The data are expressed as % quenching of DPPH*
[b] *Values are expressed as mean of triplicate determinations ± standard deviation*

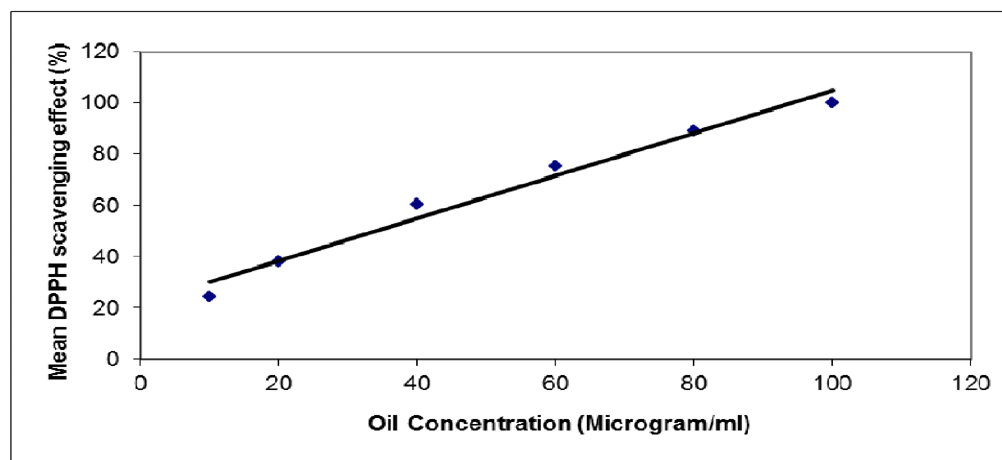


Fig. 3: Mean DPPH scavenging effect (%) against oil concentration µg/ml of the essential oil of *E. citriodora* fresh leaves

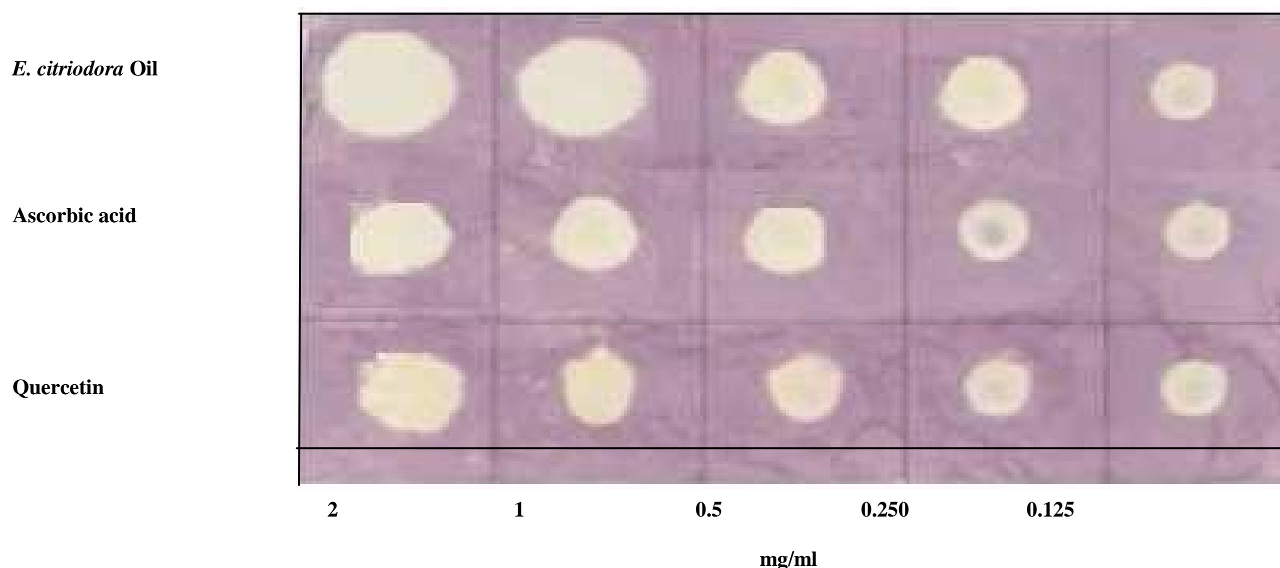


Fig. 4: Dot-blot qualitative antioxidant assay of the essential oil of *E. citriodora* on silica sheet stained with DPPH˙ solution in methanol against standards ascorbic acid and quercetin

4. Anticancer activity

Bhagat *et al.*, 2012 reported that six different extracts of *E. citriodora* leaves were tested *in vitro* against seven human cancer cell lines such as SW-620 (colon), HOP-62 (lung), PC-3 (prostate), OVCAR-5 (ovary), Hela (cervix), IMR-32 (neuroblastoma) and HePG-2 (liver), and the activity might be due to the presence of bioactive secondary metabolites like flavonoids, tannins and saponins in all the tested extracts **[53]**. On the other hand, since there are no references in literature about anticancer activity of essential oil of *E. citriodora*, so our results constituted the first report on the anticancer activity against three human cancer cell lines. The results revealed that our oil showed strongest activity against; colon carcinoma HCT-116 cell line with $IC_{50} =$ 4.75 µg/ml, followed by breast carcinoma MCF-7 cell line with $IC_{50} =$ 8.8 µg/ml and hepatocellular carcinoma hepG-2 cell line with $IC_{50} =$ 11.8 µg/ml against Doxorubicin (IC_{50} = 4 µg/ml) (Table 3 and Figure 5). In fact, the major components in the essential oils may be responsible for their biological activities, on the light of this principal, Maggi *et al.*, 2013, reported on the anticancer activity of essential oil from *Vepris macrophylla* against MDA-MB 231 (human breast adenocarcinoma) and HCT116 (human colon carcinoma) cell lines and such activity return to the presence of certain monoterpenes components one of them is citronellol **[54]**. Also, other authors reported that the anticancer and antioxidant activities of *Pelargonium graveolens* return to citronellol as a major component **[55, 56]**. So, we can conclude that citronellol as a major component in our oil may be responsible for its antioxidant and anticancer activity in collaboration with other minor ingredients.

5. Mode of action

Due to the complex chemical profile of the EOs, it is not easy to assign a unique mode of action for the anticancer activity. Also, a volatile component may be effective against certain cancer cell line and inactive against others. Moreover, various modes of actions may be predicted such as; an effect on the cell cycle, cell growth, and/or apoptosis **[57]**. The essential oil cytotoxicity might be due to its lipophilic compounds that accumulate in cancer cell membranes and increase their permeability, resulting in leakage of enzymes and metabolites **[58]**. In general, essential oils help in cell survival, proliferation and reduce the risk of cell invasion and inflammation **[59]**.

Table .3: Anticancer activity of the essential oil of *E. citriodora* fresh leaves against hepatocellular, breast and colon carcinoma cells

Sample conc. (µg)	Tested carcinoma cells (SF)[a] Hepatocellular carcinoma HepG-2 cell line	Breast carcinoma MCF-7 cell line	Colon carcinoma HCT-116 cell line
	Viability %	**Viability %**	**Viability %**
50	25.19	23.64	18.25
25	34.22	35.91	26.73
12.5	48.63	46.32	32.49
6.25	61.75	52.49	43.21
3.125	70.81	64.21	57.38
1.56	82.96	79.65	68.57
0	100.00	100.00	100.00
IC_{50} [b]	**11.8 µg/ml**	**8.8 µg/ml**	**4.75 µg/ml**

[a] *SF = Surviving fraction;* [b] *IC_{50}= Dose of the oil which reduces survival to 50%.*

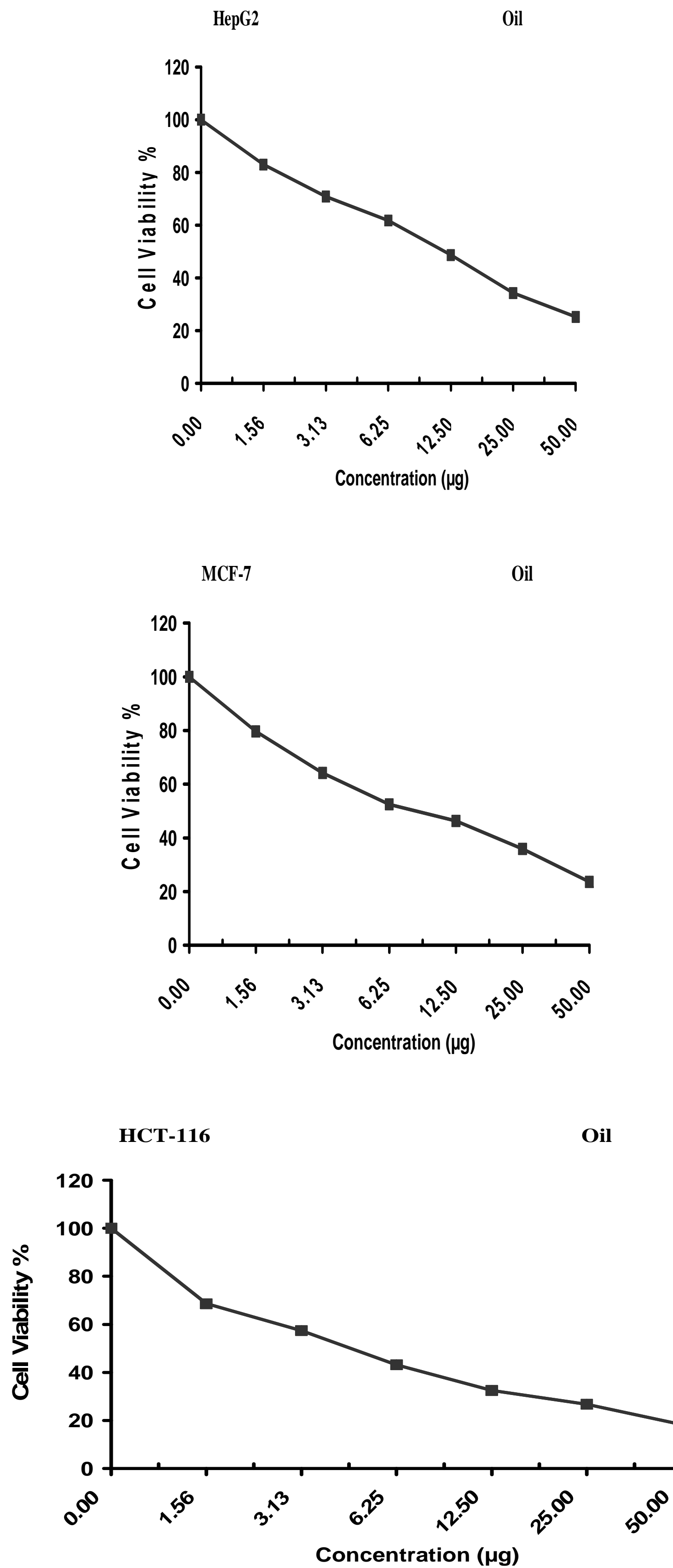


Fig .5: Anticancer activity of the essential oil of *E. citriodora* fresh leaves against hepatocellular, breast and colon carcinoma tumor cell lines

CONCLUSION

The essential oil isolated from the fresh leaves of *E. citriodora* Hook, showed synergetic biological activity return to the phenomena of co-activity or additive activity between all components of the oil, also mainly attributed to the presence of citronellol, pulegol and citronellyl acetate as major ingredients. Furthermore, the potent antioxidant and anticancer activities can recommend it to be used in certain medical applications.

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