Journal of Medicinal Plants Research Vol. 4(18), pp. 1910-1915, 18 September, 2010
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Evaluation of antioxidant and antitumour activities of lemon essential oil

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Accepted 9 August, 2010

The chemical composition of lemon essential oils, obtained by hydrodistillation, was studied by Gas Chromatography (GC) and Gas Chromatography and Mass Spectroscopy (GC–MS). The oil consisted mainly of propylphosphonic acid, fluoroanhydride, octyl ester (15.72%), decanoic anhydride (12.631%), benzene, 2,4-difluoro-1-isocyanato (11.72%), cyclopentane, 1,1'-hexadecylidenebis- (9.98%), 4-cyanocinnoline (8.58%), 4-cyanocinnoline (8.55%), cyclodocosane, ethyl- (7.58%), butanamide, N-(4-chlorophenyl)-3-oxo- (5.77%), 2-naphthuric acid (4.47%), Limonene (4.25%), (Z)-14-tricosenyl formate (3.51%), diethyl phthalate (2.94%), decanoic acid, 1,2,3-propanetriyl ester (2.91%), 13-tetradecen-1-ol acetate (2.40%). The antioxidant activity using O_2^-, OH^-, $DPPH^-$ radicals and the antiproliferative activity on a series of human cervical adenocarcinoma cell lines (LeLA) were investigated for the lemon essential oil. The result showed that the essential oil exhibited strong antioxidant activities as well as antiproliferative activity against HeLA cell line.

Key words: Lemon, essential oil, antioxidant activity, cytotoxic activity.

INTRODUCTION

Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Nadkarni and D'Souza, 1988). Electron acceptors, such as molecular oxygen, react easily with free radicals to become radicals themselves, also referred to as Reactive Oxygen Species (ROS). The ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). There are increasing suggestions by considerable evidence that the free radicals induce oxidative damage to biomolecules (lipids, proteins and nucleic acids), the damage which eventually causes atherosclerosis, ageing, cancer, diabetes mellitus, inflammation, several non-transmissible degenerative diseases in humans (Dhiman et al., 2009; Lai et al., 2010; Wang et al., 2008; Yuan et al., 2008).

In the last decades, the essential oils and various extracts of plants have been of great interest as theyhave been the sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of the foods from the toxic effects of the oxidants. Particularly, the antimicrobial activities of plant oils and extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Mantle et al., 1998; Joshi et al., 2010).Lemon (*Citrus limon*) is in the citrus family (Rutaceae). The essential oil extract of lemon has antiseptic properties which makes it find its use in treatment of infections (Fuselli et al., 2008). Because of its powerful antioxidants, it is able to fight free radicals, thus helps in strengthening of the blood capillaries (Calabrese et al., 1999; Misharina and Samusenko, 2008; Del Río et al., 2004). For the same reason it is able to boost immunity of the body (Del Río et al., 2004).

In this study, our aim was to examine the antioxidant and free radical scavenging activities of lemon essential oil prepared according to the traditional food and medical practice. Then, inhibition of the essential oil against HeLa cells growth was also determined.

MATERIALS AND METHODS

Materials

Plant materials for extraction were peal of lemon collected during summer 2009 from YanCheng area (Jiangsu Province, China).

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Obtaition of lemon essential oil

Three hundred grams of the plant were hydrodistilled for 3 h in an all-glass Clevenger apparatus. Heat was supplied to the heating mantle (50°C) and the essential oil was extracted with 5L of water for 3 h (until no more essential oil was recovered). The essential oil was collected and analyzed immediately.

Gas chromatography and mass spectroscopy analysis

GC-MS analysis was done after the extraction of the essential oil (water distillation). The fresh essential oil was collected, allowed to cool and was analyzed immediately on a Hewlet Packard HP 5973 mass spectrometer interfaced with an HP-6890 Gas Chromatograph. The column consisted of a cross-linked 5% pH ME Siloxane on 30 × 0.25 × 0.25 mm film thick and the column head pressure was 55 Kpa. The carrier gas used was Helium and the flow was 35 cm/ssplit flow 30 - 40:1. The temperatures were programmed at initial temperature of 50°C and accelerated to a temperature of 240°C at an acceleration of 3°C /min. Identification of chemical compounds was achieved by mass spectroscopy.

Inhibition of superoxide radicals

Superoxide radical generated by the xanthine–xanthine oxidase system was determined spectrophotometrically by monitoring its ability to reduce nitroblue tetrazolium (NBT) (Robak and Gryglewski, 1988). The reaction mixture consisted of 1.0 ml of 0.05 M phosphate buffer (pH 7.4), 0.04 ml of 3 mM xanthine, 0.04 ml of 3 mM EDTA, 0.04 ml of 0.15% bovine serum albumin, 0.04 ml of 15.0 mM NBT and 0.04 ml of essential oil (dissolved in methanol; 20 -120 µg/ml). After incubation at 25°C for 10 min, the reaction was started by adding 0.04 ml of 1.5 U/ml xanthine oxidase and carried out at 25°C for 20 min. After 20 min, the absorbance of the reaction mixture was measured at 560 nm. Percent scavenging of superoxide was calculated from the optical density of the treated and control samples.

DPPH assay

The hydrogen atom-or-electron donation ability of the essential oil was measured from the bleaching of the purple coloured methanol solution of $DPPH^{\cdot}$. This spectrophotometric assay uses the stable radical, 2,2-diphenylpicrylhydrazyl ($DPPH^{\cdot}$), as a reagent (Brand-Williams et al, 1995). Fifty microliters of various concentrations of essential oil (in methanol) were added to 5 ml of a 0.004% methanol solution of $DPPH^{\cdot}$. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical, $DPPH^{\cdot}$, in percent (I%) was calculated in following way:

$$I\% = [(A_{blank} - A_{sample})/Ablank] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate.

Hydroxyl radical ($OH^{\cdot}$) scavenging

$OH^{\cdot}$ scavenging was evaluated by measuring the competition between 2-deoxyribose and the essential oil for $OH^{\cdot}$ generated in a Fenton reaction. $OH^{\cdot}$ degrades 2-deoxyribose to form thiobarbituric acid reactive substance (TBARS) that could be measured at 532 nm (Bozin et al., 2006). Essential oil or monoterpene (25 - 200 µg/ml) was added into the reaction mixture containing 3 mM 2-deoxyribose, 0.1 mM $FeCl_3$, 1 mM H_2O_2, 0.1 mM EDTA, 0.1 mM ascorbic acid, and 0.02 M phosphate buffer (pH 7.4) to a volume of 3 ml and incubated for 1 h at 37°C. Then, 1 ml of TBA (1%) and 1 ml of trichloroacetic acid (2.8%) were added to test tubes and these were heated at 100°C for 20 min. After cooling the mixture, absorbance was read at 532 nm against a blank containing buffer and 2-deoxyribose. The percent inhibition (I) of deoxyribose degradation by essential oil was measured using the formula shown below:

$$\% I = [(\text{Absorbance of blank} - \text{Absorbance of sample})/\text{Absorbance of blank}] \times 100$$

β-Carotene-linoleic acid assay

In this assay antioxidant capacity is determined by indirectly measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β-carotene-linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade), 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml distilled water saturated with oxygen (30 min 100 ml/min.) was added with a vigorous shaking. 2.5 ml of this reaction mixture was dispersed to test tubes and 350 µl portions of the oils prepared at various concentrations were added and emulsion system was incubated up to 48 h at room temperature. After this incubation period absorbance of the mixtures were measured at 490 nm. Values are presented as means ± SD of three parallel measurements.

Cell cultures and anti-proliferative activity assay

HeLa (human cervical adenocarcinoma) cell lines were obtained from the institute of biochemistry, Suzhou University. All cell lines were maintained in RPMI 1640 supplemented with 10% FBS. Cells were grown in monolayer cultures. The experiments were performed in triplicate. Cell growth inhibition following exposure to essential oils was determined by MTT assay (Mosmann, 1983) with some modifications. After incubation for 24 h, the media was removed and the cells were incubated with 20 µl of media containing 5 mg/ml stock solution of MTT in PBS and 60 µl of RPMI. After incubation for 24 h at 37°C in 5% CO_2 incubator, the formazan crystal formed were dissolved by adding 120 µl/well of 0.04 N HCl and isopropanol. The optical density was measured at 570 nm with 630 nm as the reference wavelength. The number of viable cells was proportional to the extent of formazan production.

$$\text{Inhibition rate } (\%) = [(1\text{-}OD_{sample})/OD_{control}] \times 100\%$$

RESULTS AND DISCUSSION

Chemical composition of the essential oil

GC-MS of the freshly distilled oils revealed the presence of at least 55 components (Figure 1 and Table 1). The essential oils consist mainly of Propylphosphonic acid, fluoroanhydride, octyl ester (15.72%), Decanoic anhydride (12.631%), Benzene, 2,4-difluoro-1-isocyanato (11.72%),Cyclopentane, 1,1'-hexadecylidenebis- (9.98%), 4-Cyanocinnoline (8.58%), 4-Cyanocinnoline (8.55%),

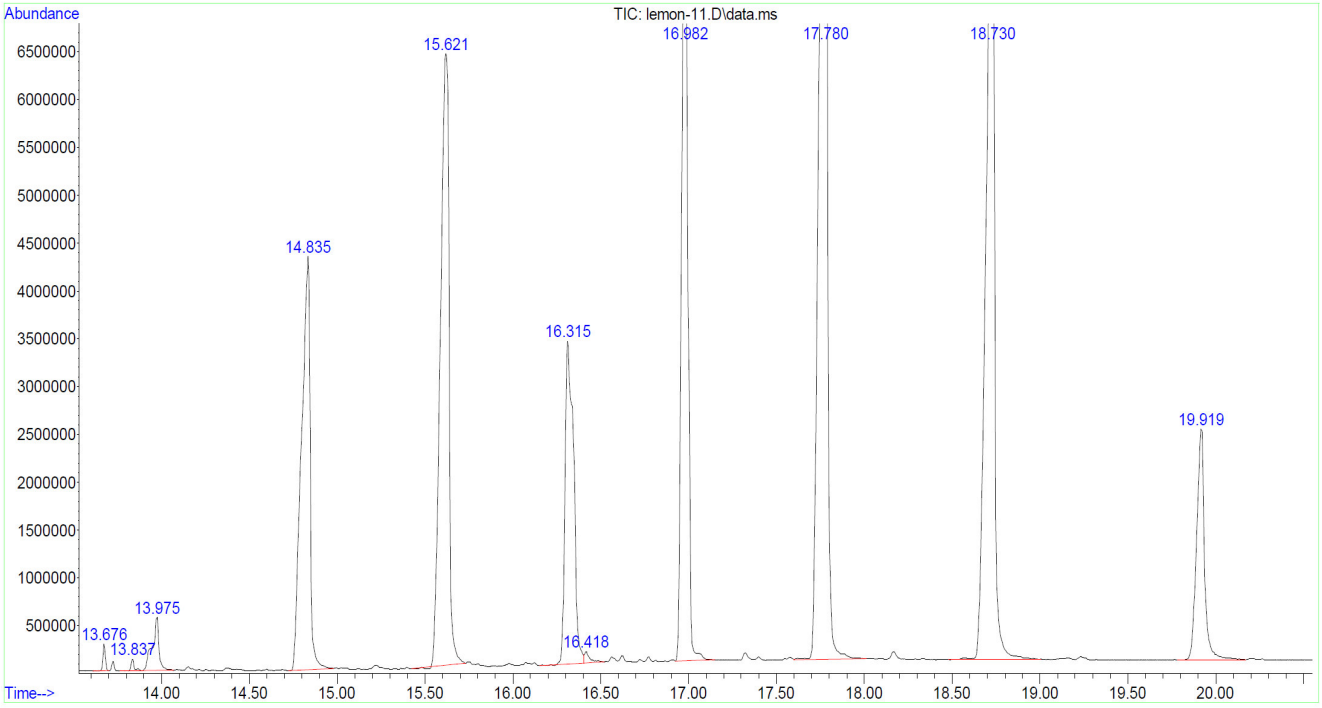

Figure 1. GC-MS analysis of lemon essential oil.

Cyclodocosane, ethyl- (7.58%), Butanamide, N-(4-chlorophenyl)-3-oxo- (5.77%), 2-Naphthuric acid (4.47%), Limonene (4.25%), (Z)-14-Tricosenyl formate (3.51%), Diethyl Phthalate (2.94%), Decanoic acid, 1,2,3-propanetriyl ester (2.91%), 13-Tetradecen-1-ol acetate (2.40%) (Figure 1 and Table 1).

In vitro free radical scavenging activities of lemon essential oil

The antioxidant ability to scavenge the radical O_2^- has been compared to the standards BHT. Figure 2 depicts a steady increase in the O_2^- radical scavenging capacity of essential oil up to a concentration of 40 µg/ ml followed by a relatively low increase, with further increase in concentration. When concentration of essential oil was 120 µg/ ml, the O_2^- radical scavenging rate reached 55%. As shown in Figure 2, the scavenging potential followed the order BHT > essential oil. The reactive oxygen radicals are unstable, and react readily with other groups or substances in the body, resulting in cell damage and hence human diseases (Halliwell and Gutteridge, 1989). Among the oxygen radicals specifically, the hydroxyl radical is the most reactive and severely damages adjacent biomolecules such as all proteins, DNA, PUFA, nucleic acid, and almost any biological molecule it touches. This damage causes aging, cancer and several diseases (Aruoma, 1998; Ehsanollah et al, 2009; Zhao and Zhang, 2009). Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. The scavenging effect against hydroxyl radicals was investigated by using the 2-deoxyribose oxidation method. Figure 3 shows the hydroxyl radical-scavenging effects of essential oil. The scavenging effect of essential oil on hydroxyl radicals was concentration-dependent. Essential oil at the final concentration of 120 µg/mL exhibited 26.7% scavenging effect on hydroxyl radical. According to the present findings, hydroxyl radical scavenging activity of essential oil was by far lower than that of BHT at the concentration range of 20 -120 µg/mL (Figure 3).

The effect of antioxidants on $DPPH^-$ is thought to be due to their hydrogen donating ability (Baumann et al., 1979). Figure 4 illustrates a significant increase in the scavenging rate of $DPPH^-$ with increase in concentration of the essential oil. Significant $DPPH^-$ radical scavenging activity was evident at all the tested concentrations. The scavenging activity increased with increasing concentration of essential oil up to 100 µg/ml and then almost leveled off with further increase in concentration. The $DPPH^-$ radical scavenging activities corresponding to the concentration of BHT and essential oil were found to be 88.6 and 70%, respectively, clearly showing that the scavenging potential followed the order BHT > essential oil (Figure 4).

Table 1. Chemical composition of the essential oil of lemon.

Pk#	RT	Library/ID	Area (%)
1	5.770	Alpha.-Pinene	0.07
2	6.234	Bicyclo[3.1.0]hexane, 4-methylene- 1-(1-methylethyl)-	0.07
3	6.280	Beta.-Pinene	0.51
4	6.783	Benzene, 1-methyl-2-(1-methylethyl)-	0.55
5	6.840	Limonene	4.25
6	7.115	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	0.18
7	7.476	1,6-Octadien-3-ol, 3,7-dimethyl-	0.24
8	7.659	Phenylethyl Alcohol	0.14
9	7.699	Benzene, butyl-	0.06
10	8.151	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-(.+/-.)-	0.10
11	8.282	1-Octanol, 2,7-dimethyl-	1.03
12	8.540	6-Octen-1-ol, 3,7-dimethyl-	1.07
13	8.666	2,6-Octadienal, 3,7-dimethyl-	0.09
14	8.752	2,6-Octadien-1-ol, 3,7-dimethyl-,(E)-	0.16
15	8.792	2-Butene, 1-bromo-3-methyl-	0.23
16	8.883	2,6-Octadienal, 3,7-dimethyl-, (E)	0.14
17	9.427	2,6-Octadiene, 2,6-dimethyl-	0.18
18	9.507	2,6-Octadien-1-ol, 3,7-dimethyl-,acetate, (Z)-	0.09
19	9.639	2,6-Octadien-1-ol, 3,7-dimethyl-,acetate	0.24
20	9.879	Neopentylidenecyclohexane	0.19
21	9.959	1,4-Methanoazulene, decahydro-4,8, 8-trimethyl-9-methylene-, [1S-(1.alpha.,3a.beta.,4.alpha.,8a.beta.)]	
22	10.028	Caryophyllene	0.15
23	10.062	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	0.12
24	10.400	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (E)-	0.26
25	10.514	Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)-	0.20
26	11.058	Diethyl Phthalate	2.94
27	11.527	Octanoic acid, 4-nitrophenyl ester	0.06
28	11.939	Octanal, 2-(phenylmethylene)-	0.05
29	12.442	Butylphosphonic acid, isobutyl 2-phenylethyl ester	
30	12.540	Octanoic acid, decyl ester	0.17
31	12.631	Decanoic anhydride	12.631
32	12.723	2,6-Octadiene, 2,6-dimethyl-	0.18
33	12.774	Propanoic acid, 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester, (E)-	0.07
34	12.900	Propanoic acid, 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester, (E)-	0.06
35	12.963	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	0.07
36	13.243	Isopropyl Palmitate	1.75
37	13.461	Butylphosphonic acid, pentyl 2-phenylethyl ester	0.09
38	13.501	Oxalic acid, isobutyl tetradecyl ester	0.13
39	13.678	6-Octen-1-ol, 3,7-dimethyl-, propanoate	0.11
40	13.838	2,6-Octadien-1-ol, 3,7-dimethyl-,propanoate, (Z)-	0.06
41	13.976	Phosphoric acid, ethyl nonyl undecyl ester	0.56
42	14.834	Butanamide, N-(4-chlorophenyl)-3-oxo-	5.77
43	15.624	4-Cyanocinnoline	8.55
44	16.316	2-Naphthuric acid	4.47
45	16.419	13-Docosenamide, (Z)-	0.10
46	16.980	Glycerol tricaprylate	8.58
47	17.781	Propylphosphonic acid, fluoroanhydride, octyl ester	15.72
48	18.731	Benzene, 2,4-difluoro-1-isocyanato	11.72
49	19.921	Decanoic acid, 1,2,3-propanetriyl ester	2.91
50	22.078	Bicyclo[10.8.0]eicosane, (E)-	0.25

Table 1. Contd.

51	24.521	13-Tetradecen-1-ol acetate	1.48
52	28.367	Cyclodocosane, ethyl-	7.58
53	33.779	Cyclopentane, 1,1'-hexadecylidenebis-	9.98
54	33.808	(Z)-14-Tricosenyl formate	3.51
55	40.835	13-Tetradecen-1-ol acetate	2.40

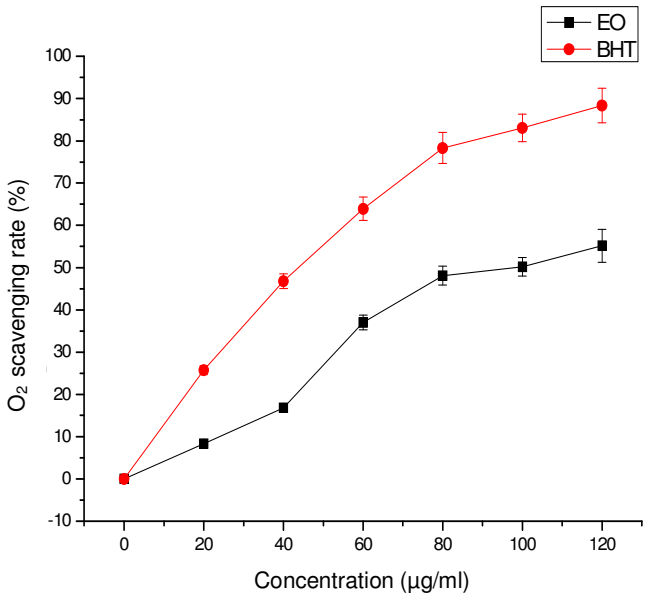

Figure 2. O_2^- radical scavenging capacity of essential oil.

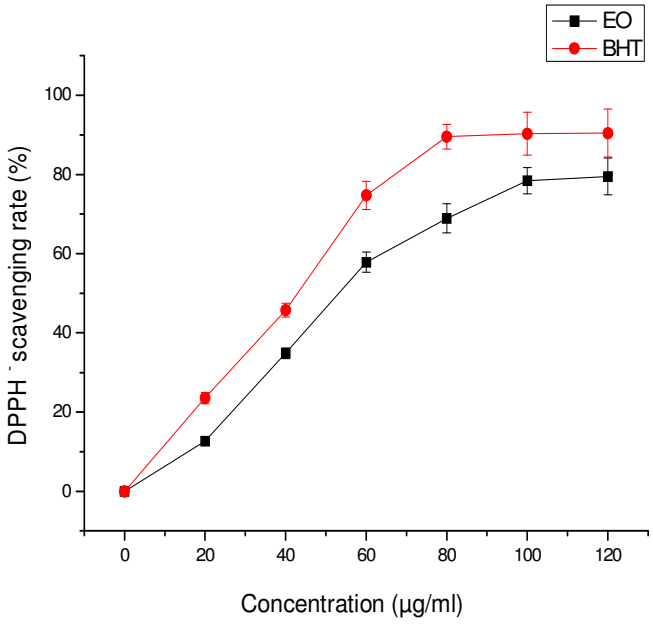

Figure 4. $DPPH^-$ radical scavenging capacity of essential oil.

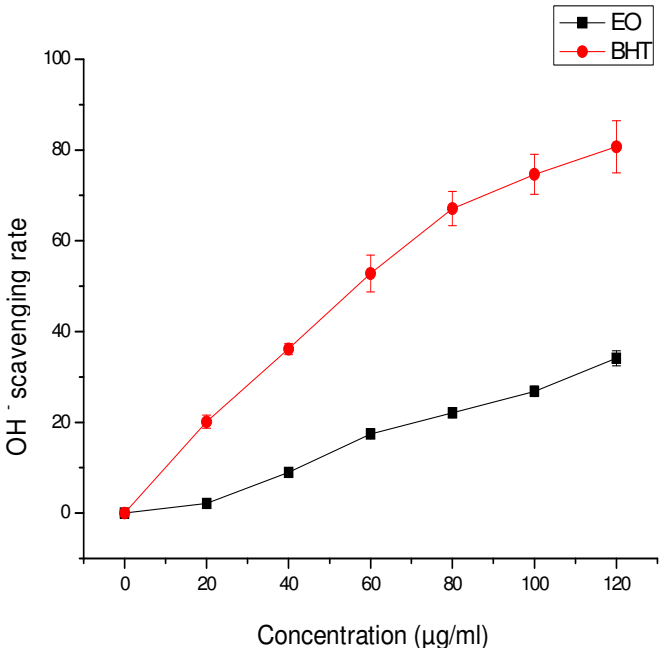

Figure 3. OH^- radical scavenging capacity of essential oil.

In β-carotene/linoleic acid system, lemon essential oil exhibited 70.3 ± 3.74% inhibition against linoleic acid oxidation at the concentration of 120 µg/ml (Figure 5). Percent inhibition of BHT calculated as 85.8 ± 6.15% at the concentration of 120 µg/ml. This indicated that lemon essential oil protect the linoleic acid against oxidation. Moreover, the antioxidant activity increased with increasing concentration of lemon essential oil. A HeLa cell (also Hela or hela cell) is a cell type in an immortal cell line used in scientific research. It is one of the oldest and most commonly used human cell lines (Gille et al., 1988). The line was derived from cervical cancer cells and found to be remarkably durable and prolific as illustrated by its contamination of many other cell lines used in research (Reiter and Demple, 2005). The cytotoxic activities of the essential oils were tested on the mammalian cancer cell lines HeLA. Essential oil showed good activities on the cell lines HeLA (Figure 6).

Inhibition rate of HeLA cells growth increased with increasing concentration of essential oil. Inhibition rate of HeLA cells growth reached 39% when concentration of essential oil was 60 µg/ml.

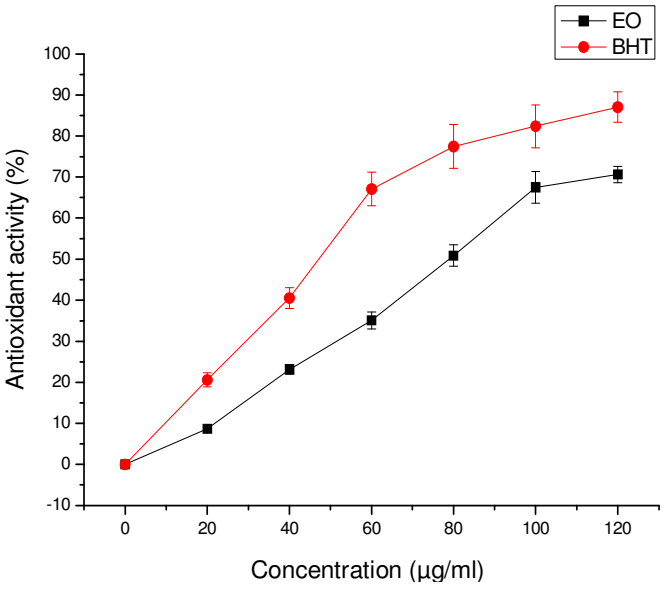

Figure 5. Percent inhibition of the linoleic acid oxidation by lemon essential oil.

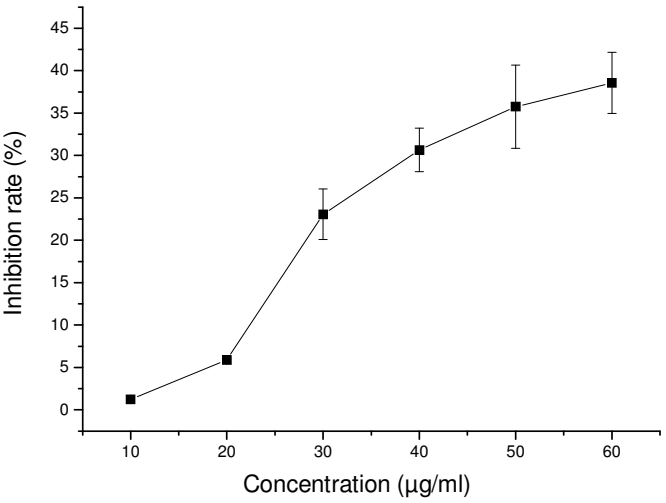

Figure 6. Percent inhibition of lemon essential oil against HeIA cells growth.

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