LEMONGRASS ESSENTIAL OIL SHOWS AN ANTIPROLIFERATIVE EFFECT ON LUNG CANCER CELLS AND ITS ROLE IN EXPRESSION OF P53 GENE

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ABSTRACT
This study was aimed to investigate the antiproliferative effect of lemongrass essential oil (LEO) from Cymbopogon citratus on A549 cell line and the effect on the expression of p53 gene in lung cancer cells. The LEO was extracted and analyzed using GC-MS analysis, the cytotoxicity of LEO against lung cancer cells was examined using MMT assay and finally the expression of p53 was determined via qRT-PCR. The chromatogram of GC-MS exhibited 14 compounds in LEO, the major one is citral (56.4%). The cytotoxicity of LEO was dose dependent which proliferation of the lung cells were significantly inhibited with increased concentration of essential oil (P≤ 0.01) as well as the IC50 was 29.13 ppm. Furthermore, it showed that cell treated with IC50 of LEO had a significant higher gene expression (P≤ 0.05) in the fold of p53 expression than untreated cells with change fold of 4.47±0.7. In conclusion, C. citratus essential oil have been found to possess antiproliferative activity against lung cancer cells and upregulation of p53 gene which can be considered a promising treatment for lung cancer patients.

Keywords: C. citratus, A549 cell line, qRT-PCR, LEO, cytotoxicity, gene regulation.

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INTRODUCTION
The p53 tumor suppressor gene which shows to be the most frequent target in which their abnormalities plays a significant role in the formation of lung epithelial cell tumors (28). The p53 tumor suppressor gene is set on the human chromosome 17p13 and its product is a nuclear phosphoprotein known as p53. It's a transcription factor regulate the transcription of target genes in response to different stress signals. The p53 describes as “the guardian of the genome” due to take part in the cell cycle controlling, apoptosis, repair of DNA and preserve the whole genome integrity (13). In lung carcinomas, the p53 converted from active to inactive protein due to missense mutations in the region of coding p53 gene which play an substantial role in oncogenic transformation of lung epithelial cells as well as development of lung carcinoma (31). Over the past years, the chemotherapy was the major standard method for treatment of lung cancer by using two drugs cisplatin or carboplatin which their cytotoxic effect due to targeting nuclear DNA (22). However, chemotherapy have side effects include vomiting, nausea, hepatotoxicity, ototoxicity, neuropathy, nephrotoxicity, suppression of bone marrow and anemia (26) as well as NSCLC may have intrinsic resistance to treatment by cisplatin. (34). Therefore, searching for other anticancer medicines with lower or no side effect for treating lung cancer is still the main goal. Through studies reports, it was found that 133 Chinese herbal plants owned anti-lung cancer effectiveness (36). Lemon-grass (Cymbopogon citratus) is a herbal plant of the Poaceae family grown in tropical countries (1). The main component of lemon grass is citral, it also contains aldehydes, esters, diterpenes, ketones and a little proportion of acids. The lemon grass essential oil (LEO) have different pharmacological activities includes, antibacterial, anti-inflammatory, insecticidal, antiangiinal, antiplatelet, antinociceptive, anxiolytic and anticancer (15). LEO found to have potent cytotoxic activities against different cancer cell lines. It shown to arrest the proliferation of KB (human mouth epidermal carcinoma), P388 (murine leukemia) (19), The colon cancer cell lines (HT-29 and HCT-116) (27) A549 (human lung carcinoma) and H1975 (human lung large cell carcinoma) cells (35). Also, the Citral was noticed to induce apoptosis and stopping the cell cycle of tumor cell lines. It was inhibited the proliferation of spontaneous tumors in mice as well as when used alone or in combination with chemotherapy, suppressed the proliferation of SCLC cell lines (LU134AM, LU135 and LU165) (20). Little studies have reported the anticancer effect of LEO on lung cancer. Therefore, this study was aimed to investigate the antiproliferative effect of LEO on A549 cancer cell line and their effect on the expression of p53 gene in lung cancer cells.

MATERIALS AND METHODS
Cell line and maintenance of cell cultures
Human lung carcinoma cells (A549 cell line) were cultured and maintained in RPMI-1640 media (Capricorn, Germany) supplemented with 10% fetal bovine serum (Capricorn, Germany), 100 µg/mL streptomycin and 100 U/mL penicillin. The passage of cell was done using Trypsin-EDTA then reseeded at 80% confluence twice a week and incubated in 5% CO2 at 37 °C (39).

Lemon grass essential oils extraction
The air dried leaves (250g) of lemon grass was applied for extraction of essential oil using hydrodistillation method in Clevenger apparatus and boiled with distilled water for 3h, then the extracted oil was kept at 4°C until use (3).

Chemical composition of lemon grass essential oil: Gas Chromatography-Mass (GC-MS) analysis for LEO was carried out in the Ministry of Science and Technology laboratories using a Shimadzu gas chromatograph GC-2010 plus (Shimadzu, Japan). Two microliter of LEO was injected to GC apparatus, the type of capillary column used was DB-5 (25m length, 0.2mm inner diameter, 0.25µm thickness). The temperature of GC apparatus was programed at 60°C for 4 min, increased to 150°C for 4 min and then elevate to 250°C. The flow rate of 1.35 ml/min and the carrier gas was helium, carrying the contents of the sample from the injector straight the column to the detector. The mass spectrometry (MS) mode was electron impact (EI) (9).
Cytotoxicity assays for lemon grass essential oil: To determine the cytotoxic effect of LEO, the viability of the cell was measured according to Jiang et al. (19) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The A549 cell line was seeded to well of 96-well plate (1x10^4 cells/well), a confluent monolayer was accomplished after 24 hrs of incubation. The cell line was treated with different concentration of LEO (6.25, 12.5, 25, 50, 100 and 200 ppm) and incubated in 5% CO2 incubator at 37 °C for 72 hrs. After removing the media, the MTT (2 mg/ml) was added and incubated the cells for another 3 h at 37 °C. The MTT solution was removed and the remaining crystals in the wells were dissolved using 130 µl of DMSO (Dimethyl Sulphoxide), incubated with shaking at 37°C for 15 min. The optical density (OD) values was determined using microplate reader (Gennex Lab ,USA) at 492 nm; the assay was performed in triplicate. The percentage of cytotoxicity was calculated by the inhibition rate of cell growth using the inhibition rate equation (4), where A and B are the OD of control and test respectively:
Inhibition rate = A-B/A ×100

Microscopic examination of A549 cells treated with LEO
For visualizing the shape of A549 cells treated with LEO under inverted microscope, the cell suspensions (200 µl of 10^4 cells/ml) was cultivated in microtitre plate and incubated at 37°C for 48 hrs. The medium was replaced with half inhibitory concentration (IC50) of LEO, incubated for 24 hr. Crystal violet (50 µl) was used to stain the cells and incubated at 37°C for 15 min, then the stain was gently washed thrice with tap water. The morphological feature of the cell was observed under 100x magnification inverted microscope (4).

Quantitative real-time PCR for expression of p53 gene
Quantitative real-time PCR was used to determine the expression of p53 tumor gene in both LEO treated and untreated A549 lung cells. Total RNA of A549 lung cells was extracted according to the manufacturer’s directions using Trizol (Invitrogen, USA). The extracted RNA was converted to cDNA using kit of High Capacity RNA to-cDNA (Applied Biosystems, USA). The primers used for expression of p53 gene were forward 5-TTGAGGTGCGTGTTTGTG-3 and reverse 5-CTTCAGTGCTGGAGTGTG-3. For normalization of qPCR, GAPDH (housekeeping gene) primers were used, forward 5-ACCACAGTCCATGCCATCAC-3 and reverse 5-TCCACCACCTGTGCTGTA-3 (33). Each reaction consisted of 7.5 µl of SYBR green (SYBR® Premix Ex Taq kit, USA), cDNA (1 µl), 0.3 µl from each related primers and the volume was completed with DW to reach 15 µl. The PCR reactions were performed using ABI PRISM 7500 sequence detection system (Applied Biosystems, USA). The quantification of p53 gene expression was carried out using the ΔΔCT calculation and the fold of gene expression was given as 2^ΔΔCT.

Statistical analysis
The data were analysed using the SPSS IBM version 20. The values were presented as the mean ± SEM of triplicate measurements. One-way ANOVA test was done to investigate the differences between the concentrations tested against lung cancer cells. T- test was used to compare between quantity of p53 gene expression in treated and untreated lung cancer cells. Values were considered statistically significant P ≤ 0.05.

RESULTS AND DISCUSSION
After extraction of LEO, GC-MS analysis was done for detection the active compounds found in the essential oil. The results of chromatogram illustrated in Figure 1, 14 compounds that the major constituent of the essential oil was citral 56.4%. The chemical composition of LEO and their percentages varies according to the habitat, genetic diversity, different plant parts and agricultural treatment of the plant (11). In addition, the maturity stages of C. citratus effects on chemical composition of essential oil and citral content (32). The cytotoxic effects of six different concentration of LEO against lung cancer cell line (A549 cell line) was investigated by MMT assay. As shows in Figure 2 (A), the cytotoxicity of LEO was dose dependent which proliferation of the lung cells were significantly inhibited with the increased concentration of essential oil (P≤
The higher rate of inhibition (84%) was seen at the concentration 200 ppm while the lowest rate (4.8%) at the concentration 6.25 ppm. The concentration that lead to 50% inhibition growth rate was 29.13 ppm.

Fig 1. Chromatogram analysis of LEO extracted from C. citratus leaves using GC-MS

The concentration that lead to 50% inhibition growth rate was 29.13 ppm. Moreover, the IC50 of LEO cytotoxicity in A549 cancer cells was confirmed by microscopic examination after staining with crystal violet. The results exhibited considerable reduction in the LEO treated lung cancer cells proliferation in comparison with untreated cells (Figure 2, B). The morphological variations in A549 cancer treated cell represented by cell proliferation stops and loss of tissue texture due to apoptosis whereas untreated cells observed unchanged in their tissue morphology. These findings supported by the results of Trang et al. (35), study which observed the leaf extracted LEO induced cycle turn off and apoptosis in A549 cells by altering the regulating proteins associated with apoptosis process such as caspase-3, Bax and Bcl-2. Lately, several studies have shown LEO was a chemoprevention promising agent provided protection for lung cancer against oxidative stress and DNA damage induced by Benzo(a)pyrene and the greatest effect was showed at 0.5% of oil. Also, revealed the LEO protect the cells from being damaged by both continuance the normal level of superoxide dismutase and catalase (anti-oxidative enzymes) as well as suppress the damaging agents like Malondialdehyde and 8-hydroxyldeoxyguanosine (19). Furthermore, LEO and their main content, citral, inhibit the survival and reproduction of four types of SCLC cancer cells resulting from suppression Src-TK from phosphorylating of Stat3 causing decreased levels of both Bcl-xL and Mcl-1. In addition, the IC50 of LEO were 17.35-23.21×10^{-4}% depending to the type of cell line treated with oil (24). Essential oils have been observed to have different anticancer mechanisms, including mechanisms for cancer prevention, acting on the cancer cell itself and interfere with the microenvironment (10,30). Recently, several studies have shown the anticancer properties of ethanol extract LEO in lymphoma and leukemia cell models (24) as well as citral which have anti-proliferative effect on tumor cells by enhancing the levels of oxygen radicals leading to oxidative stress induction and finally cell death (16, 29). In local studies, other plant extracts such as Fenugreek (Trigonella foenum-graecum) extract extract induced dose and time dependent cytotoxic effect in lung cancer cells. These extract, also, reduced significantly the expression of EGFR and decreasing mutant p53 expression (14). The ethanol extract of Radix Pulsatillae showed a strong cytotoxicity versus A549 lung cancer with low cytotoxicity to mice (6). It was also found potent anticancer and antioxidant activity after using Eriobotrya japonica against human cervical cancer (Hela) and rhabdomyo sarcoma (7). Fruits extract of Juniperus phoenicea nanoparticles against prostate cancer (18). The change in p53 gene expression in LEO treated and untraeted A549
lung cancer cells was investigated using qRT-PCR. The p53 was successfully expressed and quantify relatively to GAPDH gene which is not surprising due to GAPDH is a housekeeping gene commonly used accompany with gene expression data.

Figure 2. Cytotoxicity of LEO against lung cancer cell line (A549 cell line). A: The cytotoxicity is dose dependent, the IC50=29.13 ppm. The results were represented as the mean ± SEM of triplicate measurements. B: Antiproliferative effect of LEO in treated cells at concentration IC50, scale bar 10µm.

It is found that using GAPDH as internal gene for normalization of RT-PCR is very reliable strategy, which used for expression of 1,718 genes in 72 types of normal human tissue (5). Determination of the expression level of some genes in cancer patients by real-time RT-PCR can beneficial in its earlier stages (2). Furthermore, it showed that cell treated with LEO (IC50, 29.13 ppm) had a significant higher expression (P≤ 0.05) in the fold of p53 expression than untreated cells with change fold of 4.47±0.7 (Figure 3). These results confirmed the antiproliferative effect of LEO on lung cancer cell used in this study due to the upregulation of p53 induce apoptosis. The p53 mediated the mitochondria apoptosis pathway through stimulating caspase-8 activity which in turn release other apoptogenic factors such as cytochrome-C (25).

Figure 3. LEO upregulation of P53 expression in A549 lung cancer cells. Data are represented as mean ± SD.
Activation of upstream regulatory protein such as p53 resulting in turn activation of downstream genes which cause either repair the cell (cycle arrest) or damaged cells through apoptosis using various mechanisms, including activation of particular genes by transactivation, transcription independent mechanisms and downregulation of special genes (12). This is the first results about the upregulation of p53 in lung cell cancer following treatment with LEO. Other plant extracts reported the effect of their extract on p53 gene expression in different cancer cell lines. *Euryale ferox salisb* extract exhibited apoptotic activity and arrest cell cycle through downregulation of the Akt and upregulation of the p53 in A549 lung cancer cells (23). The methanolic extract of *Senna didymobotrya, Rhamnus staddo* and *Albizia gummifera* found to increase p53 expression in prostate cancer cells (DU145) with fold change about 15,990, 16,066 and 15,985 respectively (21). *Curcuma mangga* essential oil shown to enhance apoptosis to myeloma cell lines by upregulation of pro-apoptosis caspase-9 protein and p53 as well as downregulation of Bcl-2 and H-Ras which increased proliferation (8). The mRNA expression level of Cas-3, Bax, and p53 was higher increased treated the human grade IV glioma cells with methanolic extract of *Menyathes trifoliate* plant which induced apoptosis by disturbance of the mitochondrial membrane resulting in initiate cascade of apoptosis (17). Although many studies have examined the importance of plant extracts and essential oils as anticancer drugs for cancer promising treatment, they need other investigations including safety and toxicity studies before their participate in clinical trials. In conclusion, *C. citratus* essential oil have been found to possess antiproliferative activity against lung cancer cells and upregulation of p53 gene which might be considered a promising treatment for lung cancer patients.

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