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In-vitro evaluation of antiproliferative activity of *Cymbopogon citratus* (lemon grass) against lung cancer

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ABSTRACT

The present study describes the phytochemical and anticancer activity of *Cymbopogon citratus* (Lemon grass) extracts on lung cancer CALU6 cell line. The phytochemical analysis revealed that *Cymbopogon citratus* consists of alkaloids, tannins, terpenoids, saponins and flavanoids. The antiproliferative activity of C. citratus exhibited an IC50 value of 134.0µg/ml on lung cancer CALU6 cell line. The gene expression study was conducted on caspase 3, caspase 8 & β actin revealed that C. citratus was found to suppress the expression of Caspase-3 and Caspase-8 genes but is more effective in the treatment of lung cancer because of inhibition of cell proliferation occurred through Caspase-independent pathway.

Key words: *Cymbopogon citratus*, CALU6 cell line, phytochemicals, antiproliferative activity, Caspases.

INTRODUCTION

Lung cancer is the most common cancer diagnosed worldwide. It is the main contributor of cancer-related mortality, resulting in 1.38 million cancer deaths per year worldwide (WHO, 2013) among human population of all ages. By 2030 it has been expected that the cancer mortality rate will extend to about 30.1 million (WHO, 2013). The prevalence and pattern of smoking in India increases the incidence of lung cancer morbidity and mortality in both sexes.

Approximately, smoking contributes to 90% of lung cancer mortality in men and 70% in women around the world. As a result of constant smoking, carcinogenic stimuli, inflammation or irritation, lung cancer develops in the mucosal lining of bronchi metastasizing to other body parts. Based on histology, 4 major types of Lung cancer has been classified viz., small cell carcinomas, large cell carcinoma, squamous cell carcinomas and adenocarcinomas. The non-small cell lung cancer (NSCLC) comprises of

adenocarcinomas and large cell carcinoma, as contrasting to small-cell lung cancer (SCLC), as their behavior and management is analogous. Lung cancer can mainly be attributed to smoking (Alberg *et al*. 2016). Cigarette smoke is known to contain at least 73 carcinogens (Hecht, 2012).

Therapeutic efficiency of malign surgery, radiotherapy and chemotherapy used to cure cancer is minimal, which lead to an increase in research on alternative cancer therapy. Among researchers major interest is on natural products as an alternative and complementary medicine to many ailments. The documentation of traditional knowledge and scientific authentication of medicinal plants is the main focus of current research (Uprety *et al.* 2010).

Nowadays several natural products are employed as valuable anticancer agents which play an important role in contributing to the arsenal of the approximately 60 cancer chemotherapeutic drugs in the market. There is remarkable increased interest from the last two decades to explore novel anticancer agents from natural sources, which are the excellent sources of bioactive components exerting their beneficial health effects. Certain bioactive compounds / phytochemicals (secondary metabolites) derived from the plants are an important source of several clinical useful anticancer agents and have shown the ability to hamper cancer cytogenesis by suppressing the tumor initiation, promotion, and progression. It is estimated that approximately 50-60% of cancer patients in the United States make use of agents derived from different parts of traditional plants exclusively (Gutheil, 2012). Halabi *et al*. (2014) reported several phytochemical extracts, which have been tested for their anti-proliferative properties. Thus plants are regarded as a potential source of cancer chemoprevention drug discovery and development.

There has been considerable work conducted on the cancer chemopreventive effects of extracts and purified constituents of a few culinary herbs, fruits, spices, teas, and vegetables, which are showing the ability to inhibit the development of cancer in the laboratory animal models (Kinghorn, 2000). Few of them include curcumin from turmeric, genistein from soybean, tea polyphenols from green tea, resveratrol from grapes, sulforaphane from broccoli, isothiocyanates from cruciferous vegetables, diallyl sulfide from garlic, lycopene from tomato, rosmarinic acid from rosemary, gingerol from gingers and citral from lemon grass etc.

The genus Cymbopogon, better known as lemongrass belong to family Gramineae (grasses) has more than 55 species that grow in tropical and semi-tropical regions around the world from mountains to grasslands to arid zones (Kumar *et al.,* 2000). The plant could grow up to 6 inch high and its bulblike stems consist of glabrous linearly venated sheathed leaves with narrow base and acute apex. The leaf height is about 100 cm in length and 2 cm in width. When squeezed, the leaves usually produce yellow or amber colored, aromatic essential oil with pleasant aromas.

Cymbopogon citratus is a major crop in several tropical countries of Asia, South & Central America and Africa. It is commonly used as a culinary herb in Asian cuisine and also as medicinal herb in India. *C. citratus* is used prominently among the medicinal plants and explored commonly in folklore medicine for the treatment of various ailments. Citral being the major bioactive component with most bio-efficacy in *C.citratus* is reported (Lewinsohn *et al*. 1998).

Citral is known to activate procaspase-3 and induce apoptosis in hematopoietic cancer cell lines and hence is an important component in anti-cancer drugs. Essential oils isolated from these plants have shown the antifungal, antimicrobiol and anticancer activities. Lemongrass extracts exhibit more important therapeutic potential like anti- hypertensive, anti-inflammatory, anti-oxidant anti-mutagenicity and anti-cancer properties.

The present study intends to evaluate phytochemicals, dose response and expression profiling of apoptotic genes like CASPASE3 and CASPASE8 induced by the *Cymbopogon citratus* against CALU6 cells.

MATERIALS AND METHODS

Plant material collection

The *C.citratus* leaves were collected from Dhanvantrivana of Bangalore University. The leaves were washed with running tap water to remove pesticides and dirt. Then, the fresh sample was cut into small pieces and was placed inside the microwave reactor glass vessel (NEOS-GR Solvent-free Microwave Gravity Station) (Milestone, Italy). The extraction process required 45 minutes with the microwave power of 500W. Upon the extraction process has completed, the oil was collected in a glass vial and stored at 4^0C until use.

Phytochemical screening

The extracts were subjected to qualitative phytochemical tests for plant secondary metabolites; alkaloids, terpenoids, flavonoids, saponins and tannins according to the method described by Harborne (1973).

Cell culture

The human CALU6 (purchased from ATCC, USA) were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.03% glutamine and 1 mM sodium pyruvate (Sigma & Co.). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 incubator (Galaxy, USA).

Cell viability assay- MTT

Essential oil was dissolved in DMSO and added to medium to make the final concentration of DMSO less than 1%. Cell proliferation was determined by the MTT (3-4, 5-dimethylthiazolyl-2-2, 5-diphenyltetrazolium bromide) assay. The yellow MTT is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. HTB43 cells were plated at a density of 1×105 cells/well in a 96-well cell culture plate and treated with oil at doses of 0.5, 0.125, 0.062, 0031, 0.015, 0.0075 and 0.0039 mg/ml and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 hours in completed DMEM, 10% FBS, 2mM L-glutamine. After incubation, the old culture medium was discarded. Then, 20 µL (0.1mg) of MTT reagent was added to each well and incubated for 4 hours in CO_2 incubator. Then, the existing media was discarded and the purple substance was dissolved using 100 ml DMSO. Finally, the cells viability was analyzed using the Multimode Plate Reader (Perkin Elmer, USA), at wavelength of 570 nm.

Apoptosis evaluation

CALU6 cells were seeded at a density of 7000 cells per well in a 96-well plate. The plate was incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours in completed DMEM media. Then, the cells were incubated for a further 24 hours with lemongrass oil with concentration of 15 g/ml and Doxorubicin (0.1 M) as positive control. Prior to imaging, the cells were wash 3 times with PBS and was incubated with serum free media containing 4g/ml Hoech reagent for 45 minutes. The plate was imaged using DAPI channel (Cell Reporter, Molecular Devices, USA).

In vitro Caspase 3/8 activity assay

10^5/100 µL/well CALU6 cells were treated with the plant extracts (1.5 mg/mL, final concentration), for 24–48 h. Caspase 3 activity within the cells was assessed by Apo-ONE Homogeneous Caspase 3/8 Assay Kit (Promega). Experiments were carried out in parallel with cell viability assays.

Real-Time PCR analysis of Apoptosis-related mRNAs

0.5 × 10^6/3.5 mL cells were treated with 1.5 mg/mL (final concentration) of extracts 5, 10, and 11 for 3–24 h. Total mRNA was extracted and reverse-transcribed into cDNA using Verso cDNA kit (Thermo Specific, Epsom, Surrey, UK). Individual mRNA levels were quantified using Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Each 2 µL sample contained 1 µL primers (10 ng), 10 µL SYBR Green (Applied Biosystems), and 7 µL H_2O in a total volume of 20 µL per sample. The primers were as follows: Bax (exons 4-5, 116 bp) Sense: TCT GAC GGC AAC TTC AA CTG; Antisense: CAG CCC ATG ATG GTT CTGA; Bcl2 (exons 2-3, 134 bp) Sense: CCC CTG GTG GAC AAC ATC; Antisense: CAG CCA GGA GAA ATC AAA CAG; Caspase 3 (exons 7-8, 133 bp) Sense: GAA CTG GAC TGT GGC ATT GA; Antisense: CCT TTG AAT TTC GCC AAG AA; G6PD (exons 6-7, 283 bp) Sense: TCT ACC GCA TCG ACC ACT ACC; Antisense: GCG ATG TTG TCC CGG TTC.

The data was analyzed by the primer express program (Applied Biosystems).

Statistical analysis

IC50 values for cytotoxicity tests were derived from nonlinear regression (curve fit) based sigmoidal dose response curve (variable) and computed using Graph Pad Prism 5 (Graph pad, San Diegro, CA, USA).

RESULTS AND DISCUSSIONS

Phytochemical analysis

Phytochemical analysis of *C.citratus* revealed the presence of alkaloids, tannins, terpenoids, saponins, flavanoids, which is in accordance with previously reported by Asaolu *et al.* (2009) and there was absence of carbohydrates and steroids in the plant extracts (Table 1).

Table 1: Phytochemical constituents in *Cymbopogon citratus*

SL.NO	TESTS	Presence/Absence
1	Alkaloid	+
2	Carbohydrate	-
3	Tannin	+
4	Terpenoids	+
5	Steroid	-
6	Saponin	+
7	Flavanoid	+

+ = Present; - = Absent

Recent studies have reported that the phytochemicals of *C.citratus* or its components can cause cell cycle arrest and apoptosis in various human cancer cell lines, suggesting that their growth inhibitory effects occur through G_1/S or G_2/M arrest and subsequent apoptosis (Lai *et al*. 2010).

Phytochemicals obtained from fruits, vegetables, nuts and spices, have drawn a considerable amount of attention due to their ability to suppress carcinogenesis and selectively kill tumor cells in preclinical animal models (Aggarwal & Shishodia, 2006; Russo, 2007; Naithani et al., 2008, Kaefer & Milner, 2008; Moiseeva & Manson, 2009). A large number of these plant-derived substances have been shown to significantly prevent or delay cancer development in several high risk populations (Kris et al 2002, Riboli & Norat, 2003; WCRF, 2007).

The alkaloids are secondary metabolites biosynthesized by the plants for a defensive role. A diverse group of alkaloids are low molecular weight, nitrogen-containing compounds isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both *in vitro* and *in vivo* (Carocho& Ferreira, 2013). The alkaloids target DNA replication or protein synthesis in the mechanism of action on tumor cells, resulting in apoptosis of the neoplastic cells. Alkaloids such as camptothecin and vinblastine have been successfully developed into anticancer drugs (Lu *et al.* 2012). Anticancer agents play a major role in inhibiting the enzyme topoisomerase which is involved in DNA replication, inducing apoptosis and expression of p53 gene (Mohan *et al.* 2012).

Tannins, on the other hand are of polyphenolic nature. The importance of plant-based tannins are in binding to proteins, basic compounds, pigments, large-molecular compounds and metallic ions and they also show anti-oxidant activities (Yildirim & Kutlu, 2015). Tannins exhibited anti-tumor and anticancer activity against murine mammary carcinoma cells (FM3A), human T-lymphocyte cells (Molt4/C8, CEM/0), HeLa cell and murine leukemia cells (L1210/0) (Pizzi, 2008). The hydrolyzable tannins showed higher cytotoxic activity against human oral squamous cell carcinoma and salivary gland tumor cell lines as reported by Sakagami *et al*. (2000). Apoptotic activity is increased in response to exposure to tannin extracts on breast cancer and prostate cancer cells (Bawadi *et al*. 2005).

Terpenoids (monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids) constitute the largest class of natural products and are a rich pool of candidate compounds for drug discovery. Recent efforts into the research and development of anti-cancer drugs derived from natural products have led to the identification of a variety of terpenoids which could inhibit cancer cell proliferation and metastasis through various mechanisms (Huang *et al.* 2012). Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, antiinflammatory, and immunomodulatory properties (Rabi & Bishayee 2009; Wagner & Elmadfa, 2003; Sultana & Ata, 2008; Shah *et al.* 2009).

Saponins from a structural viewpoint are composed of one or more hydrophilic glycoside moieties along with a lipophilic and derived aglycone and finally one or more sugar chains. Saponins prevent the proliferation of cancer cells by interfering with the replication of cellular DNA. Sun *et al*. (2009) reported that Saponins have got a number of pharmacological actions, such as immunomodulatory potential via cytokine interplay. A cytostatic and cytotoxic effect of saponins on malignant tumor cells was studied by Bachran *et al*.(2008). The potent anti-proliferative activity was exhibited by the saponin against MCF-7 human breast cancer cells and HT-29 human colon cancer cell (Beit-Yannai *et al*. 2011). The saponin with anticancer property could be a new alternative source of cancer treatment (Qian *et al*. 2014).

The flavonoids are the most important group of molecules within the phenolic compounds (Carocho& Ferreira, 2013). In many molecular mechanisms of

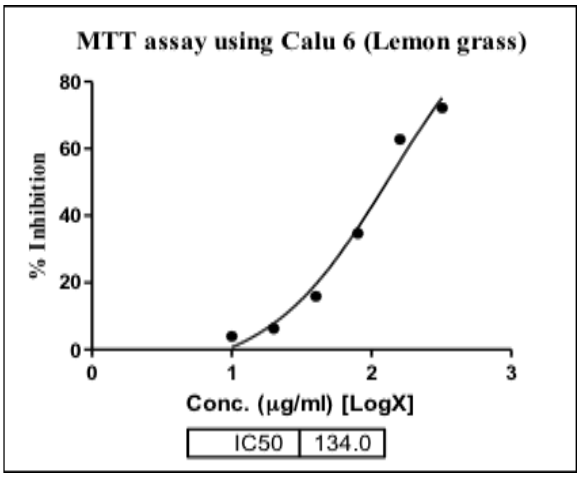
cancer prevention, flavonoids play a major role by interacting with different types of genes and enzymes. Many mechanisms of action have been identified, including antiproliferation, carcinogen inactivation, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis and reversal of multidrug resistance or a combination of these mechanisms (Chahar *et al.* 2011). The molecular mechanisms by which flavonoids induce apoptosis have not yet been clarified. Flavonoids derived from natural sources are known as angiogenesis inhibitors (Wang, 2000). The abilities of flavonoids to block solid tumor growth may be due to their inhibition of the neoangiogenic process.

MTT Assay

A preliminary assessment of the antiproliferative effect of extract of *C.citratus* on CALU 6 cells were studied and its inhibitory effects on cell growth were determined by MTT assay, which measures the metabolically live cells based on their mitochondrial dehydrogenase activity. CALU 6 cells were treated with various concentrations of C.citratus extracts (0 – 320 µg/mL) for 24 h and their growth inhibition was determined (Table 2 and graph. 1). The above extracts exhibited significant growth inhibition in a dose dependent manner in CALU 6 cells.

Table 2: Absorbance values and IC50 values of methanolic extracts of *Cymbopogon citratus* by MTT assay.

Cell line: Calu 6				
Plant Name	**Conc(µg/ml)**	**OD @590**	**%Inhibition**	**IC50**
	Control	0.57	0.00	
Cymbopogon citratus (Lemon grass)	10	0.55	3.97	134.0µg/ml
	20	0.53	6.33	
	40	0.48	15.91	
	80	0.37	34.72	
	160	0.32	62.78	
	320	0.18	72.17	



Graph. 1: Graph plotted for % Inhibition of CALU6 cells upon treatment concentration of *Cymbopogon citratus* (Lemon Grass) showing IC50 value.

The Absorbance values and IC50 values of methanolic extracts of *C. citratus* by MTT assay with percent inhibition values are shown in the Table -2. The IC50 values of of *C.citratus* were found to be 134.0µg/ml. on CALU 6 cells. The inhibition percentage ranged from 3.97 to 72.17 (Table 2). From the graph it is evident that percentage of inhibition increases with an increase in concentration. In case of *C. citratus* treatment, it showed more than 50% inhibition i.e. almost 70% inhibition at higher treatment concentration. The dose dependent inhibition of *C. citratus* on CALU6 cells increases with the concentration and reaches maximum at 320µg/ml. The inhibition activity may be due to the presence of phytochemicals like flavonoids, terpenoids etc, which play an important role for cytotoxicity having radical scavenging property (Perala *et al*. 2017). This confirms the effective anticancer activity of the plant *C. citratus* extract on CALU6 cancer cell line. A similar study was conducted by Halabi *et al.*(2014), in order to analyze the antiproliferative and antioxidant properties of *C. citratus* extract against different human cancer cell lines, human colon carcinoma(HCT-116), breast carcinoma (MCF-7 and MDA-MB-231), ovarian carcinoma (SKOV-3 and COVA) and a normal liver cell line (WRL-68).

Gene expression studies

Apoptosis is programmed cell death that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells. Initiator caspases activate

executioner caspases that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes. The morphological hallmarks of apoptosis result, including DNA fragmentation and membrane blebbing. Impaired apoptosis is one of the hallmarks of cancer. Caspase-3 and -8 are key regulators of the apoptotic response (Xuan *et al*. 2017).

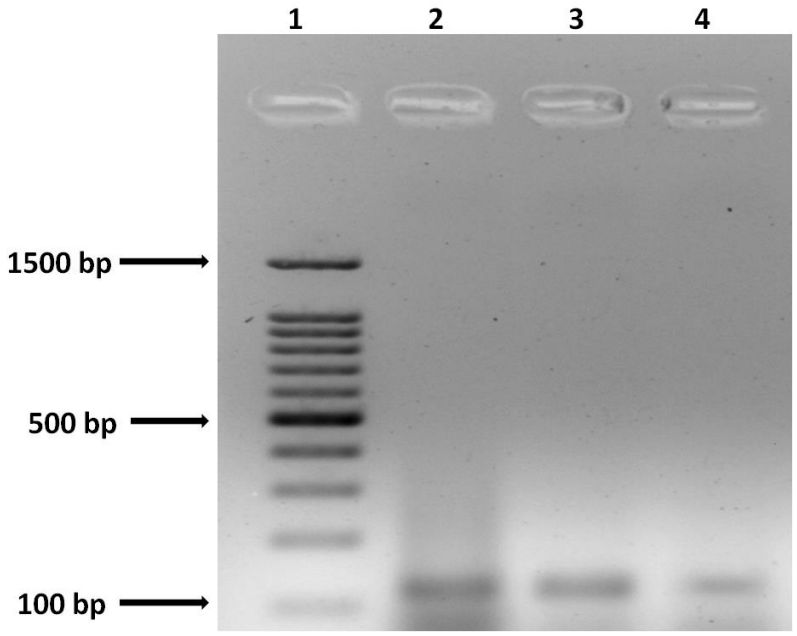


Figure 1: Gel image visualized under UV light. Amplification of CASPASE3 gene in CALU6 cells. Lane1- 1500 BP Marker; Lane2- Control Untreated Cells ; Lane3- Sample Treatment 80µg/ml; Lane4- Sample Treatment 160µg/ml.

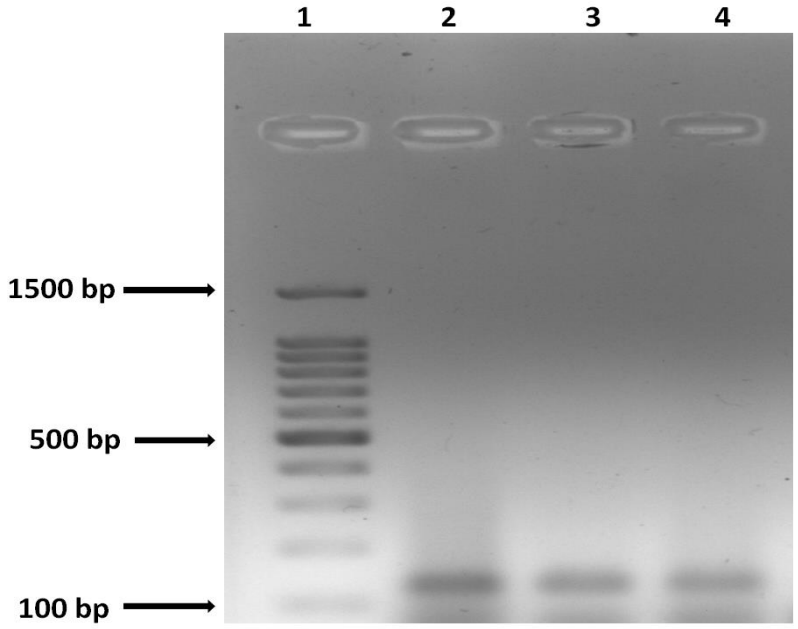


Figure 2: Gel image visualized under UV light. Amplification of CASPASE8 gene in CALU6 cells.Lane1-100 BP Marker; Lane2-Control Untreated Cells; Lane3-Sample Treatment 80µg/ml; Lane4-Sample Treatment 160µg/ml.

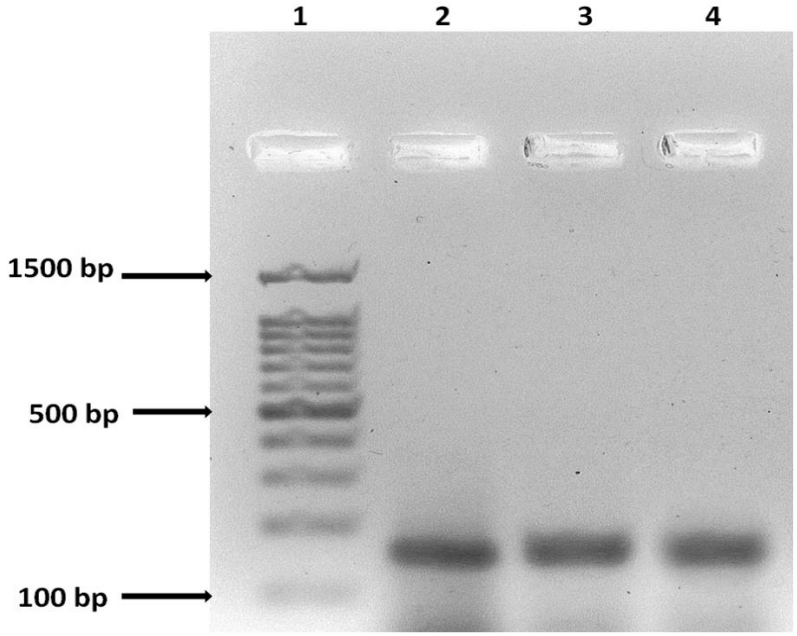


Figure 3: Gel image visualized under UV light. Amplification of β-Actin gene in CALU6 cells. Lane1- 100 BP Marker; Lane2- Control Untreated Cells; Lane3- Sample Treatment 80µg/ml; Lane4- Sample Treatment 160µg/ml.

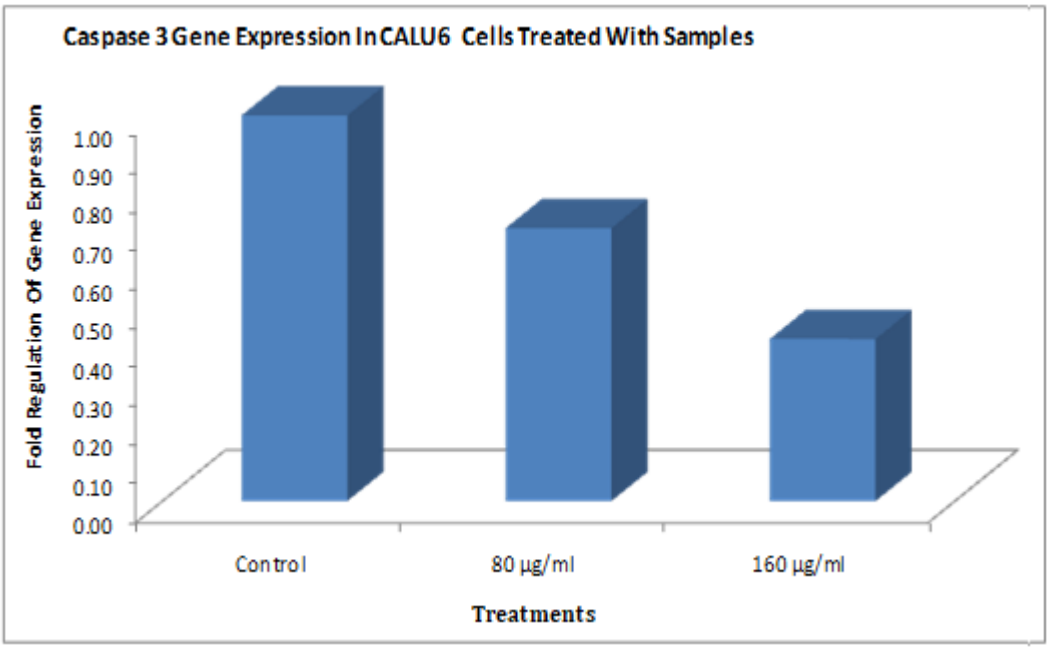
Table 3: Relative expression of CASPASE 3 in CALU6 cells treated with *Cymbopogon citratus* (Lemon Grass) and control untreated CALU6 cells.

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
	β-Actin	Caspase 3		
Control	44615.827	99741.295	2.24	1.00
80 µg/ml	39267.726	62132.902	1.58	0.71
160 µg/ml	40869.688	38413.425	0.94	0.42

β-actin is part of the cytoskeleton's microfilaments and is not supposed to be affected by experimental conditions. In Western blotting, they are run as a "loading control" to show that very similar amounts of protein were loaded in each lane. This is essential in some experimental systems where it may not be possible to always collect the exact same number of cells for each sample, such as when working with primary cells, tissue samples, or systems where you may be altering protein synthesis, cell size/number, or apoptosis.

In the present study β-actin, caspase-3 and caspase-8 are taken for the study of gene expression. Wherein β-actin is treated as the control in the experiment. Amplification of Caspase-3, Caspase-8 and β- actin genes in CALU6 cells are shown in figures 1, 2 & 3 respectively. In the gel image, first lane (1st well) contains 1500bp DNA marker/DNA ladder. DNA marker contains several segments of DNA with varying size and it is used for comparison of the bands. By such comparison we can roughly assume the band size on gel. The second lane (2nd well) contains DNA from control untreated cells, where the expression level of the genes will be normal. Third and fourth lane contains 80µg/ml and 160µg/ml treatment concentrations of *C. citratus* (figure 1 & 2). The gel images are then analyzed by software, ImageJ, which converts the bands into area plots.

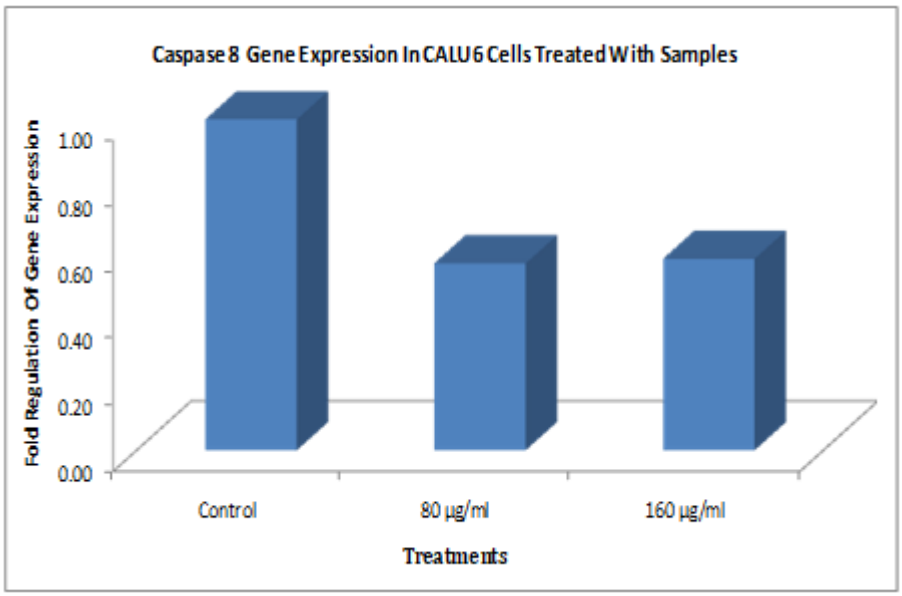
Relative gene expression of CASPASE 3 in CALU6 cells treated with *C.citratus* extract at different concentrations (80 µg/ml and 160 µg/ml) and control untreated CALU6 cells is shown in the table-3 and Graph-2 explaining the relative gene expression of caspase3 with the value of maximum 0.71 at 80 µg/ml and a minimum 0.42 at 160 µg/ml.



Graph 2: Graph showing expression levels of CASPASE3 gene in CALU6 cells treated with *Cymbopogon citratus* (Lemon Grass) and control untreated CALU6 cells.

Table 4: Relative expression of CASPASE 8 in CALU6 cells treated with *Cymbopogon citratus* (Lemon Grass) and control untreated CALU6 cell.

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
	β-Actin	Caspase 8		
Control	44615.827	89433.345	2.19	1.00
80 µg/ml	39267.726	48532.789	1.24	0.56
160 µg/ml	40869.688	56492.375	1.27	0.58



Graph 3: Graph showing the expression level of CASPASE8 gene in CALU6 cells treated with *Cymbopogon citratus* (Lemon Grass) and control untreated CALU6 cells.

Relative gene expression of CASPASE 8 in CALU6 cells treated with *Cymbopogon citratus* (Lemon Grass) at different concentrations of 80 µg/ml and160 µg/ml and control untreated CALU6 cells is shown in the table- 4 and the graph-3 explaining the lower activity of caspase-8 with the value

The results presented here on cell growth inhibition of CALU6 by *C. citratus* extracts were further supported in the light of the antiproliferative effects of the plant's phytochemical constituents as discussed before. Our results revealed that treated cells showed decrease in Caspase-3 and Caspase-8 gene expression as compared to the control/untreated cells. *C. citratus* was found to suppress the expression of Caspase-3 and Caspase-8 genes, which contradicts the previous report of Dudai *et al.*(2005), stating that the mechanism of inhibition is mostly accompanied by DNA fragmentation and Caspase-3 catalytic activity induction. According to our phytochemical analysis, MTT assay and gene expression studies, we found that *C. citratus* is more effective in the treatment of lung cancer and the inhibition of cell proliferation occurred through Caspase-independent pathway. The apoptotic effect of the extracts of *C.citratus* significantly inhibited the caspase 3 and caspase 8 activity in CALU6 cells as shown in Table 3 & 4 and Graph 2&3.

The modulation of apoptosis signaling pathways by natural compounds have been demonstrated to constitute a key event in anticancer activities (Fulda 2010). In the present study, it was established that the *C.citatus* is rich in phytochemicals which has potent antiproliferative activity on CALU 6 cells. This gives us scope for further studies to identify the specific pathway involved in the inhibitory activity of *C. citratus* and caspase independent apoptosis against lung cancer.

CONCLUSION

From the present investigation it is concluded that the phytochemicals of the *Cymbopogon citratus* (Lemon grass) have shown the effective anticancer activity on lung cancer CALU6 cell line found to suppress the expression of Caspase-3 and Caspase-8 genes, which occurred through Caspase-independent pathway. The observed efficacy could probably be due to the phytochemical constituents of the plant *C.citratus* such as flavonoids, terpenoids etc., are responsible for the antiproliferative activity. Hence, we can isolate some pure phytochemicals, which in turn can be used as lead molecules for synthesizing the novel agents having good therapeutic activity. With regard to the development of quality herbal medicine, standardization of the extracts, elucidation of the mechanism of action of the isolated compounds and their clinical trials are much needed. In the changing global scenario, the interest towards medicinal plants is increasing substantially in the primary healthcare system both in developed and in developing countries. Therefore, the information will help the scientists and researchers to elucidate the molecular mechanism of action of phytochemicals responsible for different bioactivities to treat cancer and other diseases.

Competing interests

Authors have declared that no competing interests exist.

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