Pharmacognosy Communications
Volume 3 | Issue 4 | Oct–Dec 2013
www.phcogcommn.org

Research Article

Anticancer effect of lemongrass oil and citral on cervical cancer cell lines

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ABSTRACT: **Aims:** The aim of the present study was to evaluate the anticancer effect of lemongrass oil and citral emulsion on cervical cancer cell lines (HeLa and ME-180) *in vitro*. **Settings and Design:** Citral is a very important component in lemongrass oil. It is proved to have anticancer properties in various human cancer cell lines. **Methods and Material:** DLS analysis revealed the average size of the lemongrass oil emulsion to be 267 nm and the average size of the citral emulsion to be 270 nm. The anticancer effect of both the emulsions was determined by MTT assay, DCFH-DA method, Rh-123 and AO/EtBr-staining. **Statistical analysis used:** One-way ANOVA followed by DMRT taking $p < 0.05$ to test the significant difference between groups. **Results:** The results summarize that lemongrass oil and citral emulsions initiate the cancer cell death by decreasing cell proliferation, increasing intracellular ROS, altering mitochondrial membrane potential, and initiating apoptosis in HeLa and ME-180 cell lines. The present findings of this study clearly demonstrate the involvement of oxidative mechanism for the anti-proliferative effect in HeLa and ME-180 cell lines. ME-180 being chemosensitive showed good results at lower concentrations of citral (IC_{50} 24 h 300 µg/ml), as compared to chemoresistant HeLa cells (citral IC_{50} 24 h 500 µg/ml). Whereas lemongrass oil exhibited better activity in both the cell lines (IC_{50} 24 h 200 µg/ml). **Conclusions:** All the results suggest lemongrass oil and citral emulsion could be considered as potent candidates for anticancer agents.

KEYWORDS: Lemongrass oil, Citral, Emulsion, Anticancer, HeLa, ME-180

KEY MESSAGE: Lemongrass oil and citral emulsion are potent candidates for anticancer ointment based drugs.

INTRODUCTION

Cervical cancer is the third most common cancer in women, and the seventh overall, with an estimated 530,000 new cases in 2008. More than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancers. In India, cervical cancer is the first threat after breast and ovarian cancer.[1] The options for treating each patient with cervical cancer depend on the stage of disease. The stage of a cancer describes its size, depth of invasion (how far it has grown into the cervix), and how far it has spread.[2] Early-stage cancer that is confined to the cervix, offers an excellent outlook, with a success rate of over 85%. However, if the cancer has spread to the vagina, surroundings tissues and pelvic area, or elsewhere, the outlook is less positive.[3]

Several natural products are nowadays employed as effective anticancer agents. In the last two decades the search for novel anticancer agents from natural sources has witnessed an impressive increase of interest. The genus *Cymbopogon* (family Gramineae) has many species of grasses that grow in tropical and subtropical regions around the world from mountains to grasslands to arid zones.[4] These plants produce essential oils with pleasant aromas in their leaves. Five species yield the three oils of main commercial importance: lemongrass from *Cymbopogon citratus* of Malaysian origin (West Indian lemongrass) and *Cymbopogon flexuosus* (East Indian lemongrass) from India, Sri Lanka, Burma, and Thailand; palmarosa oil from Cymbopogon martinii; citronella oil from *Cymbopogon nardus* (Sri Lanka), and *Cymbopogon winterianus* (Java). *Cymbopogon flexuosus* (also known as East India or Cochin lemongrass) is a perennial,

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DOI: 10.5530/pc.2013.4.6

multicut aromatic grass that yields an essential oil used in perfumery and pharmaceutical industries and vitamin A.[5]

The plants of lemongrass *Cymbopogon citratus* and *C. flexuosus* are medium-sized grasses that are commercially grown for essential oil distillation in Guatemala (*C. citratus*) and Cochin, India (*C. flexuosus*). The fresh grass is cut and allowed to wilt before it is used for steam-distilled oil production. Oils from different regions have somewhat different compositions, Guatemalan West Indian (W.I.) having slightly different citral content than East Indian (E.I.) Cochin origin. Lemongrass oil is mobile, pale yellow in color, with a powerful, fresh, floral-herbal odor that is quite prominent.[6]

Cymbopogon flexuosus oil helps with stress-related disorders, and has shown to have antifungal and antimicrobial properties.[7] The chemical composition of the oil has also been reported.[6] The various constituents (%) present in the oil from lemongrass variety of *C. flexuosus* such as geraniol (20.08), geranyl acetate (12.20), α-bisabolol (8.42) and isointermedeol (24.97) have been individually reported for their cancer cell cytotoxicity.[6,8]

The essential oil from a lemongrass variety of *Cymbopogon flexuosus* (CFO) and its major chemical constituent sesquiterpene isointermedeol (ISO) were investigated for their ability to induce apoptosis in human leukemia HL-60 cells, because deregulation of apoptosis is the hallmark of cancer cells. CFO and ISO inhibited cell proliferation with IC_{50} of ~30 and 20 μg/ml, respectively.[6] Two active compounds, d-limonene and geraniol, were isolated by fractionation of lemongrass (*C. citratus*) oil. These were tested for their capacity to induce activity of the detoxifying enzyme glutathione-S-transferase (GST) in several tissues of the female A/J mice. d-Limonene increased GST activity two- to three fold than controls in the mouse liver and the mucosa of the small and large intestines. Geraniol showed high GST-inducing activity in the mucosa of the small and large intestines, which was about 2.5-fold greater than controls. Induction of increased GST activity, which is believed to be a major mechanism for chemical carcinogen detoxification, has been recognized as one of the characteristics of the action of anticarcinogens.[9] The essential oil from *C. citratus* and its isolated principal citral have been tested for cytotoxicity against P388 leukemia cells. The cytotoxicity of citral, IC_{50} against P388 mouse leukemia cells was 71 μg/ml. In another experiment, IC_{50} of C. citratus oil in P388 leukemia cells was found to be 5.7 μg/ml.[10] A study also showed that the oil from *Cymbopogon flexuosus* induced differential *in vitro* cytotoxicity in 12 human cancer cell lines and *in vivo* tumor growth inhibiton in murine, Erlich and S-180 tumor models. The oil also caused dose dependent increase in apoptosis in HL-cells.[11] The lemongrass oil used for the study had density of 0.89 g/ml.

Citral (figure 1), or 3, 7-dimethyl-2,6-octadienal ($C_{10}H_{16}O$), is a mixture of two isomeric acyclic monoterpene aldehydes. The two compounds are double bond isomers. The *trans*-citral is known as geranial or citral A. The *cis*-citral is known as neral or citral B.[5] Geranial has a strong lemon odor. Neral has a lemon odor that is less intense and sweeter than geranial. Citral is an aroma compound used in perfumery for its citrus effect. Citral is also used for flavoring and fortifying lemon oil. It also has strong antimicrobial qualities[12] and pheromonal effects in insects.[13,14] Citral is used in the synthesis of vitamin A, ionone, and methylionone, and to mask the smell of smoke.[5]

Citral has been found to be a potent inducer of glutathione-*s*-transferase class of enzymes, which provide protection to healthy hepatocytes against apoptosis during chemotherapy of liver cancers.[15] A good superoxide scavenging activity (EC_{50} = 19 mcg/ml) was reported in Swiss albino mice in citral treated groups, suggesting that the antioxidant action could be responsible for the anti-clastogenic effect of citral against nickel chloride.[16] Citral, at a concentration comparable to that found in a cup of tea brewed with 1 gram of lemongrass, was found to induce apoptosis in cancer cells, without any harm to normal cells. Apoptosis was accompanied by DNA fragmentation and caspase-3 catalytic induction.[17] Citral also disrupts animal microtubules and inhibits polymerization of microtubules *in vitro*.[18] Citral was also tested on cyclooxygenase activity. Citral treatment also caused inhibition of breast cancer MCF-7 cell growth (IC_{50} -48 h: 18×10^5 m) with a cycle arrest in G_2/M phase, apoptosis induction and

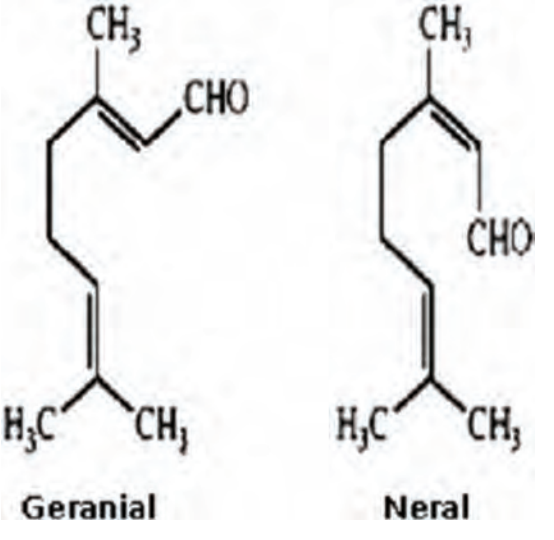

Figure 1. Citral isomers.

also a decrease in prostaglandin E2 synthesis. These findings suggested that citral has a potential chemopreventive effect.[19] The citral used for the study had density of 0.89 g/ml.

The aim of the present study was to evaluate the anticancer effect of lemongrass oil and citral emulsion on cervical cancer cell lines (HeLa and ME-180) *in vitro*.

SUBJECTS AND METHODS

Chemicals

Citral (0.89 g/ml) was purchased from Sigma chemical Co., St. Louis, USA. Lemongrass oil was purchased from Varsha Aromatics, Chennai. Other chemicals for cell cultures were purchased from Himedia, Mumbai. All other chemicals and solvents were of analytical grade and obtained from S.D fine chemical, Mumbai and Fisher Inorganic and Aromatic Limited, Chennai.

Determining the density of lemongrass oil

Density of the lemongrass oil was measured using specific gravity bottle. Density of the oil was calculated as follows: Density = Mass/Volume

Where, Mass = (Substance + Bottle Weight) – (Empty Bottle Weight)
Volume = 10 ml

Determination of citral content

The citral content in lemongrass oil was determined by sodium bisulphate method (IS 327: 1991 Oil of Lemongrass–Specification).

Preparation of emulsion

Emulsion formation method was used as described by Hart *et al.,* 2000.[20]

Particle size and size distribution

The Lemongrass oil and citral emulsion drop mean size and distribution were measured with a dynamic light scattering (DLS) instrument (Zetasizer Nano, Malvern Instruments Ltd. United Kingdom). Lemongrass oil and citral were sonicated in ultrapure water before measurement. The analysis was performed at a scattering angle of 173° at a temperature of 25 °C. The wavelength of the laser used in the Zetasizer Nano instruments for the measurement, 632.8 nm 'red' laser wavelength.

Cell lines and culture conditions

The cervical cancer cell lines (HeLa and ME-180). were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown as monolayer in MEM medium supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37 °C in 5% CO_2 atmosphere.

Dose fixation study

Cells were treated with lemongrass oil and citral in a concentration range of 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 µg/ml, and incubated for 24 h. The cytotoxicity was observed by MTT assay according to the method of Moshmann.[21] IC_{50} values were calculated and optimum dose was used for further study.

Experimental groups

Cervical cancer cells (HeLa and ME-180) were divided into three groups; in each group six samples were processed (Table 1).

Determination of intracellular ROS generation

ROS was measured by using a non-fluorescent probe, 2, 7-dichlorodihydrofluorescein diacetate (H_2-DCFDA) that can penetrate into the intracellular matrix of cells, by the method of Bhosle *et al.*, 2005.[22]

Change in mitochondrial transmembrane potential ($\Delta\psi_m$)

The change in ψm in different treatment groups was observed microscopically and determined using fluorescent dye Rh-123 by the method of Prasad *et al.*, 2010.[23]

Apoptotic morphological changes

Acridine orange (AO) and ethidium bromide (EtBr) staining of DNA allowed visualization of the condensed chromatin of dead apoptotic cells and was determined by the method of Lakshmi *et al.*, 2008.[24]

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by DMRT taking $p < 0.05$ to test the significant difference between groups.

RESULTS

Density of lemongrass oil

The lemongrass oil used for the study had density of 0.89 g/ml.

Table 1: Experimental design

Group 1: Control (HeLa)	Group 1: Control (ME-180)
Group 2: HeLa + Lemongrass oil (200 µg/ml)	Group 2: ME-180 + Lemongrass oil (200 µg/ml)
Group 3: HeLa + Citral (500 µg/ml)	Group 3: ME-180 + Citral (300 µg/ml)

Determination of citral content

78.5% of citral was found to be present in the lemongrass oil used in the study. The sample used thus conforms to I.S. 327: 1991 reaffirmed 2002 specification.

Particle size distribution and size distribution

DLS results revealed that the average size of the lemongrass oil emulsion was 267 nm (figure 2A) and the average size of the citral emulsion was found to be 270 nm (figure 2B).

Dose fixation study

Inhibitory concentration 50 (IC_{50}) value for lemongrass oil and citral was found to be 200 µg/ml and 500 µg/ml for HeLa cell line, and 200 µg/ml and 300 µg/ml for ME-180 cells respectively, and it was used for further experiments (figures 3 A and B). Figure 3 C and D shows the microscopic images showing the morphological changes of HeLa and ME-180 cells respectively, during treatment with IC_{50} concentrations of lemongrass oil and citral. At these respective IC_{50} concentrations, 50% of cell death was observed in HeLa and ME-180 cell line.

Emulsions of lemongrass oil and citral increased ROS generation in HeLa and ME-180 cells

Lemongrass oil and citral treatments significantly increased ROS level in HeLa and ME-180 cells. Among all the

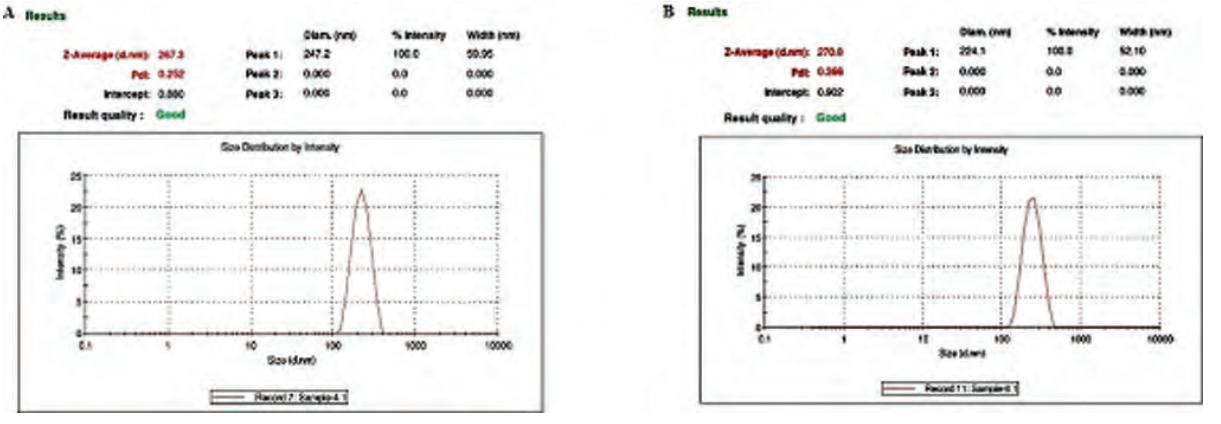

Figure 2. (A and B): Average size of the lemongrass oil and citral emulsion.

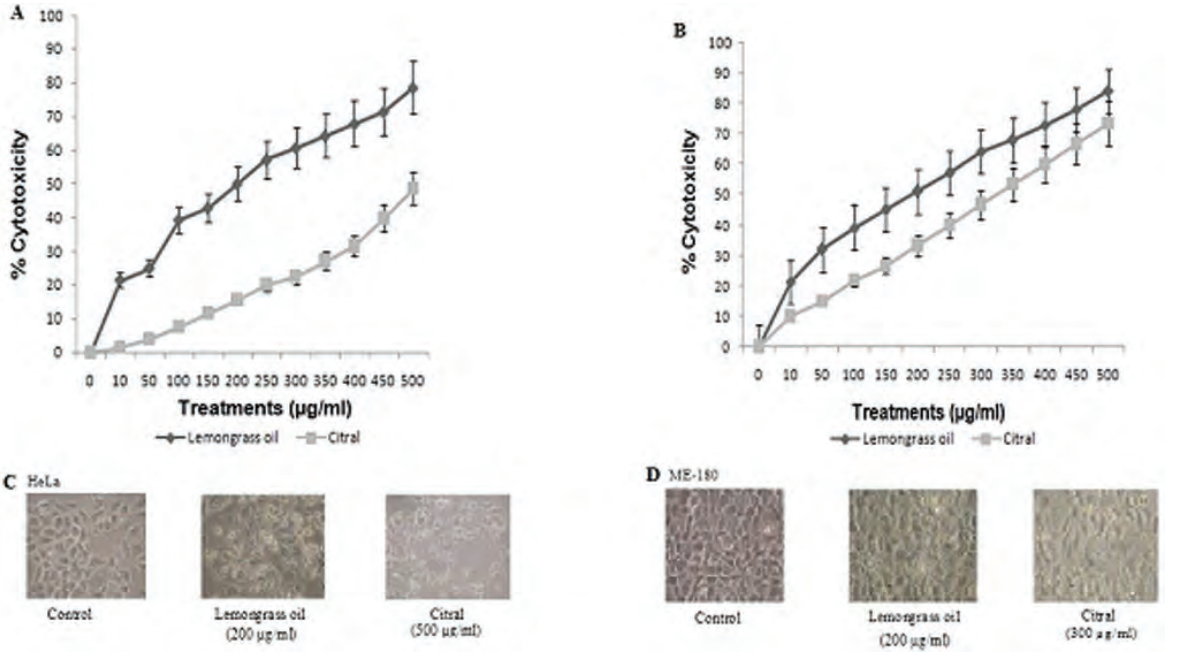

Figure 3. (A and B) Percentage cytotoxicity of lemongrass oil and citral in HeLa and ME-180 cell lines. Inhibitory concentration 50 (IC_{50}) value for lemongrass oil and citral was found to be 200 µg/ml and 500 µg/ml for HeLa cell, and 200 µg/ml and 300 µg/ml for ME-180 cells respectively, and it was used for further experiments. The values are given as mean ± SD of six experiments in each group. Figure 3 (C and D) Microscopic images showing the morphological changes of HeLa and ME-180 cells respectively, during treatment with IC_{50} concentrations of lemongrass oil and citral.

doses tested, 200 µg/ml of lemongrass oil in HeLa cells, and 200 µg/ml of lemongrass oil ME-180 cells showed maximum generation of ROS (increased fluorescence) (figures 4 A and B). Fluorescence microscopic images (figures 4 C and D) confirm the DCF fluorescence in lemongrass and citral treated groups.

Emulsions of lemongrass oil and citral modulate mitochondrial membrane potential in HeLa and ME-180 cells

Mitochondrial membrane potential was decreased in lemongrass oil and citral treated groups compared to control (figures 5 A and B). Fluorescence microscopic images (figures 5 C and D) show the accumulation of Rh-123 dye (red condensed spots) in control and the accumulation have been found to be decreased in lemongrass oil and citral groups as the membrane potential decreased.

Effect of lemongrass oil and citral emulsions on apoptotic morphology

The figure 6 (C and D) shows the photomicrographs of apoptotic morphological changes in lemongrass oil and citral treated cells. The % apoptotic cell death was increased in citral and lemongrass oil (increased orange colored cells) when compared to control. Figure 6 (A and B) show the quantitative result of apoptosis in different treatment groups. HeLa cells showed 95% apoptotic cells in lemongrass oil and 80% in citral treatment groups. ME-180 cells showed 98% apoptotic cells in lemongrass oil and 90% in citral treatment groups.

DISCUSSION

In this present study, we evaluated the anticancer effect of lemongrass oil and citral on cervical cancer cell lines (HeLa and ME-180) *in vitro*. Lemongrass oil and citral treatment (24 h incubation) significantly decreased percentage of cell viability in HeLa and ME-180 cells. This suggested that lemongrass oil and citral treatment was able to inhibit the growth of cancer cells during incubation. It was found that (IC_{50}) 200 µg/ml ($P > 0.05$) of lemongrass oil could greatly inhibit the cell growth in both the cell lines. Whereas (IC_{50}) 500 µg/ml and 300 µg/ml ($P > 0.05$) concentration of citral was required for good inhibition of HeLa and ME-180 cells growth respectively. Hence, the result indicates that concentration of phytochemicals play a vital role for cytotoxicity. Previous study supports the findings of this study, that citral inhibits P388 mouse leukemia cells[10] and oil from *Cymbopogon flexuosus* induced differential *in vitro* cytotoxicity in 12 human cancer cell lines and *in vivo* tumor growth inhibiton in murine, Erlich and S-180 tumor models. The oil also caused dose dependent increase in apoptosis in HL-cells.[11] Mitochondrial activity of cancer cells may be influenced by lemongrass and citral, and this might be the reason for the increased cytotoxicity observed in lemongrass oil and citral treated cells. This hypothesis was confirmed by evaluating the alteration in mitochondrial membrane potential in treated groups. It was observed that mitochondrial membrane potential was decreased in lemongrass oil and citral and treated groups compared to

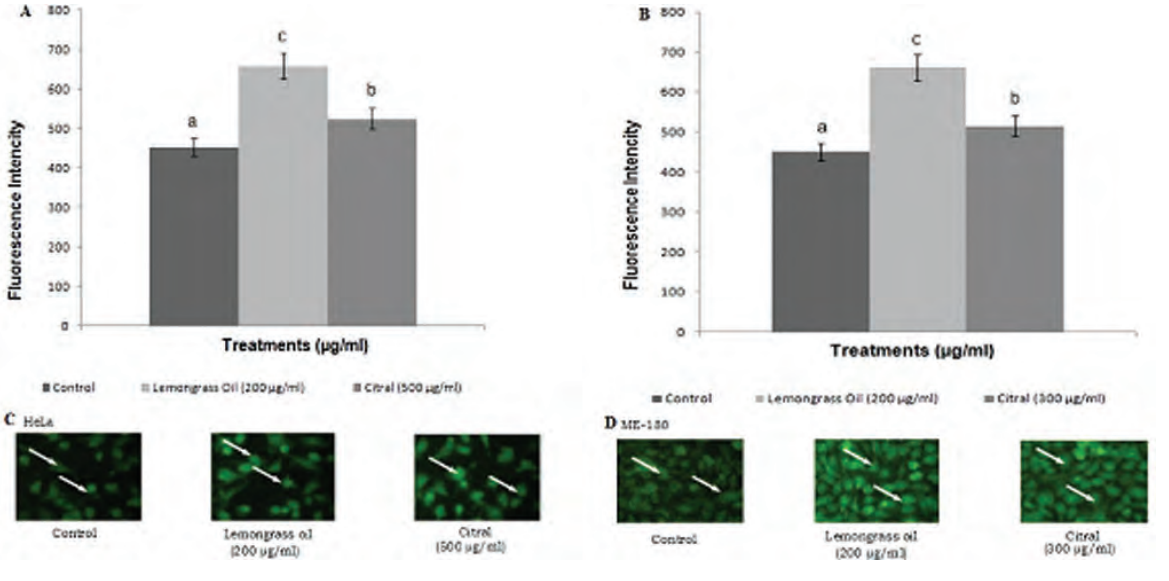

Figure 4. (A and B) Effect of lemongrass oil and citral on ROS in HeLa and ME-180 cells. The values are given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Bars not sharing the common superscripts differ significantly at $p < 0.05$ (DMRT). Figure 4 (C and D) Fluorescence microscopic images showing changes in the levels of ROS generation in lemongrass oil and citral treated ME-180 cells. Arrow mark (→) represents high DCF fluorescence.

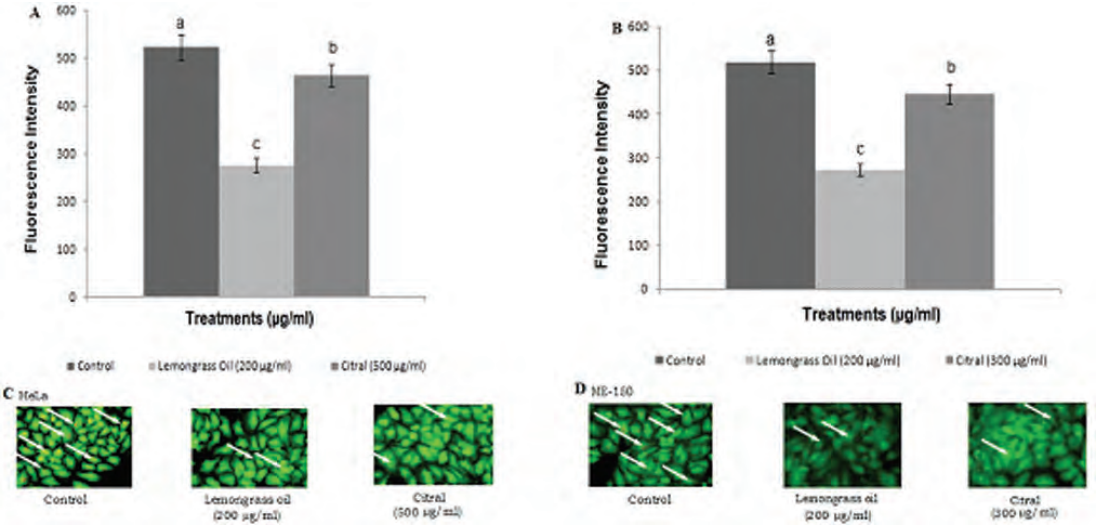

Figure 5. (A and B) Effect of mitochondrial membrane potential in lemongrass oil and citral treated HeLa and ME-180 cells respectively. The values are given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Bars not sharing the common superscripts differ significantly at $p < 0.05$ (DMRT). Figure 5 (C and D) Fluorescence microscopic images showing the alteration of MMP by Rh-123 staining in lemongrass oil, citral and cisplatin treated cells. Arrow marks (→) represents dye accumulation.

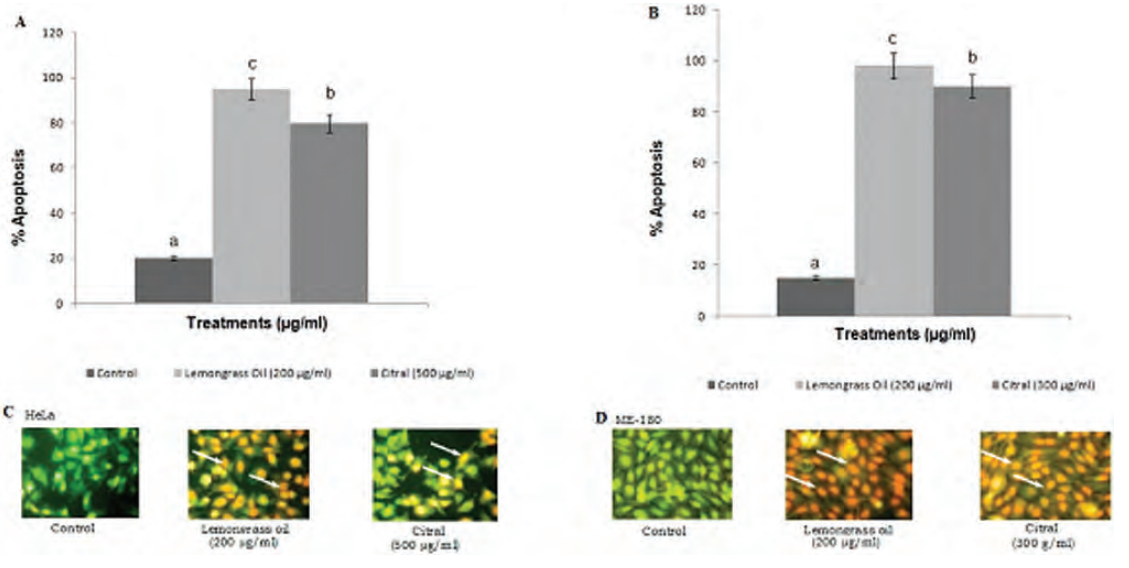

Figure 6. (A and B) Effect of apoptotic morphological changes in lemongrass oil and citral treated HeLa and ME-180 cells respectively. The values are given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Bars not sharing the common superscripts differ significantly at $p < 0.05$ (DMRT). Figure 6 (C and D): Fluorescence microscopic images showing the apoptotic morphological changes by dual staining in lemongrass oil and citral treated HeLa and ME-180 cells respectively. Arrow mark (→) represents orange-colored cells which are late apoptotic cells.

control. The results obtained in this experiment showed that lemongrass oil had higher activity than citral for both the cell lines. Further an increase in ROS production had been observed in lemongrass oil and citral when compared to control groups. ROS production is a mechanism shared by all non-surgical therapeutic approaches for cancers, including chemotherapy, radiotherapy and photodynamic therapy, due to their implication in triggering cell death. Therefore ROS are good tools to kill cancer cells.[25] Several naturally occurring antioxidants have been reported to be anti-mutagenic/anticarcinogenic in the literature such as, reservertrol.[26] It had been reported that citral, in a dose dependent manner inhibited the oxidative process involved in the formation of free radicals in nickel chloride treated mouse.[16] Increased ROS levels are thought to constitute an essential step in cell death induction by many different cytotoxic drugs. In the present work, it was found that lemongrass oil emulsion at a

concentration of 200 μg/ml in HeLa cells and ME-180 cells showed maximum generation of ROS ($p > 0.05$).

The previous experiment showed a decrease in mitochondrial potential in treated groups compared to control. Mitochondrion is one of the most important organelles in regulating cell death as well as a maker in apoptosis.[27] This was proved by observing the apoptotic morphology of treated and non treated groups using dual staining methods. The percentage apoptotic cell death was increased in lemongrass oil and citral emulsion when compared to control. HeLa cells showed 95% apoptotic cells in lemongrass oil emulsion and 80% in citral emulsion treatment groups. ME-180 cells showed 98% apoptotic cells in lemongrass oil emulsion and 90% in citral emulsion treatment groups. Citral has been found to be a potent inducer of glutathione-*s*-transferase class of enzymes, which provide protection to healthy hepatocytes against apoptosis during chemotherapy of liver cancers. A good superoxide scavenging activity (EC_{50} = 19 mcg/ml) was reported in Swiss albino mice in citral treated groups, suggesting that the antioxidant action could be responsible for the anti-clastogenic effect of citral against nickel chloride.[16] Citral, at a concentration comparable to that found in a cup of tea brewed with 1 gram of lemongrass, was found to induce apoptosis in cancer cells, without any harm to normal cells. Apoptosis was accompanied by DNA fragmentation and caspase-3 catalytic induction.[17] Another study also reported that citral disrupts animal microtubules and inhibits polymerization of microtubules *in vitro*.[18] Citral was also tested on cyclo-oxygenase activity. Citral treatment also caused inhibition of breast cancer MCF-7 cell growth (IC_{50} -48 h: 18×10^5 m) with a cell cycle arrest in G_2/M phase, apoptosis induction and also a decrease in prostaglandin E 2 synthesis. These findings suggested that citral has a potential chemopreventive effect.[19] Thus, citral seems to have the ability to turn on the apoptosis process in cancer cells, causing them to die. It also disrupts animal microtubules, acts as antioxidant in normal cells and selectively behaves as pro-oxidant in cancer cells.[16,18] These characteristics of citral make it a very potent candidate as anticancer agents.

The various constituents (%) present in the oil from lemongrass variety of *C. flexuosus* such as geraniol (20.08), geranyl acetate (12.20), α-bisabolol (8.42) and isointermedeol (24.97) have been individually reported for their cancer cell cytotoxicity.[8,6,29,30] Another report showed that the essential oil from *Cymbopogon flexuosus* (CFO) and its major chemical constituent sesquiterpene isointermedeol (ISO) induced apoptosis with in 48 h IC_{50} of ~30 and 20 μg/ml, respectively, in human leukemia HL-60 cells.[6] The activities of essential oils have always been credited to its synergistic effects of its complex mixtures of various compounds.[31] Synergistic anticancer effects have also been reported for crude extracts of *Polyalthia evecta*, which induced apoptosis in HepG2 cells.[32] Essential oils are complex mixtures of chemical constituents, thus more than one chemical constituent in lemongrass oil can cause cancer cell death and is thus a very promising candidate for anti-cancer therapies. These findings support that there may be synergy between the citral and other components with anticancer activity in lemongrass oil and also explain the reason for it to show more potent anticancer activity than citral alone.

Our results summarize that lemongrass oil and citral emulsion decreased cell proliferation, increased intracellular ROS, altered mitochondrial membrane potential, and induced apoptosis in HeLa and ME-180 cell lines. Hence, lemongrass oil and citral emulsion can be considered as potent anticancer agents and could be useful in chemotherapy of cervical cancer *in vitro*. However, further investigation warrants proving their *in vitro* anticancer efficacy in *in vivo* models, and understanding the various properties of lemongrass oil and citral emulsions for the production of ointment based anticancer drugs, which can be used *in sit*.

ACKNOWLEDGEMENT

None

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