ORIGINAL PAPER

https://dx.doi.org/10.4314/njpr.v18i1.1

Nig. J. Pharm. Res. 2022, 18(1) pp 1-9
ISSN 0189-8434 e-ISSN 2635-3555

Available online at http://www.nigjpharmres.com

Chemical Composition and Cytotoxic Activity of *Callistemon citrinus* (Myrtaceae) Volatile Oil and Active Fraction

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A – Research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Volatile oils have found use locally in the management of many diseases including tumor-related ailments. Due to the short-comings of orthodox medicine, there is a need to source for alternative drugs with better effects. *Callistemon citrinus* oil contains some bioactive compounds which are useful in treating many diseases. This study was designed to examine the chemical composition and cytotoxic efficacy of this plant oil.

Method: *Callistemon citrinus* volatile oil was extracted from fresh leaves using a Clavenger apparatus by hydro-distillation method. Preliminary cytotoxic screening was carried out with brine shrimp at 10-1000 µg/mL. The essential oil was further tested on breast (AU 565) and cervical (HeLa) cancer cell lines at 50 µg /mL using MTT assay. Column chromatography of the oil was carried out and the resulting fractions subjected to biological testing. GCMS analysis was carried out on the oil and the most active fraction.

Results: The oil produced concentration- dependent activity with an LC_{50} of 528.48 µg/mL in the brine shrimp mortality assay. The oil also produced -7.60 and +11.80 % inhibitions against HeLa and AU 565 cells respectively. Column fraction F_1 produced the highest activity against AU 565 cells with 70.44 % inhibition and an IC_{50} of 15.96 µg/mL. *C. citrinus* oil revealed the presence of cineole (36.06 %) and α-pinene (21.41 %) as the major components while 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-ethanone (24.89 %.) was the most abundant in the active fraction.

Conclusion: *C. citrinus* volatile oil has cytotoxic potential and is a good candidate for further *in vivo* studies.

Keywords: *Callistemon citrinus*, Brine Shrimp, Cytotoxic, MTT

INTRODUCTION

Cancer is a disease that has been found to be on the rise world-wide and is characterized by poor prognosis especially if it is not detected early (Ferlay *et al*., 2015). Certain orthodox treatments are applied in the management of this disease but are burdened with shortcomings such as high cost, inaccessibility and severe side effects. Other treatment alternatives which address the short comings of orthodox medicine are presently in demand (Rashid *et al*., 2002).

The use of medicinal plants in the treatment of various diseases is on the increase globally. Up to 80 % of the world's population presently uses herbs as source of medicine due to their availability, accessibility, cost effectiveness as well as safety. Phytochemical constituents naturally found in these plants have been adjudged responsible for the bioactivity they displayed either in human or animals (Sharma *et al*., 2011a;

Solowey *et al*., 2014). While the constituents are the secondary metabolites like saponins, alkaloids, various forms of glycosides with reported activities, volatile oils obtained from aromatic plants have also been documented to have a wide array of pharmacological activities including anticancer activities (Bou *et al*., 2013; Yousefzadi *et al*., 2014).

Some plants in Myrtaceae family have been reported to have anticancer effects. Leaf extract of *Psidium guajava* L. (Myrtaceae) reportedly induced cell death in prostate PC-3 cancer cell lines (Ryu *et al*., 2012), breast MCF-7, leukemia P388, cervical KB, and HeLa cell lines (Corrêa *et al*., 2016). *Eugenia stipitata* volatile oil displayed cytotoxic activity against melanoma and breast cancer cells (da Silva *et al*., 2017) while *Myrcia splendens* oil reportedly demonstrated cytotoxic activity against gastric, melanoma, and colon human cancer cells (Scalvenzi *et al*., 2017).

Callistemon citrinus (Curtis, Skeels) which is commonly known as crimson bottlebrush, red bottlebrush or lemon bottlebrush also belongs to the family Myrtaceae. It is an ornamental plant found in Asia, Queensland, New South Wales, South America and Australia (Oyedeji *et. al*., 2009). In ethnomedicinal practice in some parts of India, hot water infusion of the plant is used in treating tuberculosis, bacterial, and fungal diseases. It is also used as an anti-inflammatory agent, insecticide, for urinary incontinence and cleansing genitourinary tract (Sutar, 2014; Tabuti *et. al*., 2010). The plant has been scientifically documented to possess antimicrobial (Oyedeji *et al*., 2009), relaxant (Ali *et al*., 2011), cardioprotective (Momin *et al*., 2011) antidepressant (Pendyala and Thaarkur, 2017) effects as well as cytotoxicity against food-borne pathogens and MCF-7 cell line (Fayemi *et al*., 2019).

Lopez-Mejia *et al*. in 2019 reported that tumors of rats administered *C. citrinus* leaves extract were observed to reduce in size significantly. The essential oils from the leaves and flowers of the plant have been reported to have activity against several cancer cell lines namely human lung carcinoma (A549), rat glioma (C-6), human colon cancer (Colo-205) and human cervical cancer (SiHa) cells (Kumar *et al.*, 2015). This same study report evaluated apoptosis induction by caspase-3/7 activity which was further confirmed by western blotting. Anticancer activity of crude extracts of the plant against cancer cell lines such as A431, MG-31 and HaCaT (Sampath *et al.*, 2017) is also described in literature. The compound 1,8 cineole was found to be the major component in one of the n-hexane extracts analyzed. In addition, the anticancer activity of nanoparticles of phenolic extract of the plant against three breast-cancer cell lines have been reported (Ahmed *et al.*, 2019).

The report in literature of the cytotoxic property of the volatile oil of *Callistemon citrinus* especially on some cancer cells and reduced toxicity on non-cancerous cells suggested potential of the plant oil in some forms of cancer. Hence, fractionation of the oil is necessary to identify the active composition of the oil.

In view of this, this study was aimed at evaluating the anticancer potential of this plant oil against AU 565 and HeLa cell lines, in addition to subjecting the oil to chromatographic analysis to ascertain the active chemical constituents.

METHODOLOGY

Collection and identification of plant material

Fresh leaves of *C. citrinus* were harvested from the premises of the University of Benin, Benin City, Nigeria in September 2016. Its identity was confirmed by Dr. Akinnibosun Henry of the Department of Plant Biology and Biotechnology, University of Benin, Benin City and voucher number UBH C383 was obtained.

Extraction of plant material

The leaves (1 kg) were extracted in batches by hydro-distillation method with the aid of a Clavenger-type apparatus to obtain its volatile oil which was stored in the refrigerator at 4ºC until needed.

Determination of cytotoxic effects using brine shrimps (*Artemia salina*)

The volatile oil (20 mg) was dissolved in 2 mL acetone from which 10, 100 and 1000 µg/mL concentrations were obtained. The solution was exposed overnight for the solvent to evaporate. After 48 h when the nauplii had hatched and matured, 10 larvae were transferred to each vial using a Pasteur pipette. The volume was made up to 5 mL with seawater (38 g/L, pH 7.4) and the vials incubated at 25-27 ^{0}C for 24 h under illumination. Acetone served as the negative control while etoposide (10-1000 µg/mL) was used as the positive control (McLaughlin, 1991). The experiment was performed in triplicates.

Column chromatography of *C. citrinus* volatile oil

C. citrinus oil (20 mL) was triturated with silica (200-400 mesh) and subjected to column chromatography. Gradient elution was carried out with 200 mL each of hexane (100 %), and hexane-ethylacetate (99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8). Fractions (75) were collected, bulked according to their TLC profile (solvent system: hexane-ethylacetate (9.7:0.3)) and coded F1 (3-5), F2 (6-14), F3 (15-21), F4 (22-38), F5 (39-66) and F6 (67-75).

Determination of the growth inhibitory effects of the column fractions of *C. citrinus* volatile oil on guinea corn (*Sorghum bicolor*)

Sorghum bicolor seeds were purchased from a local market in Benin and the preservative removed with absolute alcohol after which the viable seeds were separated, dried and used for the experiment. About 10 ml of 1 mg/mL concentration of *C. citrinus* oil column fractions prepared with 2 % tween 80 in water, were poured into 9-cm-wide glass Petri dishes previously under-laid with cotton wool and filter paper (Whatman No 1). Twenty (20) viable seeds were spread on each plate and incubated in a dark environment. The length (mm) of the growing seed radicles was measured at 24, 48, 72 and 96 h. The control seeds were treated with 10 mL of 2% tween 80 in distilled water. The experiment was carried out in triplicates.

Determination of anticancer activity of oil and column fractions using cancer cell lines

The anticancer activity was done using MTT assay on human breast cancer (AU 565) and cervical cancer (HeLa) cell lines obtained from the molecular bank of the International Center for Chemical and Biological Sciences (ICCBS) at the University of Karachi, Pakistan. For the experiment, the cancer cells were seeded in 96-well plates at 10,000 cells per well density in 100 µL of the complete media and allowed to incubate for 24 h at 37 °C and 5% CO_2 to promote the healthy growth of the cells. The cells were treated in triplicates with 50 µg/mL of the oil and fractions. Doxorubicin (50 µM) was used as the standard. After treatment, the cells culture were allowed to incubate for 48 hours at the same environmental condition after which 200µL of MTT (0.5mM) dye was added to each well and then incubated for another 3-4 h. DMSO (100 µL) was used to dissolve the formazan crystals formed and the absorbance of the solution was measured at 570 nm. (Puig *et. al*., 2011; Kritsanawong *et. al*., 2016). EZ-Fit software was used to calculate the IC_{50}.

Gas chromatography-mass spectrometry (GCMS) analysis

GCMS analysis was carried out on the oil and fraction F_1. The gas chromatogram was recorded in Agilent technologies 7000 GC/MS triple quadrupole mass spectrometer with OPTIMA-5-ZB-5 column having dimensions of 30 m x 250 µm x 0.25 µm. For GCMS detection, electron ionization (EI) with ionization energy of 70eV was us ed. Carrier gas was helium (99.99 %) at a constant flow rate of 1.129 mL/min and injection volume 2 µL (split ratio 15:1). Ion-source temperature was 250°C. The oven temperature was programmed from 50°C (isothermal for 15 min.), with a reduction to 8°C/min, to a further increase to 180°C for 15 min, then 15°C/min and finally to 290°C for 5 min. Total run time was 58.58 min. Chemstation software was used to handle mass spectra and chromatogram while NIST library match was employed for the identification of the compounds.

Statistical analysis

The data obtained were expressed as mean ± SEM and analyzed with one way Analysis of Variance (ANOVA) using SPSS 21. $P< 0.05$ was regarded as significant.

RESULTS

Result of brine shrimp lethality Assay

A concentration-dependent response was observed with the shrimps. *C. citrinus* oil produced 26.66 ± 0.00 and 60 ± 3.30 % mortality at 100 and 1000 µg/mL concentrations respectively with LC_{50} of 528.48 µg/mL obtained. No mortality was observed with the negative control (Table 1).

Table 1: Cytotoxic effect of *C. citrinus* oil against *Artemia salina* nauplii

Sample	Concentration (µg/mL) / % Mortality			
	10	100	1000	LC_{50} (µg/mL)
Distilled water	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
C. citrinus	20 ± 0.50	26.66 ± 0.00*	60 ± 3.30**	528.48
Etoposide	50.30 ± 0.5**	89.10 ± 1.98***	100 ± 0.00***	10.00

Values are mean ± sem, n=3,* indicates significance compared to negative control. *P < 0.05, **P < 0.01, ***P < 0.001.

Result of the growth inhibitory effects of the column fractions of *C. citrinus* oil

All the fractions were observed to produce concentration-dependent growth inhibitory effects with fraction F_1 having the highest effect all through the period of the experiment. At 24 h, using 1 mg/mL concentration, the radicle length of the control was noticed to be 1.89 mm while fractions F_1 and F_2 produced 0.5 and 0.41 mm indicating a percentage inhibition of 73.54 and 78.31 respectively. At 96 h, the radicle length of the control was 24.05 mm while F_1 and F_2 gave 1.83 and 2.55 mm indicating 92.39 and 89.4 % growth inhibitions respectively. Fraction F_5 also produced significant growth inhibitory effect but not as effective and potent as F_1 and F_2 (Figure 1).

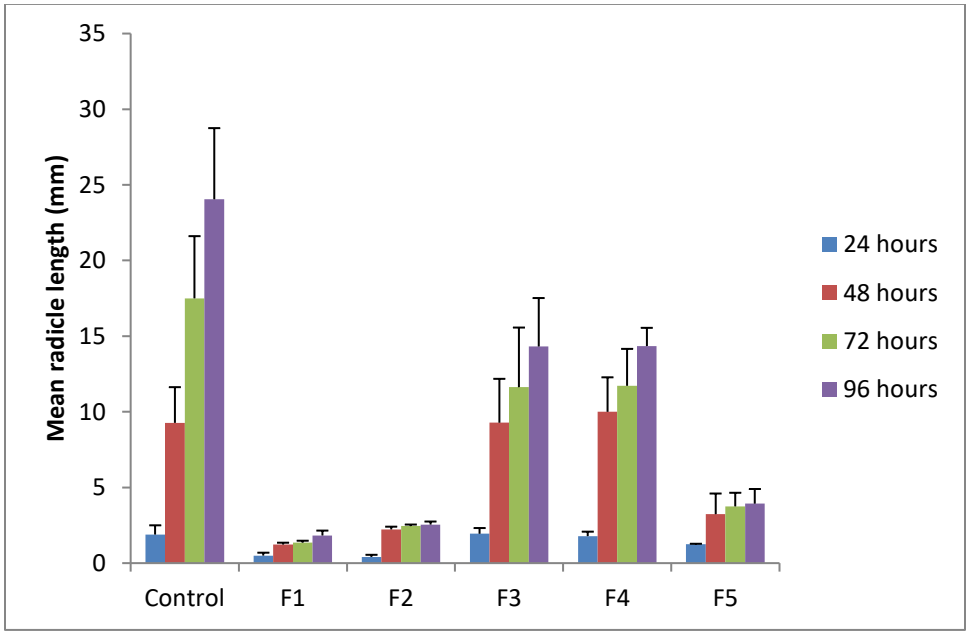

Figure 1: Growth inhibitory effect of *C. citrinus* oil column fractions on the radicle length of *S. bicolor* seeds at 24 to 96 hours. Values are Mean ± SEM, n = 3

Result of anticancer activities of *C. citrinus* volatile oil and fractions

The volatile oil of the plant produced no growth inhibitory effect against HeLa cell line as -7.60 % inhibition was obtained whereas it showed more activity on AU 565 cells with +11.80 % inhibition observed (Table 2). Almost all the chromatographic fractions elicited anticancer properties against the AU 565 cells. Fraction F_1 was discovered to be most potent producing the growth inhibition of 70.44 % with an

IC_{50} of 15.96 µg/mL. F_5 also demonstrated remarkable inhibition of 45.05 % while F_3 and F_4 produced 24.91 and 20.07 % respectively. (Table 2).

Table 2: Inhibition effects of the volatile oil and fractions on AU 565 and HeLa cell lines

Oil/Fractions	Cell line	% Inhibition/Stimulation	IC_{50}
C. citrinus oil	AU 565	+11.80	ND
C. citrinus oil	-	-7.60	ND
F_1	AU 565	+70.44	15.96±1.61
F_2	-	-	-
F_3	AU 565	+24.91	ND
F_4	AU 565	+20.07	ND
F_5	AU 565	+45.05	ND

Values are mean ± SEM, n=3, ND = not determined

Result of GC-MS analysis of *C. citrinus* volatile oil and fraction F_1

The GCMS analysis of the volatile of *C. citrinus* oil revealed the presence of cineole (36.06 %) and α-pinene (21.41 %) as the major components. Other compounds like o-cymene, α-terpineol, and 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-ethanone were also present with relative abundance of 7.54, 9.94, and 1.13 % respectively (Table 3).

Analysis of the most potent column fraction F_1 revealed 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-ethanone as the major constituent of the fraction with a percentage concentration of 24.89 %. Other components of the fraction include α-phellandrene, β-caryophyllene and isoaromadendrene epoxide which were also present with relative abundance of 2.37, 5.09, and 9.5 %, respectively (Table 4).

Table 3: GC-MS analysis of *C. citrinus* volatile oil

S/No	Retention time (s)	Compound	% Concentration
1	7.99	3-Pentanone, 2,4-dimethyl-	0.55
2	18.76	Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	1.51
3	**19.15**	**-α-Pinene**	**21.41**
4	20.99	.L-β-pinene	1.16
5	22.01	α-Phellandrene	4.47
6	22.72	o-Cymene	7.54
7	**22.99**	**Cineole**	**36.06**
8	23.71	γ-Terpinene	1.06
9	24.76	β-Linalool	1.53
10	26.72	4-Terpineol	2.8
11	27.02	α-Terpineol	9.94
12	30.49	Geraniol acetate	0.11
13	31.52	β-Caryophyllene	2.09
14	32.14	α-Caryophyllene	0.12
15	33.27	7-Isopropyl-7-methyl-nona-3,5-diene-2,8-dione	0.97
16	33.75	Ethanone, 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-	1.13
17	34.93	(-)-Spathulenol	0.73
18	35.92	β-Caryophyllene oxide	1.06
19	38.52	Flavanone	3.92
20	29.09	.Carvacrol	0.35

Table 4: GC-MS analysis of fraction F_1

S/No	Retention time (s)	Compound	% Concentration
1	22.01	α-Phellandrene	2.37
2	30.49	Geraniol acetate	1.11
3	31.52	β-Caryophyllene	5.09
4	32.14	α-Caryophyllene	1.12
5	32.30	Alloaromadendrene	4.04
6	32.93	.(+)-Ledene	3.05
7	33.81	Ethanone, 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-	24.89
8	34.75	Palustrol	4.05
9	35.17	Isoaromadendrene epoxide	9.5
10	35.75	3-Dodecyl-2,5-furandione	5.06
11	35.92	β-Caryophyllene oxide	2.15

DISCUSSION

Research into antitumor agents usually involves a series of complex procedures that sometimes produces discouraging results after much time and materials have been expended. In addition, scarcity of research funds has made the development and acceptance of simple bench-top assays necessary (Mc Laughlin, 1991). These bioassays are simple, rapid, reproducible, inexpensive, and can be used to predict the possibility of an extract having cytotoxic activity.

Brine shrimp lethality and growth inhibition test using *S. bicolor* seeds were the bench-top assay methods used in this study due to their availability. The *in vivo* lethality in simple zoologic organisms such as brine shrimps of *A. salina* can be used as a convenient method for screening natural products for cytotoxic activity which has been linked to the probable ability of such compounds to kill cancer cells in cell cultures (Mc Laughlin, 1991). In toxicity evaluation of plant

extracts by brine shrimp lethality bioassay, LC_{50} values lower than 1000 µg/mL are considered bioactive (Meyer *et al.*, 1982). Therefore, it can be inferred that LC_{50} of 528.48 µg/mL observed for the volatile oil *C. citrinus* is within the acceptable range of potency.

Meristematic tissues of seeds have the tendency to proliferate when placed under suitable conditions and the extent of proliferation is shown in the increase in length of the radicles produced in the control seeds. (Ayinde and Agbakwuru, 2010). Degree of retardation of growth of these radicles by an extract is a measure of the extracts antiproliferative ability. *C. citrinus* oil growth inhibitory potential against *S. bicolor* has previously been reported (Imade and Ayinde, 2018) where at concentrations of 5 and 10 mg/mL growth inhibitions of 44.08 and 64.40 % were observed respectively when compared with the control. The present report has revealed that separation of the components of the volatile oil through column chromatographic procedures remarkably improved the growth inhibitory effects of the volatile oil as evidenced by 92.39% growth inhibition of the seed radicles observed with fraction F_1 even at a concentration of 1 mg/mL.

The improved potency of the volatile oil occasioned by the column chromatographic exercise was further established in the anticancer evaluation results. Although the volatile oil did not produce effects on Hela cell lines, the inhibitory effects of the fractions became more pronounced on the AU 565 cell line. It is important to state that variations observed in the results of the two cell lines may be due to variations in the susceptibilities of the cell lines to components of the volatile oil. Increasing the concentration of the volatile oil may have produced more pronounced effects on the cell lines.

Variations in geographical place of collection of many plants do affect the concentrations of chemical constituents produced. The volatile oil of *C. citrinus* collected here in Benin City, Nigeria was found to contain cineole as the major constituent (36.06 %) followed by α-pinene (21.4%). The results obtained showed some close semblance to the chemical constituents of the plant reported in India where 1,8-cineole constituted 58.0% and α-pinene was 24.1%, as the major constituents of the volatile oil (Gupta *et al*., 2008). In another study, GC-MS analysis of this essential oil revealed high content of α-pinene (32.3%), limonene (13.1%) and α-terpineol (14.6%) in leaf sample (Kumar *et al*., 2015). The quantitative and qualitative differences in the composition of volatile oils may also be influenced by the climatic and soil conditions as well as the level of maturity of the plants at the point of harvest (Barra, 2009). 1, 8-cineole which is one of the main components of *C. citrinus*, has been reported to suppress the proliferation of colon cancer cells by inducing apoptosis **(**Murata *et al*., 2013).

Inhibitions of AU 565 cells by fraction F_1 was notable at 70.44%. The GCMS revealed an increase in the presence of ethanone, 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)- from 1.13% (in the oil) to 24.89 % in the fraction. This compound may have contributed to the increased anticancer activity. Other ethanone derivatives have been documented to possess cytotoxic activities. For example, a series of novel 1-(3-substituted-5-phenyl-4,5-dihydropyrazol-1-yl)-2-thio-ethanone derivatives reportedly demonstrated antiproliferative activity against SGC-7901, MGC-803, Bcap-37 and HEPG-2 cell lines (Wu *et al*., 2013). Other compounds like α-caryophllene and β-caryophyllene present in the fraction have also been reported to have anticancer properties. α-caryophllene has been reported to be cytotoxic against human breast adenocarcinoma cell line (MCF-7) and its anticancer effect was observed to be potentiated by the presence of β-caryophyllene (Legault and Pichette, 2007). β-caryophyllene reportedly suppressed PC-3—prostate cancer cell and MCF-7—breast cancer cell proliferation in a dose-dependent manner (Park *et al*., 2011) by inhibiting signaling pathways in these cells responsible for cell survival, proliferation, and angiogenesis (LoPiccolo *et al*., 2008). The remarkable activity displayed by fraction F_1 therefore, may be as a result of synergistic effects of these components found in it.

CONCLUSION

In light of the results obtained from this study, *C. citrinus* leaf oil has some bioactive constituents such as ethanone, 1,1'-(5-hydroxy-2,2-dimethylbicyclo[4.1.0]heptane-1,7-diyl)bis-, (1α,5β,6α,7α)-, 1, 8-cineole, α-caryophllene and β-caryophyllene and therefore has potential to be used as a cytotoxic agent.

CONCLUSION

We wish to acknowledge the Non-Align Movement of Science and Technology (NAMST-ICCBS) for providing the Fellowship that enabled us to carry out this study.

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Conflict of Interest: None declared

Received: December 02, 2021

Accepted: February 02, 2022