

Original Article

GC-MS Analysis and In Vitro Cytotoxic Effects of *Ocimum gratissimum* (Lamiaceae) Volatile Oil and Thymol on Cancer Cells



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ABSTRACT

Background: Cancer is one of the most prominent causes of death worldwide. *Ocimum gratissimum* Linn. (Lamiaceae) leaves are used in many countries as a spice or medicine.

Objectives: This study investigated the essential oil of the *O. gratissimum* leaves and its major constituent, thymol, for cytotoxic activity against breast (AU565) and cervical (HeLa) cancer cell lines.

Methods: Preliminary screening was carried out using bench-top assay methods for cytotoxicity involving the use of tadpoles of *Raniceps raninus* (10-40 µg/mL) and brine shrimp of *Artemia salina* (10-1000 µg/mL) and growth inhibition using radicle of *Sorghum bicolor* seeds (1-30 mg/mL). Antiproliferation was verified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Chromatographic separation of the oil resulted in fractions and sub-fractions, which were also subjected to biological testing. The components of the oil and active subfraction were further identified by gas chromatography-mass spectrometry (GC-MS).

Results: Remarkable cytotoxic activities were seen against *R. raninus* tadpoles and *A. salina* nauplii. Growth inhibitory activity on *S. bicolor* seed radicles was produced concentration-dependent. The subfraction possessed greater cytotoxic activity on the cell lines than the oil, with inhibitory action of +85.07% and +29.20% against AU565 and HeLa cells, respectively. Thymol was the major constituent of the oil (22.49%) and increased to 94.31% in the subfraction.

Conclusion: *O. gratissimum* volatile oil showed little inhibitory activity against AU565 and no inhibition on HeLa cells. However, its major component, thymol, demonstrated high potency, especially on the AU565 cell line, making it a good candidate for further studies.

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Introduction

Cancer is one of the major causes of death. Over a hundred forms of cancer have been discovered, with breast and cervical cancers being the most common in women [1]. The low success of clinical therapies, as shown by high morbidity and mortality rates, justifies the search for new and better treatment methods. In southeast Asian countries, herbs are used as spices, and help reduce the incidence of colon, prostate, breast, and other cancers [2]. They are widely used in alternative medicine to treat gastrointestinal and respiratory illnesses and have carminative, digestive, galactagogue, and diuretic effects [3]. Their use in cosmetic and pharmaceutical products is well known [4].

Essential oils have been shown to have an anti-cancer effect against various cancer cell lines. *Hypericum hircinum*, *Thymus vulgaris*, and *Solanum erianthum* leaf essential oils have demonstrated antiproliferative activity against human prostatic adenocarcinoma (PC3 cell line) [5, 6]. *Xylopiia frutescens* leaf essential oil [2] also demonstrated anti-cancer effects in vitro and in vivo on the bronchioalveolar lung carcinoma cell line (NCI-H358M).

Ocimum gratissimum L. (Lamiaceae) is an aromatic perennial erect herb with many branches that grows to a maximum height of 1 m and has a woody base. It is native to South Africa, Asia, and Brazil [7]. The plant is frequently employed in traditional medicine, primarily in the form of teas and infusions of its leaves to treat headaches, stress, colds, pruritus, and abdominal discomfort [8, 9]. Additionally, there are reports of using *O. gratissimum* to treat skin lesions, pneumonia, conjunctivitis, and upper respiratory tract infections [10].

Numerous scientific investigations have revealed some of the plant's properties, including anti-diabetic, anti-oxidant, anxiolytic, sedative, anti-inflammatory, hepatoprotective, anti-cancer, gastroprotective, and hypolipidemic [11-19]. Much research on various parts of the plant has confirmed its anti-bacterial properties [20]. *O. gratissimum* crude extract and its hydrophobic and hydrophilic fractions differentially suppress the progression of MCF10ADCIS.com xenografts and tumor growth and inhibit breast cancer cell growth in vivo [21].

The literature search reveals different constituents in the oil obtained from separate locations. Joshi [22] reported that the oil contained eugenol as the major constituent (57%) alongside β -caryophyllene, bulnesene, and others. Koba et al. [23] further documented the oil to possess mostly thymol, p-cymene, and γ -terpinene.

In light of these facts, this work was carried out to examine the chemical profile of *O. gratissimum* essential oil harvested from the southern part of Nigeria and to assess the cytotoxic activity of the oil and its major constituent against breast (AU565) and cervical (HeLa) cancer cell lines.

Materials and Methods

Collection and identification of plant material

Fresh leaves of the *Ocimum gratissimum* plant were collected in December 2016 in Benin City, Edo State, Nigeria. The plant was identified by Dr Henry Akinnibosun of the Plant Biology and Biotechnology Department, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. A voucher number UBH O34 was assigned to it.

Extraction of volatile oil

With the help of a Clevenger apparatus, the leaves (approximately 1 kg) were hydro-distilled in batches to recover the volatile oil. The oil was placed in the freezer at -18°C for any water that may have accompanied it from collection to freezing. Subsequently, it was transferred to a dry vial with a pipette and kept in the refrigerator at 4°C until further use.

Column chromatography of *O. gratissimum* volatile oil

Silica gel (200–400 mesh) was used to triturate the volatile oil (15 mL), which was then subjected to column chromatography. For gradient elution, 200 mL of C_6H_{14} (100%) and C_6H_{14} - $\text{CH}_3\text{COOC}_2\text{H}_5$ were used (99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10). Fractions (118) were collected, bulked in accordance with respective TLC (thin-layer chromatography) profiles, and assigned the following codes: F_1 (5–12), F_2 (13–28), F_3 (29–40), F_4 (4–63), F_5 (62–90), and F_6 (91–118).

As a result of the high yield and conspicuous nature, fraction F_1 (5 mL) was subjected to preparative TLC analysis on silica gel using C_6H_{14} - $\text{CH}_3\text{COOC}_2\text{H}_5$ (9.7:0.3). Two sub-fractions were obtained and coded as SF_1 and SF_2 .

Source and identification of *Raniceps raninus* tadpoles

From within the University of Benin, tadpoles (5–6 days old) were gathered from toad colonies in small water communities. They were confirmed to be *Raniceps raninus* tadpoles by Professor M. Aisien, an Animal

Parasitologist at the Department of Animal and Environmental Biology, Faculty of Science, University of Benin, Benin City, Nigeria.

Determination of the essential oil's cytotoxic effects on tadpoles

Following the method stated in a study [24], 10 tadpoles were placed in a 50-mL beaker containing 15 mL water from the tadpoles' colonies. This was made up to 49 mL with distilled water, and then 1 mL of the different concentrations of the oil (0.5, 1, and 2 mg/mL) was added to make up to 50 mL. The final concentrations were 10, 20, and 40 µg/mL. For each concentration, the experiment was carried out in triplicates. The tadpole mortality rate was observed for 24 hours.

Brine shrimps lethality bioassay (*Artemia salina*)

Brine shrimp lethality bioassay [25] was applied to determine cytotoxic activity. Then, 2 mL of acetone was used to dissolve the volatile oil, and from this solution, concentrations of 10, 100, and 1000 µg/mL were obtained in triplicates. The solvent was allowed to evaporate overnight. Using a Pasteur pipette, 10 larvae were introduced to each vial after 48 h of nauplii maturation and hatching. Seawater (38 g/L, pH 7.4) was used to make up the volume to 5 mL, and the vials were incubated at 250°C–270°C for 24 hours while being illuminated. The negative and positive controls were seawater and etoposide, respectively.

Preparation of the guinea corn (*Sorghum bicolor*)

Absolute alcohol was used to rinse the *Sorghum bicolor* seeds purchased from a market in Benin City, Nigeria. A quick viability test was conducted to remove the preservative by adding about 100 mL of water and immediately decanting, thus removing the floating compromised seeds. The submerged viable seeds were dried on filter papers before use.

Determination of growth inhibitory effects of the essential oil on *Sorghum bicolor* seeds

About 10 mL of 1, 2, 5, 10, 20, and 30 mg/mL of *O. gratissimum* oil prepared by dissolution with 2% Tween 80 in water, were poured into 9 cm wide glass Petri dishes lined with cotton wool and filter paper (Whatman No 1). Twenty viable seeds were dispersed on each plate and incubated in the dark. At 24, 48, 72, and 96 h, each seed's radicle length (mm) was measured. Then, 10 mL of distilled water with 2% Tween 80 was used to treat the control seedlings. The test was performed in tripli-

cates [24]. Chromatographic fractions F₁, F₂, SF₁, and SF₂ were also subjected to a similar procedure at 1 mg/mL concentration.

Assessment of cytotoxic activity of *O. gratissimum* oil on cancer cell lines

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the cytotoxic activity. Human breast cancer (AU565) and cervical cancer (HeLa) cell lines were utilized. They were obtained from the International Center for Chemical and Biological Sciences (ICCBS) molecular bank at the University of Karachi in Pakistan. A density of 10000 cells/well/100 µL in 96-well plates was used, and they were incubated for 24 h in a complete medium at 37°C and 5% CO₂. Next, 50 µg/mL of the oil and fractions were used to treat the cells in triplicates (F₁, F₂, F₃, F₄, and SF₂). The standard utilized was doxorubicin (50 µM). Incubation was for 48 h at 37°C and in a humidified atmosphere of 5% CO₂. Before the medium was taken out, a fresh medium was added containing 200 µL of MTT dye (0.5 mM) and then incubated for an additional 3–4 hours. The formazan crystals produced were dissolved in 100 µL of DMSO, and absorbance was measured at 570 nm [26, 27]. IC₅₀ was calculated by EZ-Fit software.

Gas chromatography-mass spectrometry analysis

The oil and fraction SF₂ were analyzed using gas chromatography-mass spectrometry (GC-MS). The Agilent technologies 7000 GC/MS triple quadrupole mass spectrometer was used to record the gas chromatogram, and it used an OPTIMA-5-ZB-5 column with measurements of 30 m x 250 m x 0.25 m. Electron ionization (EI) with an ionization energy of 70 eV was employed for GC-MS detection. Helium (99.999%) served as the carrier gas, flowing at a constant rate of 1.129 mL/min with an injection volume of 2 L (split ratio 15:1). It was 250°C at the ion source. The oven was programmed to start at 50°C (isothermal for 15 min), then decrease to 8°C/min, rise to 180°C for 15 min., then 15°C/min, and ultimately reach 290°C for 5 min. Runtime was 58.58 min in total. While NIST library match was utilized to identify the compounds, ChemStation software was used to handle the mass spectra and chromatograms.

Statistical analysis

To analyze the data, a 1-way analysis of variance (ANOVA) was performed using SPSS software, version 21, and the results were expressed as Mean±SEM. In every instance, differences were deemed significant at P<0.05.

Table 1. Cytotoxic effect of *O. gratissimum* oil against *Artemia salina* Nauplii

Sample	Concentration ($\mu\text{g/mL}$) / % Mortality			
	10	100	1000	LC ₅₀ ($\mu\text{g/mL}$)
<i>O. gratissimum</i>	13.33 \pm 0.40	26.66 \pm 0.00	80 \pm 0.03	200.34
Etoposide	50.30 \pm 0.5	89.10 \pm 1.98	100 \pm 0.00	10.00
Distilled water	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	-

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Results

Cytotoxic effects of the volatile oil on tadpoles

A concentration-dependent activity was observed in the tadpole mortality test. *O. gratissimum* oil showed no mortality at 10 $\mu\text{g/mL}$, while 70% and 100% mortality were seen at 15 and 20 $\mu\text{g/mL}$, respectively (Figure 1). An LC50 of 13.74 $\mu\text{g/mL}$ was obtained.

Cytotoxic effects of the oil against *Artemia salina* Nauplii

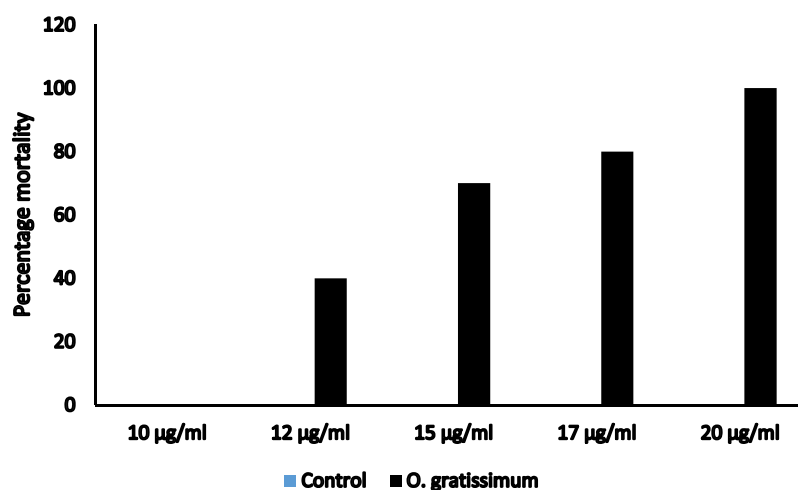
A concentration-dependent response was observed when the shrimps came in contact with the volatile oil. About 13.33 % mortality was observed at 10 $\mu\text{g/mL}$, which was increased to 80% at 1000 $\mu\text{g/mL}$ with LC50 of 200.34 $\mu\text{g/mL}$ (Table 1).

Growth inhibitory effects of the essential oil and fractions on radicle length of *S. bicolor* seeds

The volatile oil was found to greatly suppress radicle growth in a concentration-dependent manner. A signifi-

cant difference ($P < 0.05$) compared to negative control was observed at 5-30 mg/mL concentrations for 24-96 h. From 5 mg/ml at 48 h and 2 mg/mL at 72 h, the percentage reductions in radicle growth were highly significant ($P < 0.001$). Also, 10 mg/mL of the oil completely inhibited the growth of the radicles at 96 h. An average length of 9.54 \pm 1.73 mm in the controls simultaneously was reduced to 0.77 \pm 0.02 mm in seeds treated with 5 mg/mL concentration showing a 91.93% reduction (Figure 2).

The growth of the radicles was greatly retarded by the column fractions at 1 mg/mL concentration. From 72 to 96 h, the radicle growth reductions for fractions F₁, F₂, and SF₂ were highly significant compared to the control ($P < 0.001$). At 96 h, F₁ and F₂ gave 81.26% and 86.46% growth inhibitions, respectively. At the same concentration and time, SF₁ and SF₂ (obtained from F₁ through preparative TLC) produced growth inhibitions of 28.73% and 92.03%, respectively (Figure 3). The fractions and subfractions remarkably inhibited the growth of the radicles, with SF₂ showing the highest inhibitory effect ($P < 0.001$) compared to the negative control.

**Figure 1.** Cytotoxic effect of *O. gratissimum* volatile oil on *R. raninus* tadpoles**PBR**

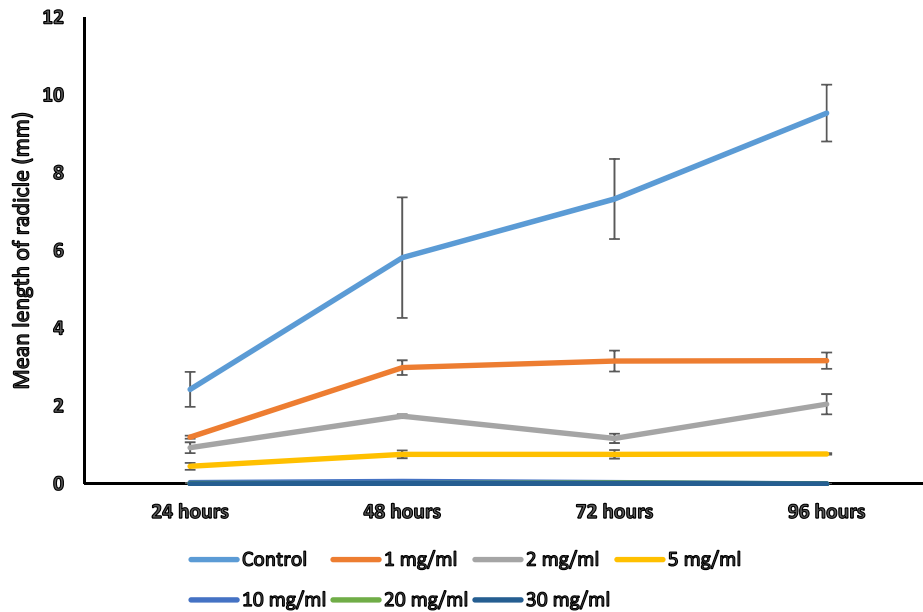


Figure 2. Growth inhibitory effect of *O. gratissimum* volatile oil on radicle length of *S. bicolor* seeds

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Inhibitory effects of *O. gratissimum* volatile oil and fractions on the cell lines

The oil demonstrated moderate inhibition against breast cancer, +17.82%, but no activity was observed with the cervical cancer cell line (HeLa). However, the inhibitory actions of the column fractions varied, with F₄ producing the highest inhibition of +34.42%. A signifi-

cant difference in activity was observed between the oil and SF₂ on both HeLa and AU565 cells (P<0.001). SF₂ gave 85.07% inhibition on AU565 cells and an IC₅₀ of 15.96 ± 1.61, while an inhibition of 29% was observed against HeLa cells (Figure 4). Doxorubicin gave 97.89% inhibition with an IC₅₀ of 0.085±0.03 μM with AU565 cells.

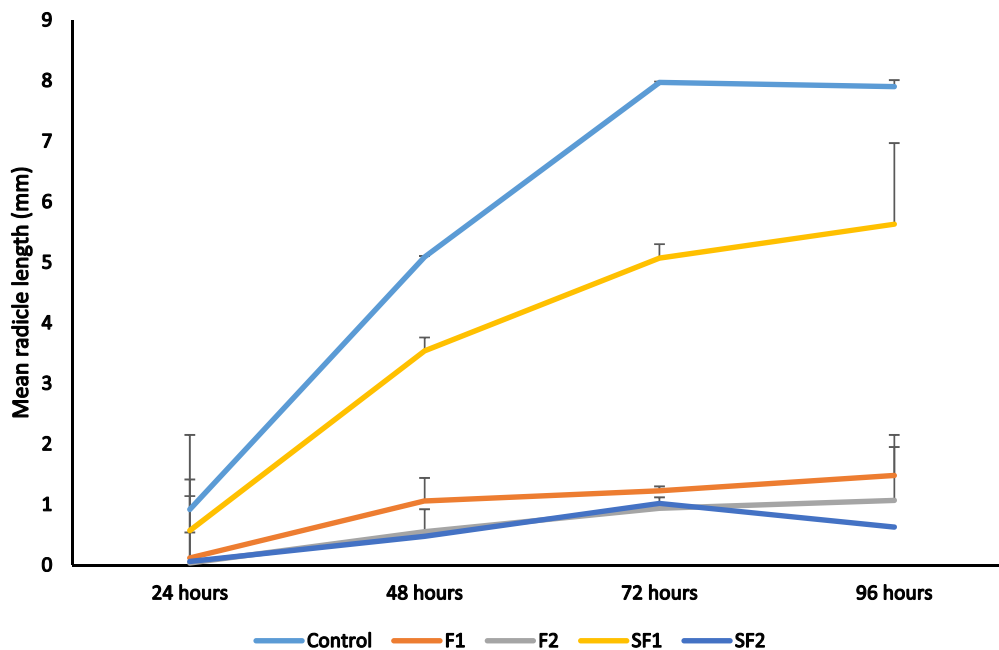
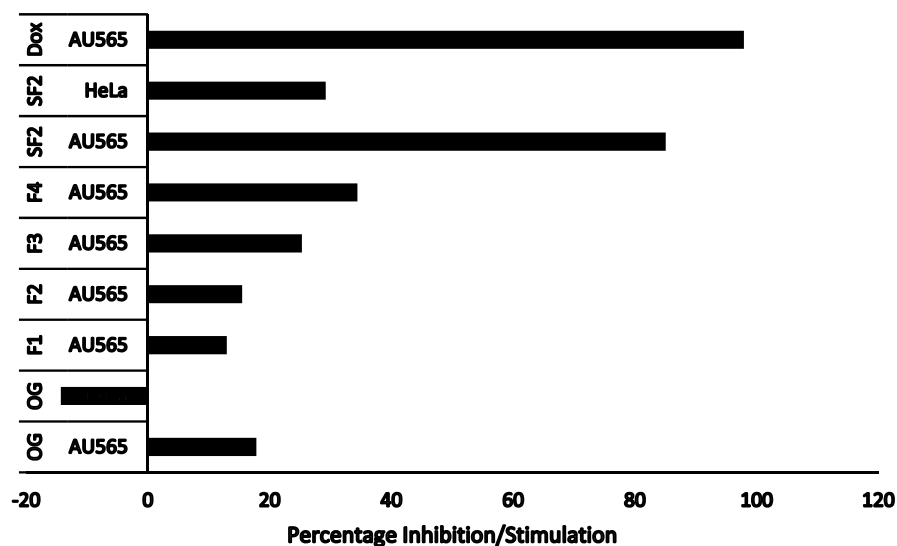


Figure 3. Growth inhibitory effect of *O. gratissimum* oil column fractions and subfractions on the radicle length of *S. bicolor* seeds

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Figure 4. Inhibition/stimulation effects of the volatile oil of *O. gratissimum* (OG), column fractions (F₁ – F₄), preparative thin-layer chromatography (TLC) fraction (SF₂), and doxorubicin (Dox) on the cell lines

GC-MS analysis of *O. gratissimum* essential oil and fraction SF₂

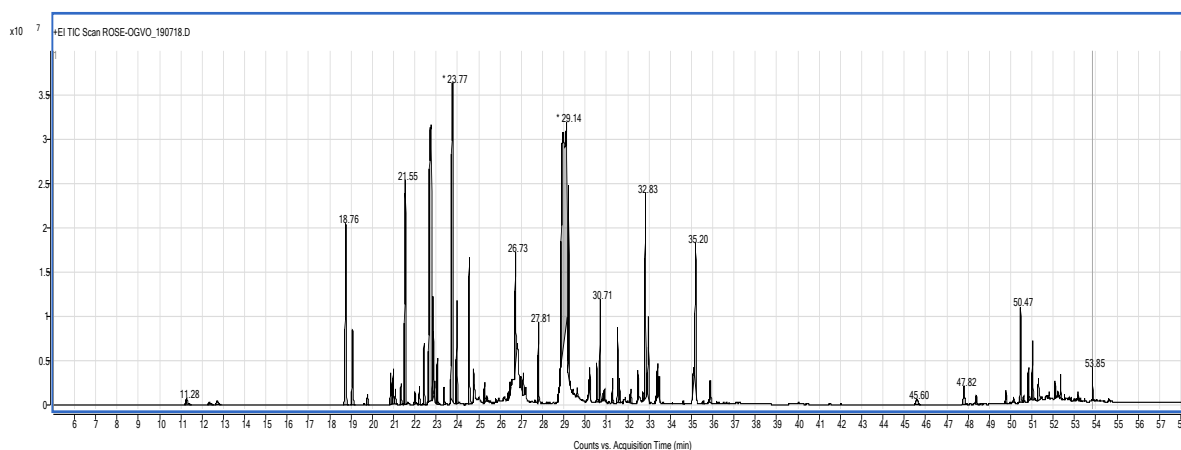
Thymol (22.49%), p-cymene (13.47%), and γ -terpinene (11.5%) were present in the highest quantities in *O. gratissimum* oil. β -Myrcene, carvacrol, eugenol, and β -selinene were also present with a relative abundance of 4.58%, 2.6%, 0.28%, and 4.15%, respectively. A total of 25 components were obtained (Figure 5, Table 2).

Thymol was the major identified component in preparative TLC fraction SF₂ from *O. gratissimum* essential oil, with a relative abundance of 94.31% (Figure 6).

Discussion

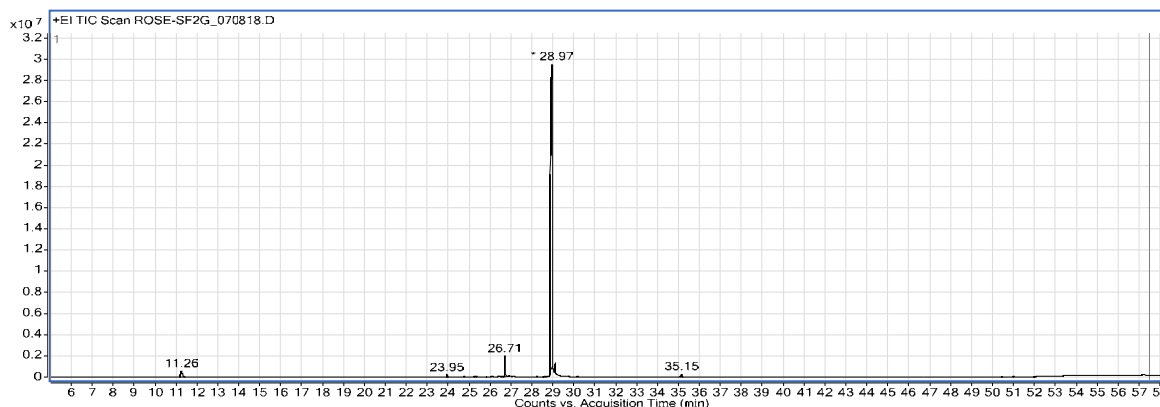
Cytotoxicity against simple organisms like the tadpoles of *R. raninus* and nauplii of *A. salina* is predictive of an extract's ability to kill cancer cells in vivo [25, 28].

The meristematic tissues of seeds naturally proliferate under favorable conditions resulting in the elongation of the radicles. Growth inhibition of the seed radicles by an extract can also be used to predict its potential antiproliferative effect against cancer cell lines in cell culture. Seeds of *S. bicolor* were used in this study due to their availability, cost-effectiveness, and small size. They are reliable as up to 90% can germinate within 24 hours [24]. Radicle growth inhibition is tied to the suppression of



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Figure 5. Gas chromatography-mass spectrometry (GC-MS) chromatogram of *O. gratissimum* volatile oil



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Figure 6. Gas chromatography-mass spectrometry (GC-MS) chromatogram of SF₂ from *O. gratissimum* volatile oil

cell division [29], thereby affecting its elongation [30]. The inhibition of the radicles could also be explained by changes in osmotic potential which prevents the development of turgor pressure in the seed cells, which is one of the essential elements for radicle development to begin during seed germination [31]. *O. gratissimum* oil and fractions retarded the growth of the radicles to varying degrees. The extent of sensitivity could be related to the biochemical resistance of the cells as a response to the constituents of the oil and fractions.

The cytotoxic potential of *O. gratissimum* oil was further observed in the brine shrimp lethality bioassay with LC50 of 200.34 µg/mL, which indicates its potential anticancer effects [32].

In vivo lethality in simple organisms can be used to analyze fractions, and this could lead to the separation and purification of biologically active components [25]. The growth inhibitory test with *S. bicolor* radicles correlated well in this regard as the active subfraction (SF₂) with growth inhibition of 92.03% at 72 h also produced 85.07% inhibition against the AU565 cell line.

Essential oils generally contain terpenes, especially monoterpenes and sesquiterpenes [33]. Thus, in conducting studies on these oils, the chemical characterization and the possibility of linking the major components with particular biological properties come into play.

Thymol, the major component isolated from this oil, has previously been noted to have a cytotoxic effect on human epidermic cell line HaCat, HL-60 (acute promyelocytic leukemia) cells, intestinal (Caco-2) cell line, AGS cells, oral squamous carcinoma (Cal27) and cervical cancer (HeLa) cells derived from the mouse xeno-

grafts [34-37]. Its activity against AU565 breast cancer cells is reported here for the first time. Several reports state that thymol exerts its activity through various mechanisms, such as inhibiting cell development, triggering apoptosis, and creating intracellular reactive oxygen species. All these effects were observed in different experimental mode [36].

Biological activities observed with volatile oils have been attributed to the quality and quantity of their individual components, especially the major ones they possess [37], and variations have been observed in the chemical compositions of these oils harvested from different locations.

In this study, thymol (22.49 %) was of the highest abundance in *O. gratissimum* oil (Table 2), but some earlier reports stated eugenol [22, 38] and caryophyllene [15] as being the most abundant. The variability in the compositions of the oils could be due to climatic and geographic factors, including the degree of maturity at the time of collection [39].

Conclusion

In this study, *O. gratissimum* volatile oil had moderate cytotoxic effects on the AU565 cell line with no inhibition observed with HeLa cells, while thymol, its major component, showed remarkable potency with more inhibition against AU565 cells. Further investigation of the oil and its main component against other cancer cell lines is required.

Table 2. Gas chromatography-mass spectrometry (GC-MS) analysis of volatile composition of essential oil of *O. gratissimum*

No.	Retention Time	Compound	% Concentration
1	19.08	1R- α -Pinene	1.69
2	20.87	Sabinene	0.57
3	20.99	L- β -pinene	0.64
4	21.55	β -Myrcene	4.58
5	22.21	1R- α -Pinene	0.32
6	22.43	α -Terpinene	1.15
7	22.77	p-Cymene	13.47
8	22.93	Cineole	0.19
9	23.77	γ -Terpinene	11.59
10	24.76	β -Linalool	0.3
11	25.28	Thujone	0.36
12	26.49	Isoborneol	0.18
13	29.14	Thymol	22.49
14	29.23	Carvacrol	2.6
15	30.18	Copaene	0.17
16	30.23	Eugenol	0.28
17	30.71	Copaene	1.42
18	30.92	β -Elemene	0.14
19	31.54	Caryophyllene	1.07
20	31.62	α -Bergamotene	0.36
21	32.16	α -Caryophyllene	0.31
22	32.83	β -Selinene	4.15
23	32.98	α -Selinene	1.35
24	33.41	δ -Cadinene	0.51
25	35.09	Caryophyllene oxide	0.37

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and Supervision: Buniyamin Ayinde; Methodology, investigation, data collection, and analysis: Rose Imade and Anam Alam; Writing the article: Rose Imade; Review and editing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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