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GC-MS analysis and *invitro* cytotoxic activity of *Ocimum basilicum* (Lamiaceae) volatile oil and active fraction composed majorly of estragole

Rose O. IMADE*, Buniyamin A. AYINDE

Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, PMB 1154, Benin City, 300001, Nigeria

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Abstract

Ocimum basilicum leaves contain some bioactive compounds and this study was designed to evaluate the cytotoxic efficacy of its volatile oil and fractions. Preliminary screening of the oil obtained by hydrodistillation was carried out using bench-top assay methods employing tadpoles of *Raniceps ranninus* (10-40 µg/mL), nauplii of *Artemia salina* (10-1000 µg/mL) and radicles of *Sorghum bicolor* seeds (1-30 mg/mL). Application of column chromatography and preparative TLC on the oil resulted in fractions that were tested alongside the oil on breast (AU 565) and cervical (HeLa) cancer cell lines at 50 µg/mL. GCMS analysis was carried out on the oil and the most active fraction. A concentration dependent activity was observed in the preliminary screening with the bench-top assays. The active fraction produced greater growth inhibition of the radicle of *S. bicolor* seeds than the oil. Inhibitions of -1.02 and +23.02 % were realized against AU 565 and HeLa cell lines respectively with the oil, and these were increased to +33.19 and +89.3 % inhibitions respectively with the active fraction. GCMS results revealed the presence of estragole (88.61 %) as being most abundant in the fraction. This result shows the cytotoxic potential of *O. basilicum* volatile oil, which was increased in its estragole-containing fraction.

Keywords: *Ocimum basilicum;* AU 565 cells; HeLa cells; Essential oil; Estragole

INTRODUCTION

Plants are useful sources of medicine for the treatment of a wide array of diseases. About 80 % of the population of developing countries depend on medicines of plant origin [1]. Claims have been made by some traditional practitioners about the potency of some herbs and this has contributed to the drive for scientific evaluation of these plants and the isolation of some bioactive compounds [2]. The use of medicinal herbs for cancer treatment alongside conventional therapies has received increasing recognition due to their anticancer potential [3]. The benefits of medicinal herbs in tumor treatment have been attributed to their phytochemical content [4–6] which include flavonoids, phenolic acids, and essential oils. Such bioactive compounds have been found to be potent immunomodulators, with the potential to minimize negative impacts from cancer treatment [2]. In particular, are flowering plants of the family Lamiaceae, which can be found in almost all parts of the world [7]. Many members of the family are used as spice, in medicinal preparations and as sources of essential oils

*Correspondence. *E-mail*: rose.jesuorobo@uniben.edu *Tel*: +234-7032444024. ISSN 0189-8442

[8]. Amongst the plants in the family is *Ocimum basilicum* which is an erect herb that grows between 0.3 and 1.3 m high. Its leaves are light green, simple, opposite, petiolate, sometimes subsessile, between 3 to 11 cm long and 1 to 6 cm wide. They are also acute, ovate, and contain numerous oil glands. The plant has loose or congested inflorescence, whorls with 6 flowers and bracts of varying sizes [9]. Its flowers are white to purple and grow from a central inflorescence arranged in a terminal spike. It has a thick central tap root.

O. basilicum (Lamiaceae) has long been utilized traditionally for the treatment of cancer, convulsion, epilepsy, gout, nausea, sore throat, toothaches, bronchitis, rhinitis, mental fatigue, cold, spasm and as a first aid treatment for snakebites and wasp stings [10-11]. Parts like the leaves, seeds and essential oils are used medicinally [12] for curing cough, pyrexia, anxiousness, gripe, worms, infective diseases, acne, diarrhea, headaches, constipation, warts, and kidney malfunction [13].

Available literature reports reveal its potent antibacterial, antifungal, antiviral and antiparasitic activities, attributed to the presence of several bioactive compounds [14-16]. The essential oil of *O. basilicum* has been reported to prevent *Aspergillus flavus* growth and aflatoxin production [17]. The plant has been reported to have anti-inflammatory actions which are attributed to compounds such as α-bergamothene, α-cadinol, linoleic acid, estragole, and methyl eugenol [18]. Its antioxidant activity has also been investigated by Silva *et al* [19]. The insecticidal activity [20-21] of the oil has been studied; its repellent properties were attributed to the compounds d-limonene, myrcene, and thymol, while eugenol and methyl-chavicol were proven to have larvicidal activity. The extract of the leaves significantly lowered blood glucose level of alloxan-induced diabetic rats [22-23]. Its gastroprotective effect [24], sedative effect [25], and anti-amnesic effect [26] have also been investigated. The analgesic effect of *O. basilicum* essential oil was assessed and found to be active in all pain models [27].

O. basilicum extract has been considered a potent cancer preventive agent and its polysaccharides are reportedly used to treat cancer [28]. Zagoto *et al* [3] showed that its extract exhibits strong cytotoxic effect against colon carcinoma (HCT116) and liver cancer cell lines (HEPG2). It also reduced cell growth of the human breast cancer cell line (MCF-7) [29] and induced apoptosis in leukemia cells [30].

O. basilicum leaves have been reported to contain chiefly volatile oil and phenylpropanoid derivatives [7]. The objectives of this present study therefore were, first to determine the chemical composition of the essential oil from the leaves of *O. basilicum* harvested from the southern part of Nigeria by GC-MS analysis and, second to evaluate the cytotoxic activity of the essential oil and its fractions on breast (AU565) and cervical (HeLa) cancer cell lines.

EXPERIMENTAL METHODS

Collection and identification of plant material. The plant *O. basilicum* was collected in November, 2016 in Benin City, Edo State, Nigeria. Its identity was confirmed by Dr Henry Akinnibosun at the Plant Biology and Biotechnology Department of the University of Benin, Benin City, Nigeria where a voucher number (UBH H462) was assigned to it.

Extraction of volatile oil. About 1 kg of the leaves was extracted in batches by hydro-distillation method with the aid of a Clevenger-type apparatus to obtain the volatile oil which was kept in the refrigerator maintained at 4ºC until required.

Source and identification of the tadpoles of *Raniceps ranninus.* Tadpoles (5-6 days old) were obtained from small water settlements around the Faculty of Pharmacy, University of Benin. They were identified as the tadpoles of

Raniceps ranninus by Professor M. Aisien, Animal Parasitologist, Department of Animal and Environmental Biology, Faculty of Science, University of Benin, Benin City, Nigeria.

Source and preparation of the guinea corn (*Sorghum bicolor*). *Sorghum bicolor* seeds were obtained from a market in Benin City and rinsed with absolute alcohol to remove the preservative and microbial contaminants. A simple viability test to separate the good seeds was carried out by adding water after which the floating seeds which were compromised were removed. The submerged seeds were dried on filter paper and used for the experiment.

Determination of cytotoxic effects of *O. basilicum* oil on tadpoles (*R. ranninus*). Following the method described by Ikpefan *et al*, [31] ten (10) tadpoles were placed in 50 mL capacity beakers containing 15 mL of water from the colonies of the tadpoles, which was made up to 49 ml with distilled water. 1 ml of the different concentrations of the oil (0.5, 1, and 2 mg/mL) prepared from a stock concentration dissolved in tween 80 and water was then added to make up to 50 mL. A final concentration of 10, 20, and 40 µg/mL respectively was obtained. The experiment was carried out in triplicates for each concentration. The mortality rate of the tadpoles was observed for 24 h.

Determination of cytotoxic effects of *O. basilicum* oil on brine shrimps (*Artemia salina*). The volatile oil (20 mg) was dissolved in 2 mL acetone and from this solution, concentrations of 10, 100 and 1000 µg/mL were obtained in replicates of three. The solvent was allowed to evaporate overnight. After 48 h of hatching and maturation of nauplii, 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 were incubated at 25-27ºC for 24 h under illumination. Other vials containing seawater and the reference cytotoxic drug (etoposide) served as the negative and positive controls respectively [32].

Determination of the growth inhibitory effects of the essential oil on *Sorghum bicolor* seeds. Concentrations (10 mL) of 1, 2, 5, 10, 20 and 30 mg/mL of *O. basilicum* oil prepared by dissolution with tween 80 and water, was poured into 9-cm-wide glass Petri dishes under-laid with cotton wool and filter paper (Whatman No 1). Twenty (20) seeds were spread on each plate and incubated in a dark environment. The length (mm) of the 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 [31].

Column chromatography of *O. basilicum* volatile oil. The *O. basilicum* oil (13 mL) was triturated with silica gel (200-400 mesh) and subjected to column chromatography to isolate the major component. Isocratic elution was carried out using 1200 mL of C_6H_{14} (100 %). The resulting fractions (67) were bulked based on their TLC profile and coded F_1 (4-8), F_2 (10-14), F_3 (15-20), F_4 (21-47) and F_5 (48-67) with 12% H_2SO_4 used as spray reagent. F_1, containing the major constituent of the oil, revealed by the large and conspicuous spots shown on TLC plate, was subjected to preparative thin layer chromatographic analysis with 0.5 mm thick Silica gel G (10-40 mesh). Multiple developments were carried out with C_6H_{14} (100 %). Two sub-fractions were obtained and coded SF_1 (40 mg) and SF_2 (1130 mg). Growth inhibition test with *S. bicolor* seeds was carried out at 2 mg/ml on the fractions (F_1, F_4, F_5, SF_1, SF_2) following the method earlier described.

Determination of cytotoxic activity on cancer cell lines. Cytotoxic activity was determined by MTT assay on human breast cancer (AU 565) and cervical cancer (HeLa) cell lines. The cancer cells were obtained from the molecular bank of the International Center for Chemical and Biological Sciences

(ICCBS) at the University of Karachi, Pakistan. The cancer cells were placed in 96-well plates at a density of 10,000 cells/well/100 µL and allowed to incubate for 24 h in complete media at 37 °C and 5% CO_2 for the healthy growth of the cells. The stock solution (20 mg/mL) of the oil and fractions (F_1, F_4, F_5, SF_1, SF_2) were prepared in sterile DMSO and later diluted to 50 µg/mL concentration which was used for the experiment. Doxorubicin at 50 µM was used as the standard and the experiment was performed in triplicates. After treatment, the cells culture was allowed to incubate for 48 hours at 37 °C and in humidified atmosphere of 5 % CO_2 after which 200µL of MTT (0.5mM) dye was added in each well and then incubated for another 3-4 h. The resulting formazan crystals were dissolved in 100µL of DMSO. The absorbance of the resulting solution was measured at 570 nm [33]. Samples which gave 50 % inhibition or more at 50 µg/mL, were considered active and further tested at lower concentrations to obtain the IC_{50} which was calculated by EZ-Fit software.

GC-MS analysis. The constituents of the oil and active sub-fraction were identified by GCMS method. The gas chromatogram was recorded using 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 used. Carrier gas was helium (99.999%) 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. 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

Cytotoxic effect of the volatile oil on tadpoles. At lower concentrations of 10 and 20 µg/mL the volatile oil produced no mortality, but 100 % mortality was obtained at 40 µg/mL.

Cytotoxicity assay on Brine Shrimp (*Artemia salina*). A dose-dependent response was observed when the shrimps came in contact with different concentrations of the volatile oil with highest mortality of 20 % observed at 1000 µg/mL, a result not significantly different ($P>0.05$) from lower concentrations. However, the positive control used, Etoposide gave 100 % mortality at 1000 µg/mL with an LC_{50} of 10.00 µg/mL (Table 1).

Growth inhibitory effects of the volatile oil on radicle length of *S. bicolor* seeds. The volatile oil of the plant was observed to remarkably inhibit the growth of the radicles in a concentration dependent manner. This inhibitory effect was sustained throughout the period of the assay. An average length of 27.85 ± 3.39 mm in the controls at 72 h was reduced to 0.88 ± 0.47 mm in seeds treated with 20 mg/mL concentration and 0.19 ± 0.05 mm in seeds treated with 30 mg/mL concentration showing 96.84 and 99.31 % reductions respectively (Figure 1).

Growth inhibitory effects of the fractions of the volatile oil on *S. bicolor* radicles. Fractions ($F_1 – F_5$) and sub-fractions (SF_1 and SF_2) produced inhibitory effects at 2 mg/mL on the radicles as shown in Figure 2. At 96 h the radicle length of the control was 6.18 mm while fraction F4 produced 1.61 mm, revealing 73.95% growth inhibition. At the same time,

F1 showed the least inhibition, 34.14% while sub-fraction SF_2 from the pTLC of fraction F_1 gave 79.29% inhibition (Figure 2).

Cytotoxic effects of the volatile oil and fractions on AU 565 and HeLa cancer cell lines. The volatile oil at a concentration of 50 µg/mL seemed not to have growth inhibitory effect on AU 565 breast cancer cell line, but the growth of HeLa cancer cells was inhibited by 23.02 %. The inhibitory activities of the column fractions against AU 565 varied with F_5 producing the highest activity of +48.41 %. The least activity was shown by F_4 (-7.78 %). Sub-fraction SF_2 obtained from the pTLC of fraction F_1 was active on HeLa cells with +89.3 % inhibition and IC_{50} of 18.70 ± 10 µg/mL realized. It also inhibited AU565 cells by +33.19 % (Table 2).

GC-MS analysis of *O. basilicum* volatile oil and active fraction. The major components of *O. basilicum* oil were observed to be estragole (52.8 %), β-linalool (16.1 %) and cineole (5 %). Sabinene and β-Farnesene were present in the lowest quantities with relative abundance of 0.16 and 0.18 % respectively. A total of 22 components were identified (Table 3). The subfraction SF_2 contained estragole (88.61%) as the major identified constituent with a retention time of 27.38 minutes.

Table 1: Cytotoxic effects of the oil on *Artemia salina* nauplii

Sample	Concentration (µg/mL) / % Mortality			LC_{50} (µg/mL)
	10	100	1000	
O. basilicum	6.66 ± 0.00	13.33 ± 0.30	20.00 ± 1.40	>1000
Etoposide	50.30 ± 0.50	89.10 ± 1.98	100.00 ± 0.00	10.00
Distilled water	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-

Values are expressed as the mean ± SEM of three independent observations.

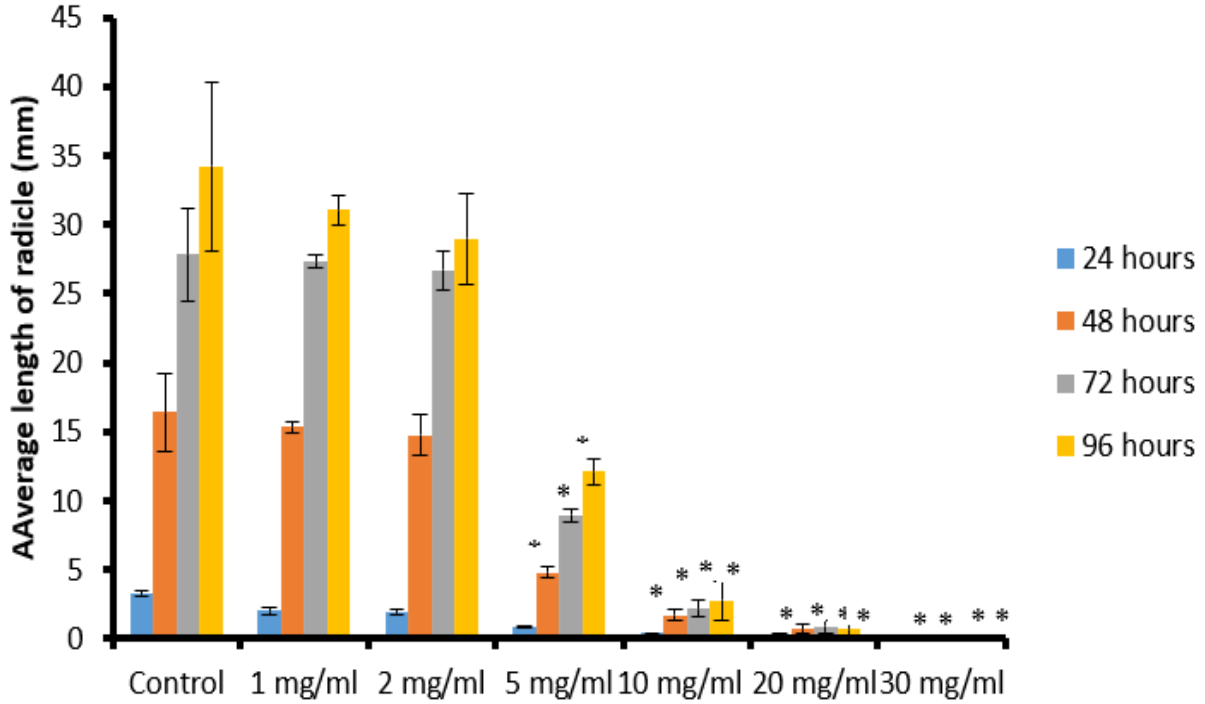

Figure 1: Growth inhibitory effect of *O. basilicum* volatile oil on the radicle length of *S. bicolor* seeds. . Values are mean ± sem, n=3. The values with superscript * indicate significant difference relative to negative control at P < 0.05.

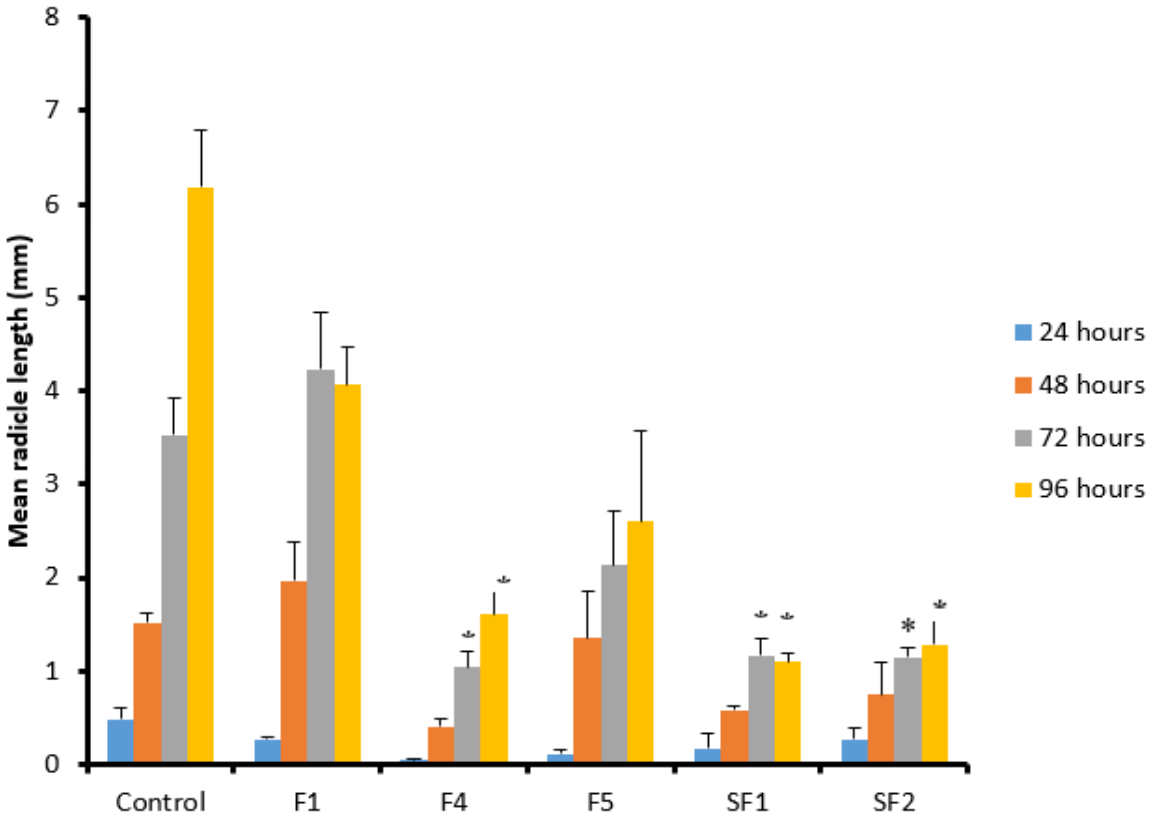

Figure 2: Growth inhibitory effect of *O. basilicum* oil column fractions and subfractions on the radicle length of *S. bicolor* seeds. Values are Mean ± SEM, n = 3. The values with superscript * indicate significant difference relative to negative control at $P < 0.05$.

Table 2: Inhibition effects of the volatile oil and fractions on AU565 and HeLa cell lines

Samples	Cell line	% Inhibition	IC_{50}
O. Basilicum oil	AU 565	-1.02	ND
O. Basilicum oil	HeLa	+23.02	ND
Column fraction F_1	AU 565	-1.17	ND
Column fraction F_2	AU 565	+14.90	ND
Column fraction F_4	AU 565	-7.78	ND
Column fraction F_5	AU 565	+48.41 *	ND
pTLC subfraction SF_2	AU 565	+33.19	ND
pTLC subfraction SF_2	HeLa	+89.3 *	18.70 ± 1.00
Doxorubicin	AU 565	+97.89*	0.085±0.03

Each value represents % mean ± SEM of three independent experiments. ND = not determined

Table 3: GC-MS Analysis of volatile composition of *O. basilicum* essential oil

S/No	Retention time	Compound	% Concentration
1	20.87	Sabinene	0.16
2	22.67	o-Cymene	0.37
3	22.81	D-Limonene	0.92
4	22.9	Cineole	5
5	23.36	.β-Ocimene	0.4
6	23.70	γ-Terpinene	0.31
7	24.55	Fenchone	0.68
8	24.79	β-Linalool	16.1
9	26.00	d-Camphor	1.08
10	26.47	α-Terpineol	0.31
11	26.72	4-Terpineol	3.75
12	27.00	Terpineol	1.09
13	27.21	Estragole	52.8
14	28.15	Chavicol	0.23
15	30.19	Eugenol	3.47
16	31.52	Caryophyllene	1.85
17	31.62	α-Bergamotene	2.2
18	31.81	β-Farnesene	0.18
19	32.14	Humulene	0.68
20	32.65	Germacrene D	0.4
21	32.96	β-Bisabolene	4.48
22	33.29	γ-Cadinene	0.47
		Unknown/unidentified	3.07
		Total	100

DISCUSSION

The predictive assays employed in this work were the growth inhibition test with *S. bicolor* seeds, tadpole mortality test, and brine shrimp lethality assays due to their availability. When seeds are exposed to favorable conditions, their meristematic tissues proliferate, and the extent of this proliferation is demonstrated by the lengthening of the radicles produced in control seeds. Because of their compact size, *S. bicolor* seeds were found to be more convenient. Furthermore, the availability is great, with up to 90% of seeds germinating within 24 hours [31]. The inhibition of radicle growth by *O. basilicum* volatile oil and its chromatographic fractions could be linked to the suppression of cell division thereby affecting its elongation, probably through reduction in mitotic index. It's also likely that the oil's contents interfere with the generation and activity of essential amino acids for cell proliferation in germinating seeds [31]. The extent of the interference may be a function of the concentration of the volatile oil constituents in the medium.

In vivo lethality in basic zoological species can be utilized as a useful monitor for screening and fractionation in the search for new bioactive natural compounds [32]. Lethality of substances to brine shrimp nauplii has been linked to the probable ability of such compounds to kill cancer cells in cell cultures [32].

Low to moderate inhibitions were observed with *O. basilicum* oil on AU565 and HeLa cancer cells in this study; it is possible that higher concentrations (beyond 50 µg/mL used) could have resulted in higher inhibitory effects. The oil has previously been reported to have *invitro* cytotoxic activity on HeLa cells with IC_{50} value of 90.5 µg/mL [10]. It has also been documented to be active against SF-767 glioblastoma and P388 murine leukemia [10,34] cancer cells.

O. basilicum oil has been reported to contain alkenyl benzenes which are presumed to be carcinogenic, however, the presence of

nevadensin or nevadensin-like compounds counteracts this side effect hence, *O. basilicum* containing drugs are considered as safe formulations [35].

Biological activity of essential oils is generally linked to their chemical compositions, or primary components. Minor compounds, on the other hand, may also be important because diverse molecules may function in concert with the large compounds [3]. The major components in *O. basilicum* oil from the results obtained was estragole (52.8 %) and β-linalool (16.1 %). GCMS analysis of essential oil of *O. basilicum* from South Africa showed that the main constituents were estragole (41.40%), 1,6- octadien-3-ol, 3,7-dimethyl (29.49%) and transalpha-bergamotene (5.32%) [34]. The plant collected from Mansoura- Egypt showed that estragole (55.95%), 1,8-Cineole (10.56%) and methyl eugenol (10.09%) were the main compounds in the essential oil [35]. Chemical analysis of the essential oil of *O. basilicum* from Algeria revealed compounds, linalool (52.1%) and linalyl acetate (19.1%) as the major constituents [10]. These quantitative and qualitative variances in oil content could be attributable to geographical, meteorological, and soil circumstances, as well as the plant's maturity during harvest time [8].

Chromatographic separation of volatile oil constituents can lead to enhanced yield of the major constituent and ultimately may also improve the activity of the compound over the oil. The chromatographic exercise in this study led to the separation of estragole (88.61%), a high boiling constituent, with almost similar Rt value as found in the volatile oil, 27.38 and 27.21 min.

The estragole content of the active fraction could probably be responsible for increased inhibitory activity on AU 565 cells from -1.02 to 33.19 %, and on HeLa cells from 23.02 to 89.3 %. The application of chromatographic techniques here remarkably improved the biological activities. Estragole, being an alkenyl benzene, is presumed to be carcinogenic but other alkenyl benzenes such as eugenol have also been reported to have anticancer activity [35].

Generally the mechanisms of action elicited by medicinal herbs against cancer include cytotoxic effect, cancer cell proliferation inhibition, efficient reduction in tumor volume, and the ability to protect DNA from threatening radiation, thus increasing survival rates [36-37]. Further anticancer studies need to be conducted with estragole against other cancer cell lines as well as to ascertain its mechanism of action and level of safety. Estragole obtained from the essential oil of *Croton zehntneri* has been reportedly tested against MCF-7 (4.62 %), HEP-2 (0 %) and NCI-H292 (7.36 %) human cancer cell lines but the inhibitions were not rewarding [38].

Conclusion. *O. basilicum* oil demonstrated potential as a cytotoxic agent especially on cervical (HeLa) cancer cells. Its active subfraction containing majorly estragole was found to have remarkably higher inhibitions, both in the preliminary test with *S. bicolor* seed radicle, and on breast (AU565) and cervical (HeLa) cancer cell lines.

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