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Original Research Article

GC-MS analysis and *invitro* cytotoxic activity of *Syzygium aromaticum* volatile oil and its major constituent - eugenol

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Abstract

Purpose: Cancer is one of the leading causes of death globally. *Syzygium aromaticum* flower buds have found application as a spice and for medicinal purposes around the world. In this research, we investigated the essential oil and its major constituent, eugenol for cytotoxic activity against breast (AU 565) and cervical (HeLa) cancer cell lines.
Methods: The oil was extracted and tested for cytotoxic effect on brine shrimp of *Artemia salina* (10-1000 µg/mL) and against the cell lines. Chromatographic separation of the oil was carried out and the resulting fractions screened for growth inhibitory activity using radicles of *Sorghum bicolor* seeds (0.25 mg/mL) as well as cytotoxicity on the cell lines with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The oil and active subfraction were subjected to GCMS studies.
Results: Our results demonstrated that the essential oil showed cytotoxic activities against *A. salina* nauplii, with LC_{50} of 143.17 µg/mL obtained. Growth inhibitory activity of the fractions against *S. bicolor* seed radicles was remarkable with subfraction 2 showing high potency. The oil and active subfraction possessed moderate cytotoxicity on AU 565 cell line but no inhibition was observed with HeLa cells. Eugenol was revealed to be the major constituent of the oil (64.84%) and was increased to 82.62% in the subfraction.
Conclusion: These results suggest that *S. aromaticum* oil and its major constituent, eugenol possess moderate cytotoxic properties against AU 565 cells but not HeLa cells. This necessitates further investigation against other cancer cell lines.

Keywords: Cytotoxic, AU565, HeLa, volatile oil, *Syzygium aromaticum*, eugenol

Introduction

Around the world, people are becoming more aware of and using plant-based drugs. Food plants used for medicinal purposes abound throughout the African continent. Although there have been advancements in Western Medicine, there are still a vast number of disorders for which conventional medications have not yet been developed. This has necessitated the continuous need for research to develop more effective medications to treat these disorders and the newly emerging challenges like cancers. The pharmacological assessment of diverse plants employed in traditional systems of treatment has advanced significantly thanks to research on medicinal plants. Plants have thus been portrayed as a major source of medicines, not merely as isolated active chemicals that are synthesized and distributed in standardized dosage form, but also as crude medications [1].

Plant essential oils have reportedly achieved a greater level of acceptance in phytomedicine and some oils have lately been examined for anti-convulsant, anti-oxidant, and anti-cancer properties due to their widespread use [2]. Cancer is a worldwide problem because it is one of the leading causes of death. It is a condition characterized by uncontrolled cell division, which results in aberrant tissue growth [3]. Currently, there are over a hundred different

forms of cancer, each with its own etiology and natural history. Breast and cervical cancers are the most common malignant diseases in women among the many cancers [4].

Several synthetic drugs used to treat the disease harm both normal and malignant cells, as well as cause other serious adverse effects [5]. As a result, new treatment options with improved outcomes are urgently required. The cost of screening extracts for direct anticancer effects is prohibitively high. This necessitates the development and application of simple, cheap predictive methods. Simple benchtop assays, such as brine shrimp lethality testing, can be used to screen and fractionate plant extracts in order to find new bioactive compounds [6].

Mosquito larvae (*Culex quinquefasciatus*) [7], tadpoles (*Raniceps ranninus*), and growth inhibition studies using *Sorghum bicolor* seed radicles [8] are also employed. In ethnomedicine, essential oils and their compounds have long been employed for the treatment or alleviation of various health conditions and applications. They have found use in the pharmaceutical, food, cosmetics, and beverage industries, among others [9].

The biological activity of their elements is determined by their composition. Terpenes derived from aromatic plants, primarily monoterpenes, and sesquiterpenes, make up the majority of these oils [10]. As a result, chemical characterization as well as the possibility of correlating main components to specific biological qualities play a role in essential oil research.

Syzygium aromaticum is a member of the Myrtaceae family. It is a genus of shrubs and trees belonging to the Myrtales order, with roughly 132 genera and 5,950 species found in tropical and mildly temperate regions of the world, including Australia, Chile, Argentina, and the Philippines. [11]. *S. aromaticum*, often known as clove, is known in Nigeria as kanafuru in Yoruba, kloovu in Igbo, and kanumfarii in Hausa. In traditional medicine, it is one of the most economically significant herbs [12]. Its dried buds have long been used as a spice. Its essential oil is used in aromatherapy and in dental emergencies to relieve toothaches [13]. Indigestion, loose stools, gas, and nausea are also treated with it. It's antibacterial, antifungal, insecticidal, and antioxidant properties have been evaluated and reported [14]. Its essential oil has been shown to have anticarcinogenic and antimutagenic properties because of its significant free radical scavenging activity [15,16].

Clove oil may have chemopreventive properties, according to preliminary research, especially in cases of lung, skin, and stomach malignancies [17]. By inhibiting two transition factors of the E2F family, eugenol derived from the essential oil was found to limit the growth of malignant melanoma WM1205Lu of both anchorage-dependent and anchorage-independent growth, lowered tumor size, and prevented melanoma invasion and metastasis [18].

Flavonoids (eugenin, rhamnetin, kaempferol, and eugenitin), triterpenoids (oleanolic acid, stigmasterol, and campesterol), and sesquiterpenes (eugenin, rhamnetin, kaempferol, and eugenitin) are among the many bioactive substances found in *S. aromaticum* [19]. Given the plant's multiple bioactivities, this study examined the cytotoxic activity of the essential oil and fractions on AU 565 (breast) and HeLa (cervical) cancer cell lines utilizing both predictive and confirmatory assays, as well as GCMS analysis of the essential oil and active sub-fraction.

Methods

Plant material collection and identification

Flower buds of *S. aromaticum* were purchased in Benin City, Edo State, Nigeria, between September and November 2016. Dr. Henry Akinnibosun of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria, confirmed the identity and assigned it the voucher number UBH C384.

Extraction of plant material

Approximately 2.0 kg of *S. aromaticum* flower buds were hydro-distilled in batches using a Clevenger-type apparatus for 3 h to obtain the volatile oil. It was kept refrigerated at 4°C until it was needed.

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

The volatile oil of *S. aromaticum* was dissolved in 2.0 mL acetone and concentrations of 10, 100,

and 1000 g/mL were obtained from this solution. The solvents were left to evaporate overnight. 10 larvae were introduced to each vial using a Pasteur pipette after 48 hours of nauplii hatching and maturation. Seawater (38.0 g/L, pH 7.4) was used to fill the vials to a capacity of 5 mL, and the vials were incubated at 25-27 °C for 24 hours under illumination. Negative and positive controls were provided by vials containing the solvent and a reference cytotoxic agent, etoposide [6].

Chromatographic fractionation of *S. aromaticum* volatile oil

S. aromaticum oil (15 mL) was subjected to column chromatography using silica gel (200-400 mesh). Gradient elution was done with 200 mL each of C_6H_{14} (100%) and C_6H_{14}-$CH_3COOC_2H_5$ (99.5:0.5, 99:1, 98.5:1.5, 98:2, 97.5:2.5, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10). The fractions (58) were collected and bulked according to their TLC profile (solvent system C_6H_{14}-$CH_3COOC_2H_5$ (9.7:0.3)) and coded F_1 (4-6), F_2 (7-11), F_3 (12-20), F_4 (21-32) and F_5 (33-58). As a result of the high yield and conspicuous spot, fraction F_1 (5 mL) was subjected to preparative TLC analysis on silica gel using C_6H_{14}-CH_2Cl_2 (9:1) to obtain two sub-fractions coded as SF_1 and SF_2.

Determination of growth inhibitory effects on *Sorghum bicolor* radicle

Guinea corn (*S. bicolor*) seeds purchased from Uselu market, Benin City, were cleaned with absolute ethanol (95%), and a simple viability test performed by adding water and quickly decanting, thereby eliminating any floating damaged seed. Those that remained submerged in water were chosen and dried before use.

The volatile oil, its chromatographic fractions, and sub-fractions were separately constituted into 0.25 mg/mL concentration using 2.0% Tween 80 in distilled water. Ten (10) mL of the resulting solutions were poured into Petri dishes covered with filter paper (Whatman No. 1) underlaid with cotton wool after which twenty (20) viable seeds were spread on each filter paper and incubated in a dark environment. The length (mm) of the radicles emerging from the seeds were taken at 24, 48, 72, and 96 hours. The control seeds were treated with 10 mL of distilled water containing 2.0% Tween 80. The experiment was carried out in triplicates [8].

Determination of cytotoxic activity using MTT assay

Cytotoxic activity was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cancer cell lines used were human breast cancer (AU 565) and cervical cancer (HeLa) obtained from the molecular bank of the International Center for Chemical and Biological Sciences (ICCBS) at the University of Karachi, Pakistan.

The cells were cultured in microplates (tissue culture treated, 96 well, flat bottoms) to the final volume of 100µL per well culture medium in a specific environmental condition such as 37 °C temperature and 5% CO_2 for the healthy growth of the cells. The next day, when the cells had adhered, the culture media was removed from each well. The cells were treated in triplicates with 50 µg/mL concentration of the volatile oil and fractions. Doxorubicin (50 µM) was used as the standard.

The cell culture was allowed to incubate for 48 hours at 37 °C and in humidified atmosphere of 5.0% CO_2. After this 200 µL of MTT (0.5mM) dye was added in each well and then incubated for another 4 hours at 37 °C and 5.0% CO_2. The resulting formazan crystals were dissolved in 100 µL of DMSO and shaken thoroughly for a minute on the shaker [20]. The absorbance of the resulting solution was measured at 570 nm against a background control which is blank using a microplate reader. The measured absorbance directly correlates to the number of viable cells [20,21].

GC-MS analysis

Gas chromatography-mass spectroscopy was carried out on the volatile oil and active subfraction. An Agilent technologies 7000 GC/MS triple quadrupole mass spectrometer with OPTIMA-5-ZB-5 column and electron ionization (EI) with ionization energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1.129 mL/min and the injection volume was 2.0 µL (split ratio 15:1). The 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. The total run time was 58.583 min. The software adopted to handle mass spectra and chromatogram was

Chemstation and compounds were identified from NIST library match.

Statistical analysis

The obtained data were expressed as mean ± SEM and evaluated with one-way Analysis of Variance (ANOVA) using SPSS 21. In all cases, differences were considered significant at $p < 0.05$.

Results

Cytotoxicity assay on brine shrimp (*Artemia salina*)

Exposure of the brine shrimps to varying concentrations of the volatile oil showed significant ($P < 0.001$) dose-dependent cytotoxicity. The volatile oil produced no effect at a concentration of 10 µg/mL. However, at concentrations of 100 and 1000 µg/mL, the mortalities increased to 26.66 and 100% respectively. The oil was found to have an LC_{50} of 143.17 µg/mL while etoposide gave an LC_{50} of 10.00 µg/mL (Table 1).

Growth inhibitory effects of the column fractions and subfractions of *S. aromaticum* oil

The column chromatographic fraction F_1 (4-6), demonstrated 94.05 % inhibition after 24 hours, while F_3 (12-20) indicated 100 % inhibition. F_1 and F_3 inhibited growth by 97.32 and 98.44 %, respectively, after 96 hours. At 24 and 96 hours, SF_2 derived by preparative TLC of F1 inhibited growth by 97.62 and 98 %, respectively (Figure 1).

Table 1: Cytotoxic effect of *S. aromaticum* volatile oil against *Artemia salina* nauplii

	Concentration (µg/mL) / % Mortality			
Test sample	10	100	1000	LC_{50} (µg/mL)
S. aromaticum	0.00 ± 0.00	26.66 ± 1.00	***100 ± 0.00	143.17
Etoposide	*50.30 ± 0.5	***89.10 ± 1.98	***100 ± 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. Standard drug; Etoposide LC_{50} = 10 µg/mL, *S. aromaticum* oil LC_{50} = 143.17 µg/mL *indicates significance compared to negative control. *$p < 0.05$, ***$p < 0.001$.

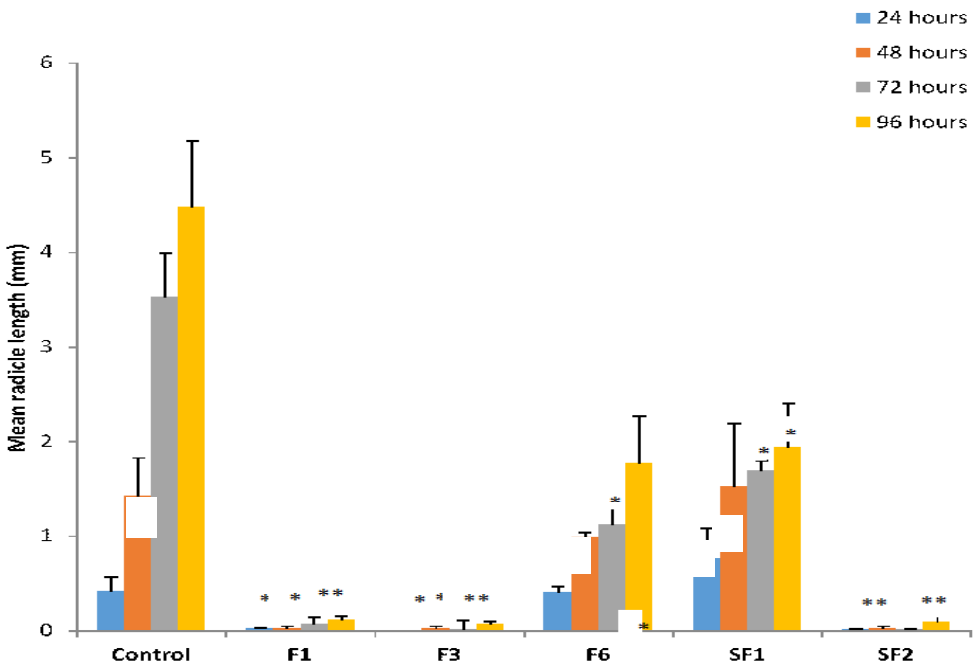

Fig 1: Growth inhibitory effect of *S. aromaticum* oil, column fractions and subfractions on the radicle length of *S. bicolor* seeds. Values are mean ± SEM, n = 20. *$p < 0.05$ compared to control

Inhibitory effects of *S. aromaticum* volatile oil and fractions on the cell lines

The volatile oil produced +16.52% inhibition on AU 565 cells but no noticeable inhibition was observed with HeLa cells. Among the column chromatographic fractions, F_4 (21-32) had the highest inhibitory activity against AU 565, with a value of +37.48%. Preparative TLC fraction SF_2 showed modest activity on AU 565 and no inhibition against HeLa cell lines; +18.82 and -1.24% were obtained respectively (Table 2).

GC-MS analysis of *S. aromaticum* essential oil and its preparative TLC fraction SF_2

The volatile oil was observed to contain eugenol (64.84%) and acetyleugenol (22%) as the major constituents. α and β-Caryophyllene were also present with relative abundance of 1.66 and 10.56% respectively. A total of 5 constituents were present in the oil (Table 3). GC-MS of the relatively active sub-fraction SF_2 showed eugenol and acetyleugenol to be the main content with relative abundance of 82.62% and 15.26% respectively (Table 4).

Table 2: Inhibition/stimulation effects of the volatile oil, column fractions, and preparative TLC sub-fractions on the cell lines

Sample	Cell line	% Inhibition/Stimulation	IC_{50}
S. aromaticum	AU 565	+16.52	-
S. aromaticum	HeLa	-11.07	-
Column fractions			
F_1	AU 565	+22.94	-
F_2	AU 565	+7.85	-
F_3	AU 565	+19.06	-
F_4	AU 565	+37.48	-
F_5	AU 565	+25.29	-
Preparative TLC fraction			
SF_2	AU565	+18.82	-
SF_2	HeLa	-1.24	-
Doxorubicin	AU565	+97.89	0.085 ± 0.03

Each value represents % mean ± SEM of three independent experiments. IC_{50} = Concentration of sample causing 50% inhibition of cells.

Table 3: GC-MS analysis of *S. aromaticum* volatile oil

S/No	Retention time (minutes)	Compound	% Concentration
1	**30.31**	**Eugenol**	**64.84**
2	31.54	β-Caryophyllene	10.56
3	32.15	α-Caryophyllene	1.66
4	33.28	Acetyleugenol	22
5	35.16	Caryophyllene oxide	0.43

Table 4: GC-MS analysis of sub-fraction SF_2

S/No	Retention time (minutes)	Compound	% Concentration
1	30.31	Eugenol	82.62
2	33.24	Acetyleugenol	15.26

Discussion

Antitumor drug research typically entails a series of complicated processes that, despite significant investment of time and resources, can provide disappointing results. Furthermore, due to a lack of research finances, the development and acceptance of simple bench-top tests have become essential [6]. These bioassays are easy to use, quick to perform, repeatable, and adaptable to a wide range of samples.

The bench-top assay methods used in this work were the brine shrimp lethality test and the growth inhibition test utilizing *S. bicolor* seeds, due to their availability. The potential of

chemicals to kill cancer cells in cell cultures is determined by their lethality toward these organisms. The drug is deemed to have anticancer efficacy if the toxicity is transferred to tumor cells *in vivo* [22].

The Brine Shrimp Cytotoxicity Test (BSCT) has been shown to correlate reasonably well with cytotoxicity and other biological characteristics [6]. Plant extracts with LC_{50} values less than 100 g/mL are deemed bioactive in the brine shrimp lethality test [23]. *S. aromaticum* oil had an activity that was comparable to the standard ($p > 0.05$), especially at the highest concentration employed where 100% mortality was obtained for both the oil and etoposide.

When seeds are exposed to favorable conditions, their meristematic tissues have a tendency to multiply, and the extent of this proliferation may be observed in the lengthening of the radicles produced in control seeds. Due to their compact size, *S. bicolor* seeds were found to be more convenient for this evaluation and up to 90% of the seeds germinate within 24 hours [8].

S. aromaticum essential oil has been reported to cause a concentration-dependent reduction in the length of radicles emerging from *S. bicolor* seeds. At a dosage of 1.0 mg/mL, the seeds showed a 94.96% reduction in growth. The oil was also cited to possess cytotoxic effect against tadpoles of *Raniceps ranninus* where it was observed to produce mortality of 23.33 ± 13.33% at a concentration of 10 µg/ml, which increased to 100% with 20 µg/ml [24]. According to our results, separation using column chromatographic methods resulted in significant increase in the activities of the fractions on the radicles, with SF_2 inhibiting growth at 0.25 mg/mL concentration by 97.99% after 96 hours.

The fractions hinder radicle growth by suppressing cell division [25] which affects radicle cell elongation [26], most likely by decreasing mitotic index and causing changes in the osmotic potential that prevent turgor creation in the seed cells, which is one of the major biological activities necessary for the commencement of radicle growth during seed germination, hence inhibition of the radicles elongation [27]. It is also likely that the contents of the fractions obstruct the production and activity of amino acids required for cell proliferation in growing seeds [28].

The biological activities of essential oils are generally related to their chemical compositions or the primary elements of the oils. The less abundant compounds, on the other hand, may be important because the diverse molecules may interact synergistically with the larger chemicals [29].

As revealed by SF_2, chromatographic separation of eugenol content of *S. aromaticum* oil using preparative TLC did not significantly improve its cytotoxic potential. Testing the cancer cells with higher concentrations of the oil and fractions beyond 50µg/mL used in this study may have increased the activity, although a chemical may have cytotoxic effect on one form of cancer but not on others. For example, 1,8-cneole [30] causes apoptosis in human colon cancer, but has no activity on either prostate cancer or glioblastoma cell survival [31]. Due to its high free radical activity, *S. aromaticum* oil has been reported to have cytotoxic properties against a variety of cancer cell lines [16, 17]. Eugenol, on the other hand, has been reported to induce cell death in several tumors and cancer cell types such as breast adenocarcinoma [32], melanoma cells [33,34], leukemia [33,35], and colon carcinoma [36].

The principal components of the oil, according to the GCMS results, are eugenol (64.84%) and acetyleugenol (22%). The quantity and quality of components contained in *S. aromaticum* oil obtained from other places have showed variances. According to one report [37], the essential oil obtained from the plant harvested in Algeria contained mainly eugenol (80.00 %) followed by eugenyl acetate (5.01%) and β-caryophyllene (2.9%). The essential oil from Brazil also contained eugenol (52.53%), caryophyllene (37.25%) and copaene (2.05%) [38]. 3-Allyl-6-methoxyphenol (87.98%), eugenol (7.38%) and caryophyllene (7.38%) have also been cited in a different study [39] as the main constituents in the oil.

Geographical, climatic, and soil conditions as well as the level of maturity of the plants at the point of harvest have been identified as some of the causes of the quantitative and qualitative variances in the composition of volatile oils [40], which could lead to disparities in their biological activities.

Conclusion

This study revealed that *S. aromaticum* essential oil has moderate cytotoxic activity on AU 565 cancer cells with no effect against HeLa cells, though significant cytotoxicity was observed with *A. salina* nauplii. Pooling and testing of its major constituent, eugenol through chromatographic techniques did not significantly improve its cytotoxic activity on the cell lines at the concentration used.

Conflict of Interest

No conflict of interest is associated with this work.

Contribution of Authors

We declare that this study was done by the authors stated in this article and all liabilities pertaining to claims relating to the content of this work will be borne by us. BAA conceived, designed, and supervised the study, and ROI carried out the laboratory work. ROI and BAA wrote and reviewed the manuscript.

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References

1. Adaikan PG, Gauthaman K., Prasad RNV. History of herbal medicines with an insight on the pharmacological properties of *Tribulus terrestris*. Aging Male 2001;4(3):163-169.
2. Mimica-Dukic N, Bozin B, Sokovic M, Simin N. Antimicrobial and antioxidant activities of *Melissa officinalis* L. (Lamiaceae) essential oil. J Agric Food Chem 2004;52: 2485-2489.
3. Vogelstein B., Kinzler K. The genetic basis of human cancer. McGraw Hill Professional, Medical. 2002; pp. 1-821
4. Jedi-Agba E, Curado M. P., Ogunbiyi O., Oga E., Fabowale T, Igbinoba F, Osubor G, Olu T, Kumai H, Koechlin A, Osinubi P, Dakum P, Blattner W and Adebamowo CA. Cancer incidence in Nigeria: a report from population-based cancer registries. Cancer epidemiology. 2012;36(5):e271-e278.
5. Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. Chem Rev 2009;109:3012-3043.
6. McLaughlin J. L., Chang C. J., Smith D. L. "Bench-top" bioassays for discovery of bioactive natural products: An update. (Atta-Ur-Rahman), Studies in Natural Products Chemistry. Elsevier Science 1991;9: 101-103.
7. Moshen ZH, Jawad AL, Al-Chalabi BM, Al-Naib A. Biological activity of Callistemon lanceolatus against *Culex quinquefasciatus*. Fitoterapia 1990;31:191-196.
8. Ayinde BA, Agbakwuru U. Cytotoxic and growth inhibitory effects of the methanol extract of *Struchium sparganophora* Ktze (Asteraceae) leaves. Pharmacogn Mag 2010; 6(2):12-21.
9. Nogueira de Melo GA., Grespan R, Fonseca JP, Farinha TO, da Silva EL, Romero AL, Bersani-Amado CA, Cuman RKN. Inhibitory effects of ginger (*Zingiber officinale* Roscoe) essential oil on leukocyte migration *in vivo* and *in vitro*. J Nat Med. 2011;65:241-246.
10. Daferera DJ, Tarantilis PA, Polissiou MG. Characterization of essential oils from Lamiaceae species by Fourier transform Raman spectroscopy. J Agric Food Chem. 2002;50:5503-5507.
11. Christenhuz MJM, Byng JW. The number of known plant species in the world and its annual increase. Phytotaxa. Magnolia Press. 2016;261(3):201-217.
12. Amla MH, Ertas M, Nitz S, Kollmannsberger H. Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum* L.). BioResources. 2007;2(2):265-269.
13. Kim HM, Lee EH, Hong SH, Song HJ, Shin MK, Kim SH, Shin TY. Effect of Syzygium aromaticum extract on immediate hypersensitivity in rat. J Ethnopharmacol. 1998; 60:125-131.
14. Niwano Y, Saito K, Yoshizaki F, Kohno M, Ozawa T. Extensive screening for herbal extracts with potent antioxidant properties. J Clin Biochem Nutr. 2011;48(1):78-84.
15. Zheng GQ, Kenny PM, Lam KT. Sesquiterpenes from clove (*Eugenia carrophyllata*) as potential anticarcinogenic agents. J Nat Prod. 1992;55:999-1003.

16. Miyazawa M, Hisama M. Suppression of chemical mutagen induced SOS response by alkylphenols from clove (*Syzygium aromaticum*) in *Salmonella typhymurium* TA153/pSK1002 umu test. J Agric Food Chem. 2001;49:4019-4025.
17. Schonfelder I, Schonfelder P. *Syzygium aromaticum* (L.) Merr & Perr (*Eugenia caryophyllata* Thunb Jambosa caryophyllus (Spreng.) (Niedenzu). J Das neue Handbuch der Heilpflanzen, Kosmos-Verlag. 2004; 431-432.
18. Ghosh R, Nadiminty N, Fitzpatrick JE, Alworth WL, Slaga TJ, Kumar AP. Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. J Biol Chem 2005;280(7):5812-5819.
19. Park IK, Shin SC. Fumigant activity of plant essential oils and components from garlic (*Allium sativum*) and clove bud (*Eugenia caryophyllata*) oils against the Japanese termite (*Reticulitermes speratus* Kolbe). J Agric Food Chem 2005;53:4388-4392.
20. Kritsanawong S, Innajak S, Moto M, Watanapokasin R. Antiproliferative and apoptosis induction of α-mangostin in T47D breast cancer cells. Int J Oncol 2006;48:2155-2165.
21. Puig T, Aguilar H, Cufí S, Oliveras G, Turrado C, Ortega-Gutiérrez S. A novel inhibitor of fatty acid synthase shows activity against HER2+ breast cancer xenografts and is active in anti-HER2 drug-resistant cell lines, Breast Cancer Res. 2011;13: R131.
22. Obuotor EM, Onajobi FD. Preliminary evaluation of cytotoxic properties of *Raphia hookeri* fruit mesocarp. Fitoterapia. 2000;71:190-192.
23. Meyer BN, Ferrigni NR, Putnam JT, Jacobsen LB, Nichols DE, McLaghlin JL. Brine shrimp: A convenient general bioassay for active plant constituent. Planta Med. 1982;45:31-34.
24. Imade RO, Ayinde BA. Cytotoxic and growth inhibitory effects of the volatile oils of *Eugenia caryophyllus* (Myrtaceae), *Callistemon citrinus* (Myrtaceae) and *Zingiber officinale* (Zingiberaceae). Journal of Science and Practice of Pharmacy. 2018;5(2):249-255.
25. Ray-Gallet D, Quivy JP, Scamps C, Martini EM, Lipinski M, Almouzni G. HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol Cell. 2013;9:1091-1100.
26. Fusconi A, Repetto O, Bona E, Massa N, Gallo C, Dumas-Gaudot E. Effects of cadmium on meristem activity and nucleus ploidy on roots of *Pisum sativum* L. cv. Frisson seedlings. Environ Exp Bot. 2006;58:253-260.
27. Maia A, Rainer L. Changes in water relations, solute leakage and growth characters during seed germination and seedling development in *Trigonella coerulea* (Fabaceae). J Appl Bot. 2001;75:144-151.
28. Alabadi D, Carbonell J. Differential expression of two spermidine synthase genes during early fruit development and in vegetative tissues of pea. Plant Mol Biol. 1999;39:933-943.
29. Bakkali F, Averbeck S, Averbeck D, Zhiri A, Baudoux D, Idaomar M. Antigenotoxic effects of three essential oils in diploid yeast (*Saccharomyces cerevisiae*) after treatments with UVC radiation, 8-MOP plus UVA and MMS. Mutat Res. 2006;606:27-38.
30. Murata S, Shiragami R, Kosugi C, Tezuka T, Yamazaki M, Hirano A, Yoshimura Y, Suzuki M, Shuto K, Ohkohchi N.. Antitumor effect of 1, 8-cineole against colon cancer. Oncol Rep. 2013;30:2647-2652.
31. Bayala B, Bassole IHN, Gnoula C, Nebie R, Yonli A, Morel L, Figueredo G, Nikiema JB, Lobaccaro JMA,. Simpore J. Chemical composition, antioxidant, anti-inflammatory and anti-proliferative activities of essential oils of plants from Burkina Faso. PloS One. 2014;9(3):e92122.
32. Jaafari A, Tilaoui M, Mouse HA, Mbark LA, Aboufatima R, Chait A, Lepoivre M, Zyad A. Comparative study of the antitumor effect of natural monoterpenes: relationship to cell cycle analysis. Rev Bras Farmacogn. 2012;22(3):534-540.
33. Satooka H, Kubo I. Effects of eugenol on murine B16-F10 melanoma cells in Proceedings of the 238th ACS national meeting. 2009; pp. 16-20, Washington, DC, USA.
34. Pisano M, Pagnan G, Loi M, Mura ME, Tilocca MG, Palmieri G, Fabbri D, Dettori MA, Delogu G, Ponzoni M, Rozzo C. Antiproliferative and pro-apoptotic activity of eugenol-related biphenyls on malignant melanoma cells. Mol Cancer. 2007;6:8.
35. Fujisawa S, Atsumi T, Satoh K. Radical generation, radical-scavenging activity, and cytotoxicity of eugenol-related compounds. In vitr Mol Toxicol. 2000;13(4):269-279.

36. Slameňová D, Horváthová E, Wsólová L, Šramková M, Navarová J. Investigation of anti-oxidative, cytotoxic, DNA-damaging and DNA-protective effects of plant volatiles eugenol and borneol in human-derived HepG2, Caco-2 and VH10 cell lines. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 2009;677(1-2):46-52.
37. Boughendjioua H. "Essential Oil Composition of *Syzygium aromaticum* (L.)", Int Res J Pharm Med Sci. 2018;1(3):26-28.
38. Teles AM, Silva-Silva JV, Fernandes JMP, Abreu-Silva AL, Calabrese K, Filho NEM, Mouchrek AN, Almeida-Souza F. GCMS Characterization of Antibacterial, Antioxidant, and Antitrypanosomal activity of *Syzygium aromaticum* Essential oil and eugenol, Evid-Based Complementary Altern Med. 2021, Article ID 6663255, 12 pages.
39. Mohammed NH, Ahmed MH, Hussien MO. Qualitative analysis of the essential oil of *Syzygium aromaticum* (L.) (Clove) using gas chromatography-mass spectrometry (GC-MS). Int. J Res Pharm Chem. 2015;5(2):350-354.
40. Barra A. Factors affecting chemical variability of essential oils: a review of recent developments. Nat Prod Commun. 2009;4(8):1147-1154.