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The Cytotoxic Potential for *Syzygium Aromaticum* and *Nigella Sativa* Essential Oil Compared to Doxorubicin against Pancreatic, Colonic, and Cervical Cancer Cell Lines

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Abstract

Syzygium Aromaticum (clove) and *Nigella sativa* (black seed), traditional medicinal herbs, display broad anticancer activity. The observations supported these oils' anti-proliferative and cytotoxic activities by MTT assay. The cold-pressed extracted oil was evaluated against cervical (HeLa), pancreatic (Panc), and colon (HCT) carcinoma cells compared to doxorubicin. Clove oil had the lowest IC_{50} values against HeLa, HCT, and Panc cells, which were 0.25, 0.38, and 5.26 µg/ml, respectively. Black seed oil was next, with IC_{50} values of about 5.51, 4.24, and 22.7 µg/ml, respectively. These oils were extra potent than the reference drug, doxorubicin, which had IC_{50} values of about 101, 188, and 244 µg/ml. According to a flow cytometry study, clove oil causes cell death in HeLa cells more than the medication doxorubicin. Clove oil has proven to possess strong inhibitory activity of the expression of anti-apoptotic protein Bcl-2 than doxorubicin. The Diphenylamine method demonstrated that clove oil causes DNA damage and increases DNA fragmentation rate more than doxorubicin. GC/MS results showed that the clove and black seed contain many valuable anticancer compounds, such as p-cymene, a-Longipinene, Idebenone, and caryophyllene for black seed and eugenol for clove. Clove oil is therefore regarded as an excellent natural anticancer agent superior to chemotherapy medications with long-term side effects.

Keywords: Anticancer; Doxorubicin; Clove; Black seed; Essential oil

1. Introduction

In recent decades, cancer has become a global health crisis. People with Cancer die frequently. Due to a lack of diagnostic tools, standard treatment methods, and increased cure costs, economically developing countries have greater difficulty. Synthetic medications help scientists solve these issues. These medications target fast-dividing tumor cells. These medications target fast-dividing tumor cells while simultaneously harming natural cells, causing other permanent severe side effects [1].

Because there are currently no effective and safe treatments, there is an immediate need to research and develop natural and novel chemotherapeutic medicines. The medicinal natural field depends heavily on using bioactive natural compounds. It has been suggested that plants have significant untapped promise in treatment cancer research [2,3]. Among those fixed constituents that have been isolated and branded, vinca alkaloids (for example, vincristine and vinblatine) obtained from *Catharantus roesus*, the taxanes (for example, paclitaxel and docetaxel) obtained from Taxus brevifolia, and the camptothecin obtained from *Campotheca acuminata*. These constituents were obtained from their respective plants [4,5]. Essential oils have been demonstrated to exhibit cytotoxic and anticancer properties after selection of plant-derived products in many laboratory models. The chemical properties of such molecules can provide a better knowledge of the possible pathways involved in their antitumor activity. Essential oils are a complex combination of chemical substances distinguished mainly by their

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volatility, fragrance, low molecular weight, and density. Terpenes and terpenoids (primary contributors), phenolics, and aliphatic chemicals are the three principal classes of EOs discovered at present [6].

The plant known as clove, *Syzygium aromaticum*, is a member of the Myrtaceae family. According to different reports, the essential oil content of *S. aromaticum L.* ranges from 15–20% by weight. The essential oil of clove has a high concentration of phenolic compounds, including eugenol, eugenol acetate, and beta-caryophyllene. These chemicals possess a variety of biological actions, such as antibacterial, antifungal, insecticidal, anticancer, and antioxidant effects. Because clove essential oil is generally considered safe (GRAS) by the FDA. it can be found in various products, including cosmetics, pharmaceuticals, and nutritional products [7,8].

Nigella sativa (Ranunculaceae family), often known as black cumin and "sinouj" in Tunisia, is an annual herbaceous plant cultivated worldwide. The seeds are widely used for flavoring and therapeutic uses. *Nigella sativa* (black seed) should be a relevant tool for health promotion regarding its low toxicity and numerous modes of action. Most recent studies reveal that the oil extracted from *N. sativa* has antibacterial and anti-inflammatory activities, as well as anticancer and antioxidant. In addition, , thymoquinone, (TQ), a principal component of black seed oil, has anticancer effects on many tumor cell lines, including ovarian, colorectal, leukemia, larynx, breast, lung, and osteosarcoma.

The main objective of this study was to compare the effectiveness of essential oils extracted from *Syzigium aromaticum* (clove) and Nigella sativa (black seed) against the viability of specific cancer cell lines, including HCT (Human colorectal carcinoma), Panc (Human pancreatic carcinoma), and HeLa (Human cervix carcinoma), in comparison to the standard anticancer drug doxorubicin, and then to determine the mode of action of the most potent oil against cancer cells.

2. Material and Methods

Essential oil extraction

Cold-pressed black seed oil and clove oil were purchased from the National Research Center (Egypt). The clove and black seeds were subjected to mechanical pressing at room temperature (25 °C) and were not heated in any way during the process. The oil phase was separated from the fiber phase by keeping the crushed seeds at room temperature for one night to allow the oil phase to separate and then filtering the oil with Whatman #4 filter paper [10].

Gas Chromatography/Mass Spectrometry (GC/MS) analysis and conditions

Gas chromatography-mass spectrometry (GC-MS) is one of the so-called hyphenated analytical techniques. It is composed of double combined techniques to form a single method for analyzing mixtures of organic chemicals. Gas chromatography separates the components of a mixture, and mass spectrometry characterizes each of the components individually. The combination of the two techniques allows for both qualitative and quantitative evaluations of a sample containing a number of organic compounds [11]. A quadrupole mass spectrometer, including a capillary DB-1 column 15 m × 0.25 mm, was used to examine the extracted oil (J&W Scientific). The temperature program for the oven was from 115°C (1 min) to 280°C (3 min) (7.5°C/min), while the injection port temperature was kept at 200°C. The mass spectra were obtained at 70 eV with helium as the carrier gas moving at a 1.5 ml/min rate. The chemical components were known by comparing the chemical components' mass fragmentation patterns to those of the WILEY MASS SPECTRAL DATABASE's standard reference data.

MTT assay

The extracted oils of clove and black seed, compared with the standard drug doxorubicin, were examined for antitumor effect against three cell lines, namely; HeLa (Human cervical cancer cells), Panc (Human pancreatic cancer cells), HCT (Human colon cancer cells) which purchased from vacsera. Those Cell lines were treated with oils ranging from 500 to 0.24 µg and incubated for 24 hours at 37 ºC after being allowed to adhere for the first 24 hours till confluence. The fresh medium was added, and 100 µl of MTT solution (5 mg/ml) was applied for 4 hours at 37 °C. A microplate reader detected the absorbance at 570 nm [12].

Microscopic Studies

After staining the cells with crystal violet, the photographs were obtained using a camera (digital one) which was connected to an inverted microscope ((CKX41; Olympus, Japan).

Quantitative detection of the apoptotic cell by flow cytometry (ANNEXIN V- FITC – apoptosis detection kit)

Flow cytometric analysis was done by performing a dot plot assay to examine the type of cell death induced by samples. HeLa cells were treated with the extracted clove oil and doxorubicin for 24h (as previously mentioned). Untreated cells

(control) were also included in the experiment design. Approximate 1×10^6 cells were harvested, washed with ice-cold phosphate buffer twice, and centrifuged for 15 minutes at 500 ×g at 4ºC. The supernatant was discarded, and the cell pellets were resuspended in ice-cold 1X Binding Buffer and then incubated on ice. After that, 1 µl of annexin V-FITC solution and 5 µl of PI solution were added to 100 µl of the cell suspensions. The stained cells were quietly mixed and then incubated for 15 minutes in a dark ice box. After the incubation period, 400 µl of ice-cold 1X binding buffer was added to each tube, with gentle mixing for 5 minutes, then analyzed by flow cytometry (FACS Caliber (BD FACS Caliber) within 30 min [13].

Western blotting analysis

In 6-well plates, HeLa cells were planted at an intensity of $4x10^5$ cells/well. Clove oil (0.49 µg/ml) and doxorubicin (250 µg/ml) at a concentration greater than the IC_{50} value or vehicle control were added to the culture medium (2 ml) after 24 h in culture before harvesting. Lysis buffer [100 mM NaCl, 10 mM Tris, 25 mM EDTA, 25 mM EGTA, 1% (v/v) Triton X-100, 1% (v/v) NP-40 (pH 7.4), which is cold, with 1:300 protease inhibitor cocktail (Sigma) and Phosphatase inhibitor cocktail Tablet (Roche)] was used to lyse the cells.

The cells were stored at −20 °C for 1 hour to facilitate further lysis, then collected using a cell scraper and sonicated for 2 minutes. The total protein content was determined using colorimetric analysis. Equal amounts (20 µg) of protein samples were combined and boiled with SDS loading buffer for 10 minutes, then allowed to cool on ice before being loaded into an SDS-polyacrylamide gel and separated by a Cleaver electrophoresis unit (Cleaver, UK). The separated proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and electroblotted for 30 minutes using a Semi-dry Electroblot.

For two hours at 37 ºC, the membrane was blocked with 5% fat-free dry milk in TBS-T to lessen non-specific protein interactions between the membrane and the antibody. Each primary antibody was used on the membrane at the appropriate dilution for overnight incubation at 4°C. Cell Signaling Technology's Bcl-2 primary antibodies (1:1000) were utilized. The blots were rinsed three times with TBS-T for ten minutes. The membrane was washed three times (10 min) with TBS-T before incubating with the matching horse radish peroxidase-linked secondary anti-rabbit antibodies (Dako) for an additional hour at room temperature. The chemiluminescent Western ECL substrate (Perkin Elmer, Waltham, MA) was applied to the blot according to the manufacturer's instructions. ECL solutions A and B were combined in equal amounts, and the membranes were incubated for 1 minute. A CCD camera-based imager was used to record the chemiluminescent signals (Chemi Doc imager, Biorad, USA). Then, the band intensities were assessed using -actin as an internal reference protein [14].

DNA fragmentation analysis (DPA assay)

HeLa cells were planted at a density of $4x10^5$ cells/well in 6-well plates. Clove oil (0.49 µg/ml) and doxorubicin (250 µg/ml) at a concentration greater than the IC_{50} value were added separately to the culture medium for 24 h before harvesting. After that, 1 ml of cell suspension $5x10^5$ was transferred to tubes labeled B. The cells were Centrifugated at 200xg at 4°C for 10 min. The supernatants were put carefully in new tubes labeled S (supernatant); then,1.0 ml of TTE solution was vigorously added to the pellet in tube B and vortex. After cell lysis (caused by the presence of Triton X-100 in the TTE solution) and nuclear structure disruption (after Mg++ chelation by EDTA in the TTE solution), this approach enables the release of fragmented chromatin from nuclei. Tube B was centrifuged at 20,000xg for 10 minutes at 4°C to remove fragmented DNA from intact chromatin. The supernatants were transferred into freshly labelled tube T, and 1.0 ml of TTE solution was added to the little pellet in tube B. After adding 1.0 ml of 25% TCA to tubes T, B, and S and aggressively vortexing, the precipitation occurred at 4°C overnight. DNA precipitated during incubation was extracted by pelleting the sample at 4 °C for ten minutes with a centrifuge set to 20,000 x g, and the supernatants were removed by aspiration. DNA hydrolysis required 160 ml of 5% TCA to each pellet, heating them for 15 minutes in a heating block set to 90 °C. A blank was made by adding 160 ml of 5% TCA to a separate tube.

Each tube was filled with 320 ml of newly made DPA solution and vortexed. Two 200 m l-aliquots of colored solution (ignoring black particles) were transferred from each tube to a well of a 96-well microtiter plate after incubation for roughly 4 hours at 37°C or during the night at room temperature. At 600 nm, a multiwall spectrophotometer reader measured optical density with a blank set to 0. Finally, the percentage of fragmented DNA can be calculated using the formula:

% Fragmented DNA = $S + T$/S+T+B *100

Where S, T, and B are the OD_{600} (optical density) of fragmented DNA in the S, T, and B fractions, respectively [15].

3. Results and Discussion

Chemical composition of black seed (*Nigella sativa)* oil by GC/MS

Syzygium aromaticum (clove) and the *Nigella sativa* (black seed) essential oils, which were purchased

from a National Research Center, were extracted using the cold-pressed process (Fig.1). The extracted oils were analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) to separate and identify the extracted oil [16].

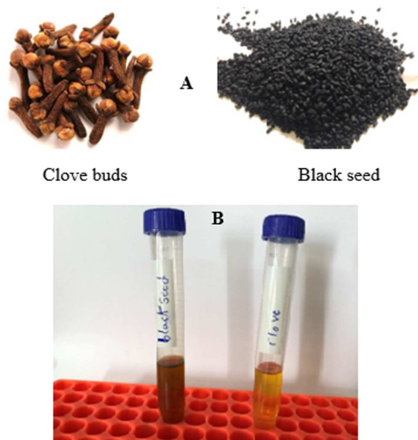


Figure (1): *Syzygium aromaticum* (clove) and the *Nigella sativa* (black seed) (A) essential oils (B) extracted by cold pressing

As shown in Table (1) and Fig. (2), Longifolene was the main compound(6.99%), followed by p-cymene(6.88%), bicyclo[4.1.0]HEPT-3-ene,3,7,7-trimethyl –(3.83),2-cyclohexene-1-carboxylic acid, 6-methylene -,methyl ester(3.38%), trans-4-methoxy thujane(3.34%), Cholestan-3-ol, 2-methylene-,(3á,5à)-(3.25%), Hexadecanoic acid,2,3-Dihydroxypropyl ester(2.37%), Caryophyllene (2.22%), Pentane, 3-methyl –(1.43%), a-Longipinene(1.38%), Bicyclo[3.1.1]hept-3-ene-spiro-2,4'-(1',3'-dioxane), 7,7-dimethyl- (1.13%), Phenanthrene,7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-, [4aS-(4aà,4bá,7á,10aá)]-(1.11), Idebenone(1.04%), Benzene, (1-butyloctyl)-(0.97%), Ethyl iso-allocholate(0.3%), Cholestan-3-ol,2-methylene-,(3á,5à)-(0.14%), Hi-oleic safflower oil(0.04%), 9,12-Octadecadienoic acid (Z,Z)-(0.03%) and finally 2-Ethyl-oxetane was the minor compound had been found in *Nigella sativa* essential oil (0.02%). The Black seed oil contains many antitumor-active compounds like p-cymene, a-Longipinene, and caryophyllene. Different *N. sativa* varieties have yielded a variety of active compounds that have been separated and identified. In addition to TQ, these include thymohydroquinone, thymoquinone, and thymol, as well as p-cymene, nigeglanine, nigellimine, nigellidine, t-anethol, and 4-terpineol, as well as carbohydrates, vitamins, minerals, and proteins. Some of the main parts of *N. sativa* are also excellent sources of fatty acids, the most important of which are linoleic and oleic acids [17].

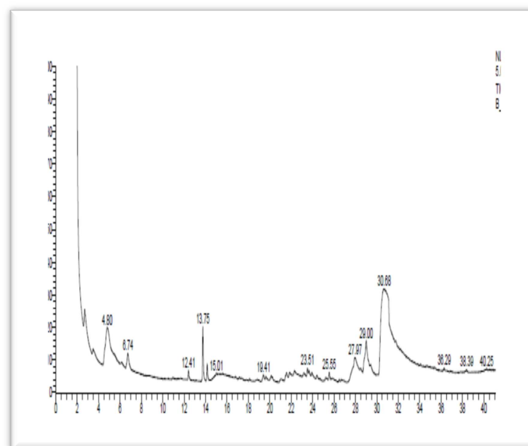


Figure (2): GC–MS chromatogram of black seed (*Nigella sativa*) oil

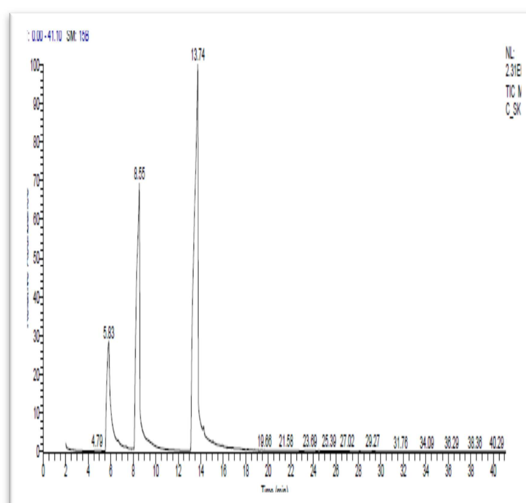


Figure (3): GC–MS chromatogram of *Syzygium aromaticum* (clove) oil

Chemical composition of *Syzygium aromaticum* (clove) oil by GC/ MS

The GC–MS analysis of clove oil identified ten compounds in **Table (2)** and **Fig. (3)**. The major compounds are Benzene methanol (36.32%), Acetic acid, Phenyl methyl ester (14.84%), Eugenol (12.89%), Phenol,2-methoxy-4-(2-propenyl)-(12.89%), Caryophyllene (2.76%). The minor compounds are 3-Pentanone,1-hydroxy-2-methyl-1-phenyl- (0.02%), Benzyl alcohol (0.01%), Caryophyllene oxide (0.15%), Phenol, 2-methoxy-5-(1-propenyl)- (0.01%), (E)-, 10-Heptadecen-8-ynoic acid, methyl ester, (E)- (0.01%), Dotriacontane (0.08%) and Isochiapin b %2<,1-Heptatriacotanol (0.01%) (Fig.3). It was noticed that the extracted

clove oil has bioactive compounds like Eugenol, which was present in a high percentage, reaching 12.89 % and 12.89 % of Phenol,2-methoxy-4-(2-propenyl). At least 30 different compounds have been isolated from clove essential oil [18], but eugenol is the primary one and accounts for at least half of the total. Eugenyl acetate, beta-caryophyllene, and humulene comprise 10–40% of the compound. Less than 10% of clove, including diethyl phthalate, caryophyllene oxide, cadinene, -copaene, 4-(2-propenyl)-phenol, chavicol, and -cubebene [19,20, 21].

Effect of black seed, clove oil, and doxorubicin on the viability of HCT, Panc, and HeLa tumor cell lines

The extracted black seeds, clove, and doxorubicin were estimated for potential cytotoxic effects against HCT, Panc, and HeLa cell lines. The cells were treated with two-fold serial dilutions of active compounds ranging from 500 to 0.24 µg/ml, and cell viability was measured by MTT colorimetric assay. Dilutions-dependent growth inhibition was observed in all tested tumor cell lines.

Table (1)
Chemical composition of black seed oil using GC–MS

RT	Compound name	Area %	Molecular formula	M.WT
2.33	2-Ethyl-oxetane	0.02	$C_5H_{10}O$	86
2.70	Bicyclo[4.1.0]HEPT-3-ene,3,7,7-trimethyl -	3.83	$C_{10}H_{16}$	136
2.70	2-cyclohexene-1-carboxylic acid, 6-methylene -, methyl ester	3.38	$C_9H_{12}O_2$	152
3.49	Pentane, 3-methyl -	1.43	C_6H_{14}	86
4.81	Benzene,1-methyle -4-(1-methylethyl)-{p-cymene}	6.88	$C_{10}H_{14}$	134
6.73	trans-4-methoxy thujane	3.34	$C_{11}H_{20}O$	168
12.41	a-Longipinene	1.38	$C_{15}H_{24}$	204
13.75	Longifolene	6.99	$C_{15}H_{24}$	204
14.15	Caryophyllene	2.20	$C_{15}H_{24}$	204
19.41	Bicyclo[3.1.1]hept-3-ene-spiro-2,4'-1',3'-dioxane), 7,7-dimethyl-	1.13	$C_{12}H_{18}O_2$	194
21.56	Benzene, (1-butyloctyl)-	0.97	$C_{18}H_{30}$	246
23.51	Idebenone	1.04	$C_{19}H_{30}O_5$	338
25.55	Phenanthrene,7-ethenyl1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-, [4aS-(4aà,4bá,7á,10aá)]-	1.11	$C_{20}H_{32}$	272
27.95	hexadecenoic acid, 2,3dihydroxypropyl ester	2.37	$C_{19}H_{38}O_4$	330
29.01	Cholestan-3-ol, 2-methylene-,(3á,5à)-	3.25	$C_{28}H_{48}O$	400
30.58	9,12-Octadecadienoic acid (Z,Z)-	0.03	$C_{18}H_{32}O_2$	280
36.28	Ethyl iso-allocholate	0.30	$C_{26}H_{44}O_5$	436
39.53	Hi-oleic safflower oil	0.04	$C_{21}H_{22}O_{11}$	450
40.53	cholestan-3-ol,2-methylene-, (3á,5à)-	0.14	$C_{28}H_{48}O$	400

Table (2)
Chemical composition of clove oil using GC–MS

RT	Compound name	Area %	Molecular formula	M.WT
4.71	3-Pentanone, 1-hydroxy-2-methyl-1-phenyl-	0.02	$C_{12}H_{16}O_2$	192
5.97	Benzene methanol	36.32	C_7H_8O	108
6.32	Benzyl alcohol	0.01	C_7H_8O	108
8.59	Acetic acid, phenylmethyl ester	14.84	$C_9H_{10}O_2$	150
13.27	Eugenol	12.89	$C_{10}H_{12}O_2$	164
13.27	Phenol,2-methoxy-4-(2-propenyl)-	12.89	$C_{10}H_{12}O_2$	164
14.29	Caryophyllene	2.76	$C_{15}H_{24}$	204
18.16	Caryophyllene oxide	0.15	$C_{15}H_{24}O$	220
20.99	Phenol, 2-methoxy-5-(1-propenyl)-,(E)-	0.01	$C_{10}H_{12}O_2$	164
23.16	10-Heptadecen-8-ynoic acid, methylester, (E)-	0.01	$C_{18}H_{30}O_2$	278
30.12	Dotriacontane	0.08	$C_{32}H_{66}$	450
38.56	Isochiapin b %2<	0.01	$C_{19}H_{26}O_6$	350
39.54	1-Heptatriacotanol	0.01	$C_{37}H_{76}O$	536

The results showed that the viability percentage of HCT cells after treatment with the black seed oil reached 6.45, 8.49, 9.34, 11.54, 11.97, 14.23, and 21.95% at concentrations of 500, 250, 125, 62.5, 31.25, 15.63, and 7.82 µg/ml, respectively. The viability percentage was decreased after decreasing the concentration from 3.91 to 0.24 µg/ml. However, the viability percentage of the Panc cells had been measured at 23.93, 24.46, 25.32, 26.80, and 31.42% at 500, 250, 125, 62.5, and 31.25 µg/ml, respectively. The viable cells were increased after decreasing the concentration from 15.63 to 0.24 µg/ml (Fig. 4A). Also, the cells of HeLa showed viability percentages of about 12.95, 13.69, 14.82, 15.39, 17.61, 18.98, and 19.95% at concentration of 500, 250, 125, 62.5, 31.25, 15.63, and 7.82 µg/ml, respectively. The viability percent was decreased after decreasing the concentration from 3.91 to 0.24 µg/ml (Fig.4 A). The inhibitory activity of the extracted black seed oil against HCT, Panc, and HeLa cells confirmed the potent antitumor activity of the tested oil that appeared at 500 µg. It reached 93.55, 87.05 and 76.07% on HCT, HeLa, and Panc cell lines, respectively.

Our results of GC/MS showed that black seed oil contains many antitumor-active compounds like p-cymene, a-Longipinene, Idebenone, and caryophyllene. Black seed has many antiproliferative characteristics against many different kinds of cancer, including cancer of the liver, colon [22,23], breast, cervix [24,25], lung, pancreas, and prostate [26-28]. Numerous studies have shown that the anticancer activity of black seed oil is mediated through the modulation of various events, such as the proliferation process, cell cycle, cell death (apoptosis), angiogenesis, carcinogenesis, and metastasis.

It was evident from the results shown in Fig (4 A) that the viability activity of the HCT cells after treatment with clove oil measured 8.34, 9.23, 12.31, 13.02, 20.14, 22.31, 24.29, 25.32, 27.7, 29.42, 37.80, and 66.92% at 500, 250, 125, 62.5, 31.25, 15.63, 7.82, 3.91, 1.96, 0.98, 0.49 and 0.24 µg/ml, respectively, under these experimental conditions. Similarly, the viability activity of HeLa cells measured 5.64, 7.84, 8.15, 9.02, 9.58, 10.14, 12.85, 13.94, 15.98, 20.56, 21.34, and 50.99 % at 500, 250, 125, 62.5, 31.25, 15.63, 7.82, 3.91, 1.96, 0.98, 0.49 and 0.24 µg/ml, respectively. Also, the viability activity of Panc cells reached 31.43, 32.01, 34.98, 35.21, 39.78, 40.98, 42.76, 53.82, 64.35, 75.48, 96.31 and 100.00% at 500, 250, 125, 62.5, 31.25, 15.63, 7.82, 3.91, 1.96, 0.98, 0.49 and 0.24 µg/m respectively. It was clear that the potent antitumor activity of the clove oil on all investigated cell lines, even at a low concentration, reached about 95% in the case of Hela cells,92% in HCT, and 70% in Panc at the first concentration.

From GC, it was noticed that the extracted clove oil has bioactive compounds like Eugenol, which was present in a high percentage, reaching 25.78 %. Eugenol is a phenylpropanoid volatile compound. Eugenol has shown potential anticancer efficacy against colonic, stomach, breast, prostate, skin cancer, melanoma, and leukemia [29].

Until now, Eugenol has been shown to induce apoptosis and inhibit metastasis in various cancer cell lines

and is considered a potent natural candidate in cancer treatment [30]. Eugenol is highly cytotoxic in cell culture models, such as osteoblasts (human source), fibroblasts, and endothelial cells [31,32,33].

The current clinically used antitumor drug, doxorubicin, was used as positive control drug. Hence, the reference compounds revealed inhibitory activity against the viable cells of HCT, panc, and HeLa at about 9.92, 30.89, and 35.96%, respectively, at 500 µg/ml (Fig .4A). The HCT, panc, and HeLa cell lines treated with 250 µg/ml of doxorubicin showed 12.82, 49.68, and 43.12% viability, respectively, and 25.93, 56.48, and 56.89% viability at 125 µg/ml, respectively. The cytotoxic activity was decreased by decreasing the concentration of doxorubicin from 62.5 to 0.24 µg/ml (Fig. 4A).

The antitumor activity of the extracted oils (black seed and clove oils) and doxorubicin were compared by calculating the IC_{50} values representing the extracts' efficiency as antitumor agents (Fig. 4 B). The best IC_{50} values were recorded from clove oil against Hela, HCT, and Panc cells, represented about 0.25, 0.38, and 5.26 µg/ml, respectively, followed by black seed oil at about 5.51, 4.24, and 22.7 µg/ml, respectively. According to our study, the inhibitory activity of the clove oil followed by black seed oil activity, was more potent in potency than the doxorubicin drug used in this study, doxorubicin, in which the IC_{50} values against HCT, HeLa, and panc represented about 101,188, and 244 µg/ml.

Flow cytometric analysis of HeLa cell line treated with clove oil and standard doxorubicin drug

The clove oil was the most successful oil tested against several cancer cell lines (HCT, Panc, and HeLa). As a result, this oil was chosen to discover the mode of action of the clove oil on HeLa cells in comparison to the usual doxorubicin treatment. The results of a flow cytometric examination showed that after 24 hours of treatment with a concentration of clove oil that was higher than the IC_{50} value of 0.49 µg/ml, approximately 1.8% of HeLa cells were in the early apoptotic state, while 78.9% of HeLa cells were in the advanced apoptotic state (Fig. 5C).

The treatment of HeLa with 250 µg/ml of doxorubicin, around 28.7% of cells were in the early apoptotic phase, and 42.9% were in the late apoptotic phase, as shown in Fig. (5B).

On the other hand, most untreated control cells, which comprised 66.5% of the total, were viable and showed up in the lower left quadrant of Figure because they did not bind the Annexin V or the PI dyes (Fig. 5A).

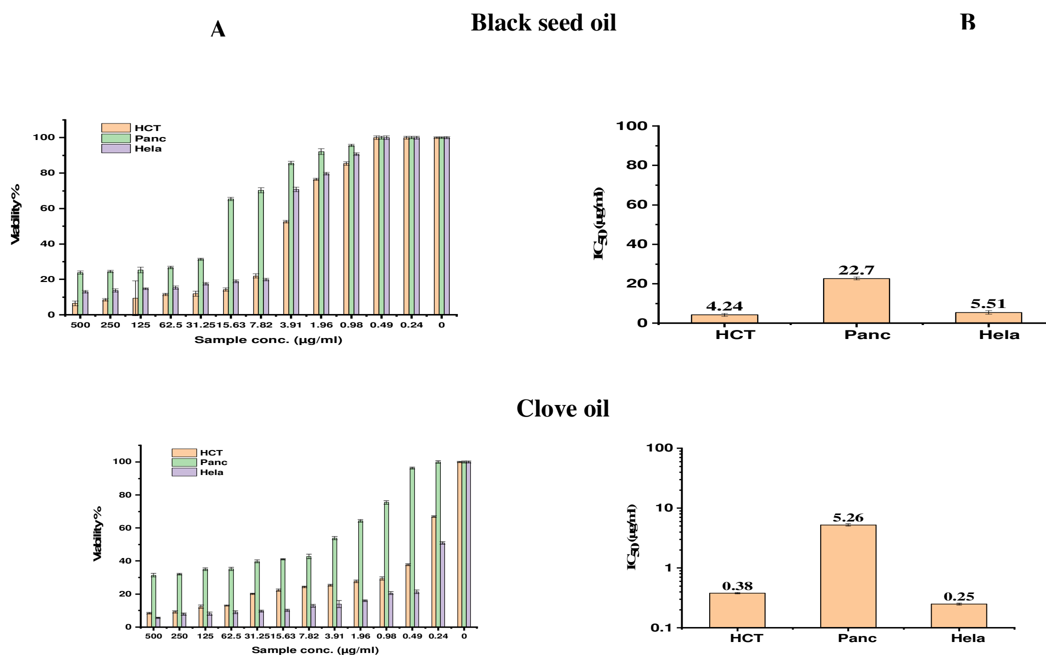
From the previous finding, it has been confirmed that clove oil has a dramatic effect that induces cell apoptosis. Also, it could precede the doxorubicin drug's effectiveness in the antitumor activity, despite the difference in the concentration used in the two cases. The higher activity of clove returned to Eugenol, an essential compound of clove oil, a phenolic compound with promising activity against cervical cancer. In addition to eugenol, Cloves' major oil, caryophyllene, has been found to inhibit the growth of tumors in many types of cancer, including prostate, breast, pancreatic, skin, cervical, as well as leukemia, and lymphoma, by increasing reactive oxygen species (ROS) levels and promoting cell death. Also, Previous studies showed that adding methyl eugenol to myricetin increased the inhibition of the activity of cancer cells by causing robust apoptosis [8, 34- 43].

According to Fouad *et al.*, 2021, the mixture of eugenol and astaxanthin generated 1.25% early apoptotic cells, much higher than the 0.87% of cells induced to undergo apoptosis by DOX treatment alone [44].

Western blot analysis of the expression of Bcl-2 protein in HeLa cells treated with clove oil and doxorubicin

Unlike oncogenes that cause the cell to divide and multiply uncontrollably, Bcl-2 increases the total number of cells by preventing apoptosis (cell death) rather than increasing the cell division rate. Apoptosis is the process of programmed cell death, which has a role in preventing cancer. However, if apoptosis is stopped, it can cause uncontrolled cell division and the subsequent development of a tumor [45]. Once the pro-apoptotic proteins are activated or anti-apoptotic ones are suppressed, apoptosis is initiated [46]. The current study explores whether clove can affect the expression of anti-apoptotic protein Bcl-2 in the HeLa cell line by isolating the protein and analyzed with a western plot assay compared with the doxorubicin drug. The concentrations used in this experiment for doxorubicin and clove oil above the IC_{50} value were calculated from the viability test.

From the result in Table (3), it was noticed that treatment of HeLa cells with clove oil could inhibit Bcl-2 expression in the 26 KDa bands to a relative intensity of about 0.49 µg/ml compared to the relative intensity of about 0.64 when treated with doxorubicin drug and relative intensity of about 1 of untreated cells. However, the big difference in the concentration of doses used in the two cases (0.49 µg/ml for clove oil and 250 µg/ml for doxorubicin drug), clove oil has proven to possess strong inhibitory activity of the expression of anti-apoptotic protein Bcl-2 even with the use of low concentrations (Fig. 6).



Doxorubicin

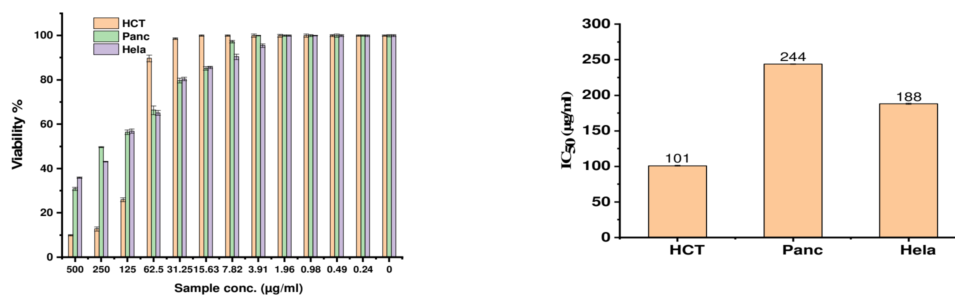


Figure (4): The antitumor activity **of Black seed oil, clove oil and DOX drug** on the viability of HCT, Panc, and HeLa cell lines; (A) Viability % (B) IC_{50} value

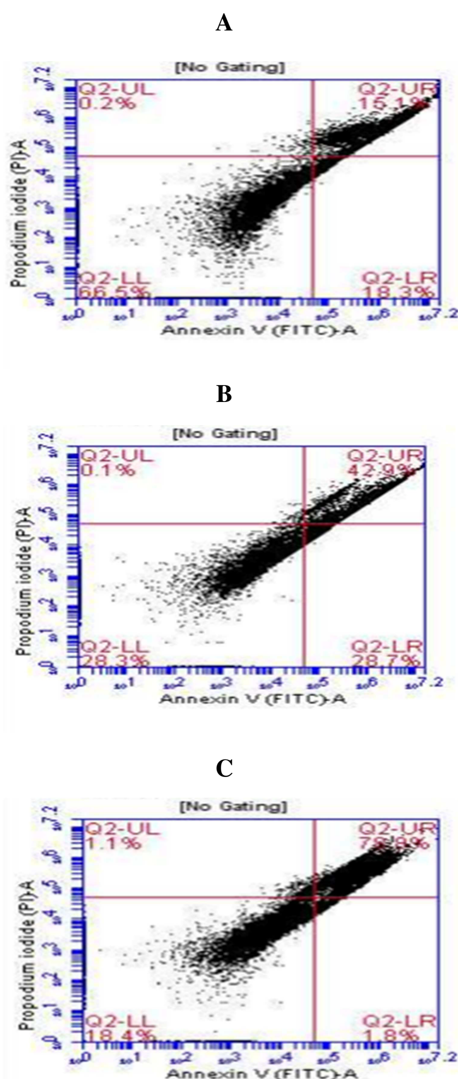


Figure (5): Flow cytometric analysis of untreated control HeLa cells (A), HeLa cells treated with 250 µg of **DOX** drug (B), and treated HeLa cells with 0.49 µg of clove oil and (C)

Our results align with earlier research, which shows that eugenol upregulated IL-1 and Bcl-2 in cervical cells by increasing gemcitabine's antiproliferative and apoptotic capabilities [43]. Additionally, a decrease in Bcl-2 was observed in eugenol-treated HL-60 cells, which are human promyelocytic leukemia cells [44]. In another recent study, Western blot assays were applied to investigate the effect of eugenol on the gene and protein expression levels of autophagy and apoptotic genes. Treating cells with different concentrations of eugenol significantly inhibited cell proliferation of Triple-negative (MDA-MB-231) and HER2 positive (SK-BR-3) breast cancer cell lines [47].

Our results align with earlier research, which shows that eugenol upregulated IL-1 and Bcl-2 in cervical cells by increasing gemcitabine's antiproliferative and apoptotic capabilities [48]. Additionally, a decrease in Bcl-2 was observed in eugenol-treated HL-60 cells, which are human promyelocytic leukemia cells [49]. Other researchers found that combining eugenol and cisplatin in a treatment led to the activation of apoptotic reactions in human melanoma G361 cells by decreasing Bcl-2 [50]. Also, the reduction in the expressions of Bcl-2 protein and COX-2 in HeLa cells after treatment with eugenol was detected by Western blotting assay [51].

Table (3)
Bcl-2 expression levels in untreated and treated HeLa cells with clove oil and doxorubicin

Samples	Relative intensity
Control	1.00± 0.1
Doxorubicin	0.64± 0.04
Clove oil	0.49±0.02

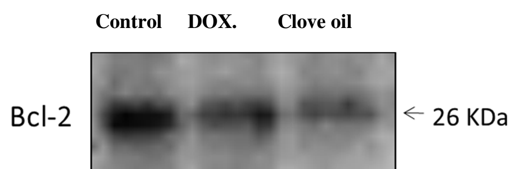


Figure (6): Western blotting analysis of anti-apoptotic protein (Bcl-2) expression in untreated and treated HeLa cells with clove oil (0.49 µg/ml) and doxorubicin drug (250 µg/ml)

DNA fragmentation analysis (DPA assay) in HeLa cells treated with clove oil and standard doxorubicin drug

Many studies use natural and risk-free compounds to reduce the serious adverse effects caused by doxorubicin and boost its overall activity. Among these natural products, the clove oil. Eugenol from clove exerted anti-inflammatory and pro-apoptotic properties in human cervical cancer cells [48], [52–54].

According to the current study, the clove oil (0.49 µg/ml) and doxorubicin drug (250 µg/ml) were evaluated for anti-apoptotic activities against the cervical carcinoma cell line (HeLa) at a concentration above the IC_{50} value. Fragmented DNA in response to treatment was measured by the diphenylamine method. The diphenylamine method helps investigate apoptosis by quantifying DNA fragmentation to oligonucleosome segments. According to our results (Fig.7), treatment of HeLa cells with 0.49 µg/ml of clove oil appeared to increase the percentage of fragmented DNA of HeLa cells to 30.561%, after being 4.958% in untreated control HeLa cells. This investigation was also applied to treated HeLa cells with 250 µg/ml of doxorubicin, and the resulting percentage of fragmented DNA was 25.204%. Thus, it was clear that even low doses of clove oil can cause significant damage to DNA and increase the rate of DNA fragmentation hence the apoptosis as well, more than the doxorubicin dose. Park *et al.*, 2011 showed that when eugenol and cisplatin were used together, they triggered apoptosis in human melanoma G361 cells by disrupting their DNA, decreasing their MMP and proteasome activity, reducing their DNA content, releasing cytochrome c into the cytosol, and activating caspase-9, -7, and -3 [50, 51].

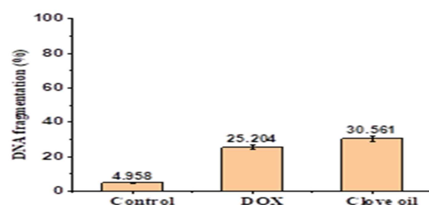


Figure (7): DNA fragmentation percentage of HeLa cells treated with 0.49 µg/ml of clove oil and 250 µg/ml of **DOX** drug compared to the control (untreated HeLa cell)

Morphological studies of cancer cells treated with clove oil

The morphology of HeLa cells after treatment with 0.49 g/ml clove oil and Dox is shown in (Fig. 8). The photographs taken with the microscope (inverted one) revealed a substantial difference between the treated and control cancer cells. The control cells grew adherently and produced more polygonal cells. However, the number of unattached cells in HeLa was considerably reduced after incubation with 0.49 g/mL clove oil. On the other hand, doxorubicin had no impact at the same concentration (Fig. 8). According to the photo of treated cells, clove oil causes death to most viable cells in contrast to standard, even at low doses. Eugenol has several therapeutic properties, including antioxidant, antibacterial, anticancer, and anti-inflammatory action against human osteoblasts, fibroblasts, or cancer cells, even at low doses [32,33,55,56].

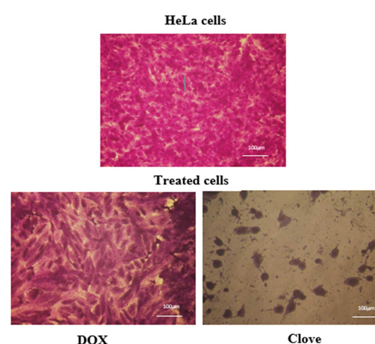


Figure (8): Morphological characteristics of HeLa cells treated with and without clove oil and Doxorubicin at 0.49 µg/ml concentrations were observed under an inverted microscope. The cells were stained with crystal violet stain. Magnification: × 40

4. Conclusion

The long terms side effects of cancer chemotherapeutic drugs can be reduced by using natural products such as essential oils. Our results demonstrated that clove oil exerts strong anticancer against all tested cell line events at low concentrations, followed by black seed. While, standard doxorubicin exerts moderated cytotoxic effects against all tested cell lines compared to the tested oil. GC/MS revealed that eugenol is the major oil in the clove responsible for the higher activity, as well as p-cymene, a-Longipinene, Idebenone, and caryophyllene for black seed. In the present study, clove oil inhibits the growth of different cancer cell lines by induced apoptosis, increasing the percentage of fragmented DNA and inhibiting the expression of anti-proapoptotic protein Bcl-2 more than doxorubicin. So, we must highlight the natural oils to enhance the body's bioavailability and synergistic effect together or with a standard anticancer drug.

5. Declaration

The authors declare the work is not published anywhere else.

6. Ethics approval and consent to participate

There are no needs as clinical trials are not involved in study.

7. Consent for publication:

The authors gave their consent for publication.

8. Availability of data and material

The data is available but not attached with manuscript.

9. Competing interests

No competing interests.

10. Funding

No funding for the research work was carried out.

11. Authors' contributions

All the authors have equally contributed to the paper.

12. Acknowledgment

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13. Conflicts of Interest

The authors declare no conflict of interest.

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