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Cell based *in-vitro* evaluation of Chia Seeds (*Salvia hispanica* L.) extract and Alpha-Linolenic Acid on human breast Cancer

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Abstract

Breast cancer (BC) is the most common malignancy and the main cause of cancer death among women globally, with a considerable increase in BC cases. Today, alternative treatment approaches have become necessary to improve rate of BC patients. Therefore, the present study investigates the anticancer effect of (*Salvia hispanica* L.) Chia seeds extract (CSE) and one of the most bioactive components (alpha linolenic acid) on MCF7 cell line. The results showed that CSE and ALA were specifically able to inhibit proliferation with IC_{50} CSE=74.1µg/ml and ALA=133.3µg/ml. CSE and ALA induced morphological changes related to apoptosis, inhibition cell adhesion, migration and reduced spheroids volumes of MCF7 cells depending on concentration. CSE was more active on cytochrome C in cytosol, increased caspase3 and decreased in protein expression Bcl2 in MCF7 cells.

Keyword: *Salvia hispanica*, MCF7, Apoptosis -ALA- apoptotic related genes.

1. Introduction

Breast cancer (BC) ranks among the foremost causes of mortality and morbidity in women [1]. Breast cancer is the most prevalent malignancy in both developed and developing nations. that affects women. In actuality, it is expected that 1.7 million more instances of breast cancer will be diagnosed in the poor world over the next few years, and the significant disparity in mortality rates will persist, with the developing world accounting for 70% of breast cancer deaths. Because of the increasing death rate and the bad consequences of anticancer medicines, researchers were compelled to create new and effective drugs with as little side effects as acceptable [2-3]. The development of BC is a complex, multi-stage process that includes oncogene activation and tumor suppressor gene inactivation.

BC progression is a multi-factorial and multistage process that involves the activation of oncogenes and the inhibition of tumor suppressor genes. P53 down regulates the pro-survival Bcl-2 and increases expression of the Bcl-2 (B-cell lymphoma-2) family proteins. that promote apoptosis, usually Bax, which causes the outer mitochondrial membrane to permobilize and ultimately triggers the apoptosis and cell death process [4-5]. Currently available chemotherapeutics for BC have a number of drawbacks, including high cost, toxicity, and poor efficacy. These drawbacks emphasize the necessity of finding novel therapeutic drugs that are both safe and effective against BC. These shortcomings highlight the necessity to identify safe and effective new treatment agents against breast cancer. The development of novel medications and therapeutic agents has benefited greatly from the use of natural resources, such as medicinal plants and herbs. Numerous physiologically active compounds found in pharmaceutical plants have the potential to be improved as medicinal medicines [6]. Medicinal plants contain phytochemicals such as flavonoids, polyphenols, polysaccharides, triterpenoids, alkaloids, glycosides, phenols, and luteolin glycosides. These phytochemicals were discovered to inhibit tumor cell proliferation and induce apoptosis [7, 8]. So, the current study aimed to regulate the therapeutic effects of chia seeds and their active component alpha linolenic acid on breast cancer cells, as well as to shed light on the molecular mechanisms that supports breast cancer's medicinal capabilities, in order to provide the groundwork for potential pharmacological targets for future breast cancer treatment research.

2.1. Material and methods

2.1.1. Materials

2.1.1.1. Collection of plant material

Chia seeds (*Salvia hispanica* L., family: Lamiaceae) were purchased and authenticated from an herbal-specialized company (Arafa Company, Cairo, Egypt). Pure alpha-linolenic acid (ALA, $C_{18}H_{30}O_2$, molecular weight = 278.4) is purchased from (Santa Cruz Company, California, United states). ALA were dissolved in sterile dimethylsulfoxide (DMSO, Sigma Aldrich Company) at stock solutions of (50mg/ml) and kept at -20°C until use.

2.1.1.2. Preparation of chia extract:

Salvia hispanica dried seeds were grinded to a fine powder using a porcelain mortar and pestle (Simax, Czech Republic). Chia seeds powder was extracted by maceration overnight in 80% methanol [10]. At 40°C, the filtered methanol was evaporating in

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a rotatory evaporator with a vacuum. The extract was then lyophilized and preserved at –20ºC. Chia seed extract (CSE) was kept at -20°C until it was used after being dissolved in sterile DMSO at a stock solution of 50 mg/ml.

2.1.2. Methods

2.1.2.1. Cell lines and culture conditions:

The Karolinska Institute's Oncology and Pathology Department in Stockholm, Sweden's Professor Stig Linder kindly donated the American Type Culture Collection (ATCC, Manassas, VA, USA) of human breast carcinoma (MCF7) and skin normal human (BJ-1) cells. In culture flasks, cell lines were grown using Dulbecco's Modified Eagle Medium (DMEM), a complete culture medium. supplemented with 10% foetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 1% L-glutamine (all antibiotics and L-glutamine, Biowest, France), and 1% antibiotic/antimycotic solution (10000 U/ml penicillin, 10000 µg/ml streptomycin, and 25 µg/ml amphotericin B). The culture flasks were kept at 37°C with 5% CO_2 and 95% humidity in a water-jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Cell cultures were cultivated in a biosafety class II sterile laminar flow cabinet (Baker, SG403INT, Sanford, ME, USA).

2.1.2.2. Cytotoxicity on MCF7 breast cancer line Monolayers.

In Vitro cytotoxic activity of chia extract was evaluated on human breast MCF7 cancer cell and normal skin cell line (BJ1), The cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] colorimetric test technique was performed according to [11,12]. MCF7 and BJ-1 cell lines were plated in 96-well plates at a concentration of (2×10^4) cells/well. After 24 hours of seeding, the media was aspirated and replaced with serum-free culture media with extracts. at several concentrations (1000-500-250-125-62.50-31.25-15.60-7.80-3.90-1.95 and 0.40 µg/ml) for 48 hours in triplicate. Doxorubicin was employed as a positive control, with DMSO (0.5%) as a negative control. The cytotoxicity was determined using the MTT technique test and the following equation: % cytotoxicity = [1 - (AVx / AVNC)] x 100.
The average absorbance of a sample well (AVX) and the average absorbance of a negative control well (AVNC) were measured at 595 nm with a reference wavelength of 690 nm [13].

2.1.2.3. Bioassay for cytotoxicity on BJ-1 and MCF7 multicellular spheroids:

Although chia seed extract and alpha linolenic acid exhibit great activity in 2-D bioassays, they may not have adequate penetrating power across multi-layer cells. Furthermore, its selectivity for cancer cells over normal cells is significant. So our next two steps were to test the CSE and ALA on the 3-dimensional spheroid model breast cancer cell line (MCF7) and human normal fibroblast cell line (BJ1). The 3D multicellular spheroids model was created using the technique of [14] with slight modification. To prevent cell adhesion to the surface, the plate was covered with poly-hema. In 96-round-bottom plates coated with poly-hema, a cell suspension ($10x10^3$ cells/well) was centrifuged for 10 minutes at 1000 ×g. After five days of incubation in a conventional growth medium, cells formed compact spheroids with a diameter of around 500 µm. The medium was replaced every 48 hours. Spheroids were incubated in fresh medium with/without IC_{50} of CSE or ALA for further 48 hours at 37°C, 5% CO_2 and 95% humidified atmosphere. After incubation, cell images were obtained with a digital camera connected to an Olympus light microscope and the spheroid diameters (d) were analysed by Olympus Cell Sense Software. The spheroid volume was calculated using the following equation **(**Vinci *et al*., 2012): Spheroid volume = $4/3\pi(d/2)^3$.

2.1.2.4. Assessment of the morphological changes 2dimension cell lines.

MCF7 cells were cultured for 24 hours at 37°C, 5% CO_2, and 95% humidity after being seeded at a density of 1×10^6 cells/ml in a 75 cm^3 tissue culture flask. to assess the effects of CSE or ALA on the morphological changes (%) of tumor cells (until reached 70-80% of confluence). Then, the cells were incubated again in fresh medium without/with IC_{50} concentrations (IC_{50} =74 µg/ml of CSE and IC_{50} =133.3 µg/ml of ALA). After 48 hours of incubation, the cells with the morphological changes related to apoptosis such as cell shrinkage, condensed chromatin, apoptotic bodies and cellular debris [15, 16] was photographed at 100X magnification by using a digital camera light microscope (Olympus).

2.1.2.5. Assessment Cell migration.

Cell migration was investigated using a wound healing assay. Six-well tissue culture dishes were seeded with MCF7 cells at a seeding density 5×10^5 cells/ml and leave it to adhere and grow at 37ºC, 5% CO_2 and 95% humidified atmosphere for 24 hours (until make confluent sheet) . To create a wound gap, a scratch was produced through the MCF7 monolayer using sterile plastic pipette tips. After creating this gab, the dishes were rinsed twice with medium to eliminate the detached cells from plates. Cells were incubated again in fresh medium either in the absence (control) or presence of IC_{50} of CSE or ALA. After 48 hours, cell images were taken by digital camera connected to an Olympus Cell Sense Software (Münster, Germany). Cell migration was calculated using the following equation: Cell migration (%) = $[(GapT_0\text{-}GapT_{48})/GapT_0] \times 100$ .Where T_0 is gap (µm) immediately after scratching and T_{48} is the gap (µm) at 48 hours after scratching [17].

2.1.2.6. Cell adhesion assay.

To show the effect of CSE or ALA on the adhering properties of MCF7 cells, Cells were planted into six-well tissue culture plates at a density of 1×10^6 cells/ml, with control and IC_{50} of CSE or ALA. After 48 hours of incubation. At 37 °C, 5% CO_2, and 95% humidity, after removing the medium, sterile PBS solution was used twice to wash the adhering cells (to removed detached cell), then the adherent cells were detached by trypsinization, and the viable cells were counted.

2.1.2.7. The comet assay is used to evaluate DNA damage.

The genotoxic damage of MCF7 by plant extract CSE and ALA was evaluated using the comet formation test at IC_{50} values. The comet test was done using the method of [18] with slight modification. A 6-well cell culture plate was seeded with MCF7 cell suspension at a concentration of ($2x\ 10^5$ cells/ml), and the plate was incubated for 24 hours. Cells were then treated with IC_{50} for each CSE and ALA then incubated for 48 hours. Following the incubation period, the cells were washed twice with

PBS and trypsinized. The solutions were centrifuged at 1750 rpm for 5 minutes. The supernatant containing fragmented DNA was extracted with cell suspensions (10ml), combined with 90ml of 0.6% low melting agarose, and put on slides coated with 1% normal melting agarose [19]. Before staining, the slides were treated with ethanol for an additional five minutes after being cleaned three times with a neutralizing buffer (0.4 mol/l Tris, pH 7.5) for five minutes at 4°C. Ethidium bromide (2 mg/ml in distilled H2O; 70 ml/slide) was applied to dried microscope slides, which were then covered with a coverslip and examined with a fluorescence microscope (Leica DM 1000, Solms, Germany) set to 200 magnification, 546 nm excitation wavelength, and 580 nm barrier.

2.1.2.8. Quantitative real-time PCR analysis cytochrome c, Bcl-2, Bax and caspase 3 gene expressions in MCF7 treated cells.

Following 48 hours of exposure to IC_{50} of CSE or ALA as mentioned previously, the MCF7 cells were collected from the culture flasks by trypsinization and counted to harvest 5×10^6 cells. Following three rounds of cell washing with 1 ml of ice-cold PBS and centrifugation at 10000 rpm, cell pellets were used for the measurement of cytochrome c, Bcl-2, Bax and caspase 3 genes expression by qPCR.

2.1.2.8.1. Quantitative polymerase chain reaction (qPCR):

The qPCR was carried out using specific primers for the required genes and glyceraldehydes-3-phosphate dehydrogenase (GAPDH, an endogenous control). The primers were obtained from Thermofischer Scientific. The SensiFASTTM SYBR® Hi-ROX kit (Bioline, London, UK) was used for all qPCR reactions. The qPCR reaction included: 10 µl of 2 X SensiFAST SYBR Hi-ROX Mix, 0.8 µl of 10 µM for each primer, 1 µl cDNA and 7.4 µl nuclease-free distilled water. The qPCR conditions were performed as the reaction program was divided into three steps. The first involved 3 minutes at 95.0 °C. The second step consisted of 40 cycles, with each cycle broken into three steps: (a) 95.0°C for 15 seconds, (b) 55.0°C for 30 seconds, and (c) 72.0°C for 30 seconds. Starting at 60.0°C, the third step's 71 cycles raised the temperature by roughly 0.5°C every 10 seconds until it reached 95.0°C. To assess the quality of the primers used, a melting curve analysis was carried out at 95.0 °C at the conclusion of each qRT-PCR. Every experiment used the StepOne Plus Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA) with distilled water as a control:

The fractional cycle number required to achieve a quantifiable fluorescence for the amplified target gene is called a threshold cycle (Ct), and it is used to compute the relative quantification of target gene (RQ) compared to a housekeeping gene and a calibrator (control) sample as follows [20].

Gene symbol	Primer sequence from 5'-3'
Cytochrome c	F: GCTGAACCAGGAAGAGATGG R: TGAAGAGCTGGGAAGGAAGA
Bcl-2	F: CAA GTG TTC CGC GTG ATT GA R: CAG AGG AAA AGC AAC GGG G
Bax	F: GTTTCA TCC AGG ATC GAG CAG R: CATCTT CTT CCA GAT GGT GA
Caspase-3	F: GCTGAACCAGGAAGAGATGG R: TGAAGAGCTGGGAAGGAAGA
GAPDH	F: TCCACCACCCTGTTGCTGTA R: ACCACAGTCCATGCCATCAC

Ethical approved: To ensure ethical cell lines using, the research protocol (Code: ASU-SCI/ZOOL/2025/1/1) was approved by Ain Shams University/ Faculty of Science/ Zoology Department. Date 6/1/2025

3. RESUIT:

3.1. Cytotoxicity on MCF7 breast cancer line Monolayers

The results indicated that CSE and ALA showed have high cytotoxic effects against MCF7 cell line where, the IC_{50} values equal 74.1µg/ml for CSE and 133.3µg/ml for ALA after 48 hours of cell culture. Regarding non-cancerous cell line (BJ-1), the result showed that IC_{50} values greater than of the MCF7 where 176.4µg/ml for CSE and 219.3µg/ml for ALA after 48 hours of cell table (1).

Table (1): IC_{50} of CSE and ALA on breast cancer cell line (MCF7) after 48 hours of cell culture.

IC_{50} µg/ml	MCF7	BJ1
CSE	74.1±2.5	176.4±4.3
ALA	133.3±4.2	219.3±3.8

CSE: Chia seeds extract, **ALA:** Alpha-linolenic acid.

The cytotoxic effects were measured by MTT cell viability assay. IC_{50} values were determined by a non-linear regression.

3.2. Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on morphological changes of breast cancer cell line (MCF7):

The investigation of the MCF7 cells under the inverted microscope revealed that the incubation with IC_{50} of CSE, and ALA for 48 hours induced morphological changes (related to apoptosis) in the MCF7 cells. These morphological changes included the reduction of cell to cell contact through enhancing cell shrinkage (with short or fewer filopodia), as well as a chromatin condensation and apoptotic bodies that led to a cellular destruction (Figure 1).

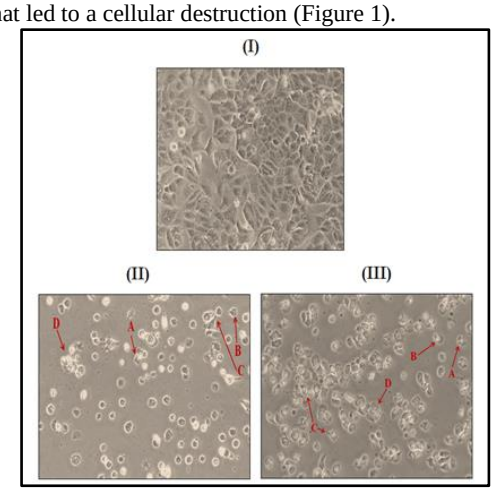

Figure (1): Effects of IC_{50} of CSE (**(II)**, ALA **(III)** and control **(I)** on morphological changes of MCF7 after 48 hours incubation.
(A) Cell shrinkage, (B) Condensed chromatin, (C) Apoptotic bodies and (D) Cellular debris.

3.3. Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on tumour spheroid volume (mm^3) of breast cancer cell line (MCF7):

The examination of the MCF7 spheroid cells under the inverted microscope showed that the spheroids of the untreated cancer cells composed of heterogeneous cellular aggregates and distinguished by a necrotic core, an internal quiescent zone brought on by restrictions in the delivery of nutrients and oxygen, and an exterior proliferative zone. On the other hand, the spheroids of IC_{50} of CSE and ALA treated MCF7 cells were smaller and irregular in shape Figure (2). The results showed that the effect of IC_{50} of CSE and ALA reduced significantly, (P<0.001), the MCF7 spheroid volume compared with the untreated cells. The MCF7 cells treated with IC_{50} of ALA showed no significant difference (P>0.05) in their spheroid volumes compared with those treated with IC_{50} of CSE table (2) and figure (2).

Table (2): Effects of IC_{50} of CSE and ALA on the tumour spheroid volume (mm^3), cell adhesion ($\times 10^4$ cells) and cell migration (%) of breast cancer cell line (MCF7) after 48 hours of incubation.

Groups / Parameters	Control	CSE	ALA
Tumour spheroid volume (mm^3)	0.95±0.01	0.43±0.01***	0.45±0.04***

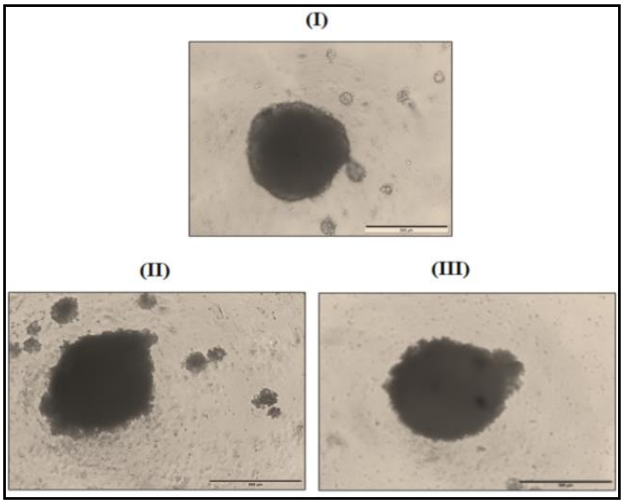

Figure (2): Effects of IC_{50} of CSE **(II),** ALA **(II)** on tumor spheroid volume (mm^3) of MCF7 and control **(I)** after 48 hours incubation. Scale bar: 500 μm. The data represent the mean ± SE of three independent experiments P < 0.001 (compared with the control group).

3.4. Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell adhesion (1×10^4 cells) of breast cancer cell line (MCF7)

As shown in Table (3) and Figure (3), our results revealed that treatment with IC_{50} of CSE, and ALA for 48 hours, there was a significant decrease ($P<0.001$) in comparison to untreated (control) cells in the quantity of adhering MCF7 cells. There was no significant difference ($P>0.05$) in the number of adherent cells between MCF7 cells treated with IC_{50} of ALA and those treated with IC_{50} of CSE.

Table (3): Effects of IC_{50} of CSE and ALA on the cell adhesion (10^4 cells) of breast cancer cell line (MCF7) after 48 hours of incubation.

Groups / Parameters	Control	CSE	ALA
Adherent cell (10^4)	209.90±1.86	12.02±0.77***	27.56±1.55***

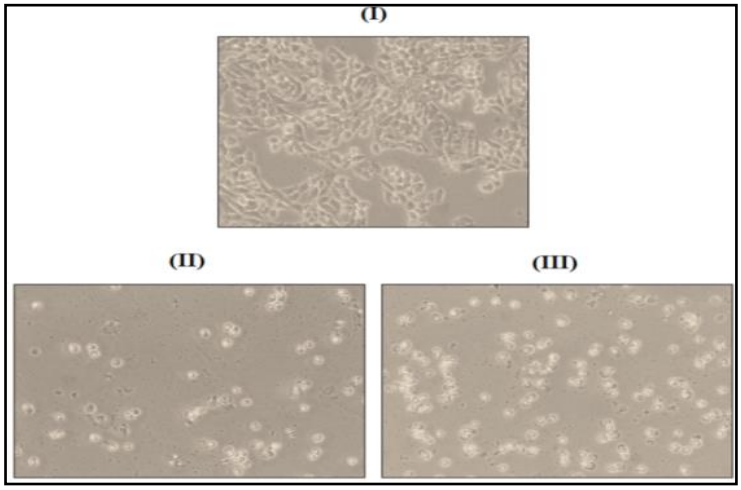

Figure (3): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell adhesion of breast cancer cell line (MCF7) after 48 hours incubation (Magnification: 40X). **(I)** Control (untreated cells), **(II)** IC_{50} CSE, **(III)** IC_{50}ALA.

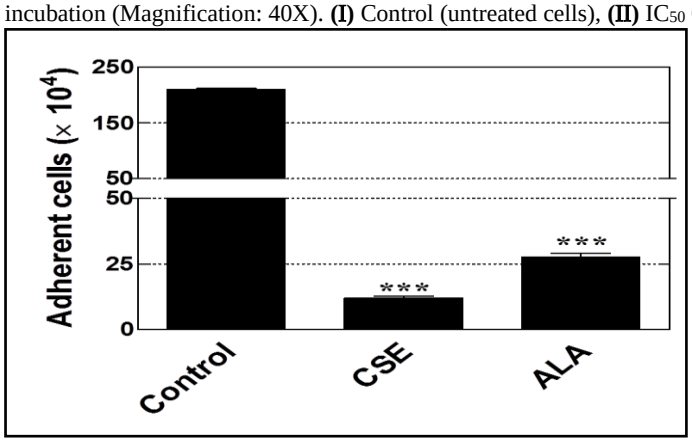

Figure (4): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell adhesion ($\times10^4$ cells) of breast cancer cell line (MCF7) after 48 hours incubation. The data represent the mean ± SE of three independent experiments ***P < 0.001 (relative to the control group).

3.5. Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell migration (%) of breast cancer cell line (MCF7)

The examination of the MCF7 cells under the light microscope revealed that the incubation of the cells with IC_{50} of CSE and ALA for 48 hours inhibited significantly ($P<0.05$) their migration comparative to the untreated cells. On the other hand, the wound gap was mostly occupied by the migrating cells after 48 hours of scratching in the untreated MCF7 (Table 4 and Figure5). When compared to the cells treated with IC_{50} of CSE, the MCF7 cells treated with ALA exhibited a substantial decrease ($P<0.01$) in the number of migrated cells.

Table (4): Effects of IC_{50} of CSE and ALA on cell migration (%) of breast cancer cell line (MCF7) after 48 hours of incubation

Groups / Parameters	Control	CSE	ALA
Cell migration (%)	16.41±2.21	8.17±0.89*	4.41±0.39**

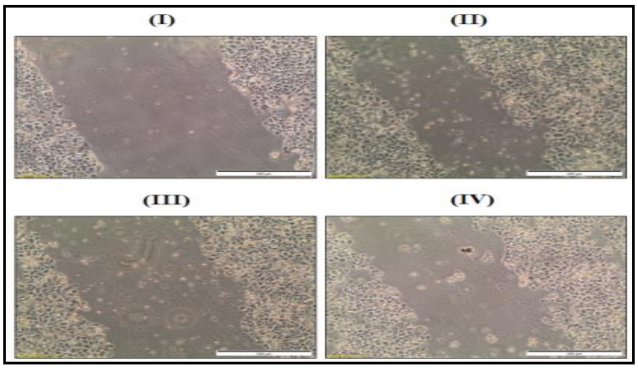

Figure (5): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell migration of breast cancer cell line (MCF7) after 48 hours incubation. Scale bar: 200 µm. **(I)** Control (untreated cells) at zero time, **(II)** Control (untreated cells) after 48 hour, **(III)** CSE and **(IV)** ALA.

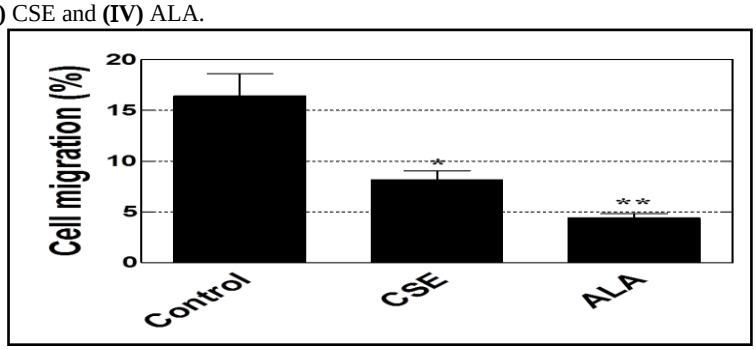

Figure (6): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on cell migration (%) of breast cancer cell line (MCF7) after 48 hours incubation. The data shown is the mean ± SE of three independent studies. *P<0.05; **P<0.01 (compared to control group).

3.6. The effects of IC50 of chia seed extract (CSE) and alpha linolenic acid (ALA) on the genotoxic activities of breast cancer cell line (MCF7)

For the evaluation of genotoxicity, at Frist step, we investigate the effects of the CSE or ALA on breast cancer cells using comet assay. The results of comet assay showed that the activity of CSE and ALA have high significant effect for both CSE and ALA with *P*<0.001 table 5 and figure 7. At second step we evaluate their effect by using DNA fragmentation on MCF7 cell line, the results showed obvious DNA fragmentation, which appeared as a smear of fragmented DNA on gel electrophoresis. CSE and ALA significant increase (*P*<0.001) in the percentage of DNA damage of MCF7 cells compared with the untreated (control) MCF7 cells (Table 5 and Figure 8&9).

Table (5): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on the DNA damaged cells (AU) and DNA fragmentation (%) of breast cancer cell line (MCF7) after 48 hours of incubation.

Groups / Parameters	**Control**	**CSE**	**ALA**
DNA damaged cells (AU)	0.95±0.01	0.43±0.01***	0.45±0.04***
DNA Fragmentation (%)	209.90±1.86	12.02±0.77***	27.56±1.55***

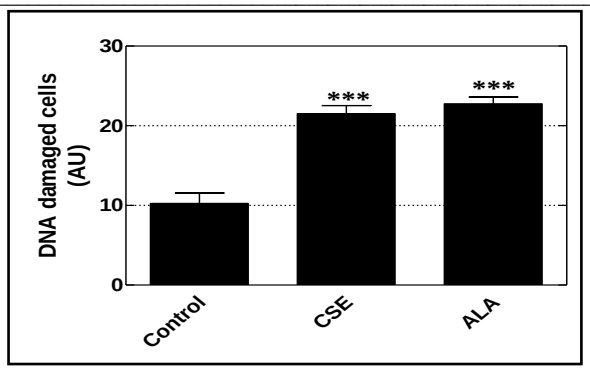

Figure (7): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on DNA damaged cells (AU) of breast cancer cell line (MCF7) after 48 hours incubation. AU: arbitrary unit. The data indicate the mean ± SE of three independent experiments. ***P < 0.001 (compared to the control group).

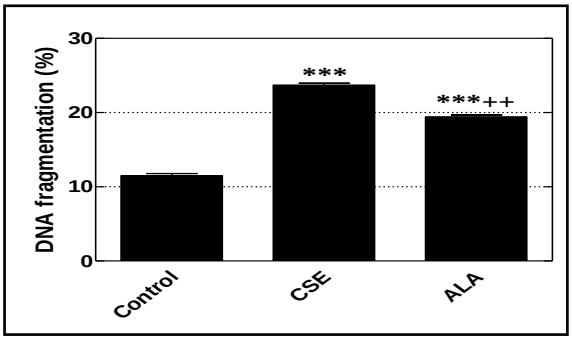

Figure (8): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on DNA fragmentation (%) of breast cancer cell line (MCF7) after 48 hours incubation. The data are mean ± SE consists of three independent experiments.
***P<0.001 (relatedwith the control group).
$^{\dagger\dagger}P$<0.01 (related with the IC_{50} of CSE).

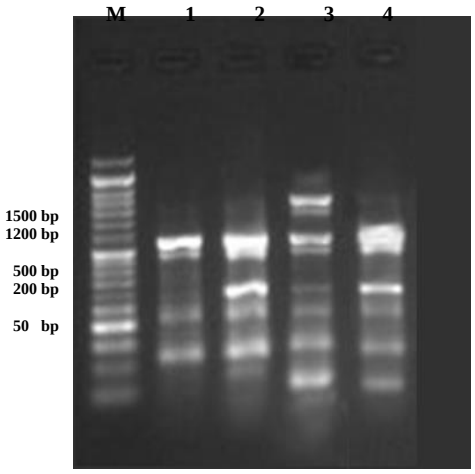

Figure (9): Agarose gel for breast cancer cell line fragmentation. M: 100 bp (base pairs) DNA Step Ladder, Lane 1:Control (untreated cells), Lane 2:CSE, Lane 3:ALA, 4: postive control.

3.8. Effects of the IC50 of chia seed extract (CSE) and alpha linolenic acid (ALA) on the gene expression of cytochrome C, Bcl-2, Bax, and caspase 3 of the breast cancer cell line (MCF7)

The results showed that CSE and ALA induced significant increase in their Bax and caspase3 genes expression compared with the untreated MCF7 cell line. Cytochrome C was increased in cells treated with ALA more than chia extract and untreated cell line.

In addition, BCL2, it's showed that a significant decrease with in both chia extract and ALA in treated MCF7 cells P<0.001 (Table6 &fig.10).

Table (6): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on the genes expression of cytochrome C, Bcl-2, Bax and caspase 3 of breast cancer cell line (MCF7)

	BCl_2	Bax	Cytochrome -c	Caspase-3
Chia	0.55±0.07	1.57±0.15	2.3±0.07	2.87±0.04
ALA	0.69±0.02	1.43±0.15	4.68±0.11	2.34±0.04
-ve	1±0.04	1.83±0.25	1.00±0.05	1±0.02

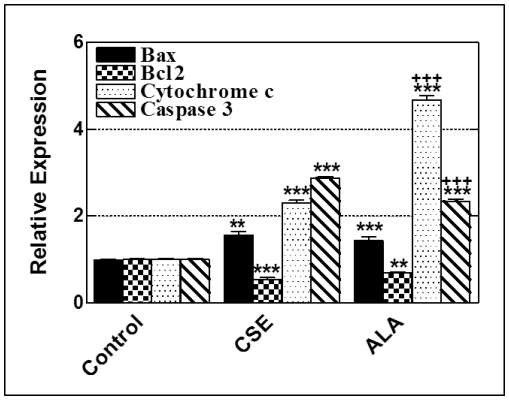

Fig (10): Effects of IC_{50} of chia seed extract (CSE) and alpha linolenic acid (ALA) on the gene expression of cytochrome C, Bcl-2, Bax and caspase 3 of breast cancer cell line (MCF7) after 48 hours of incubation.

4. Discussion:

Finding new, potent anticancer medications is difficult for both patients and researchers. Drug resistance and diverse effect development are still issues that need to be resolved. Plant-based anticancer medicines continue to attract the attention of most scientists since they are regarded to be more effective in cancer treatment [21].The methanolic extract of chia seeds (CSE), a medicinal plant, and its most potent ingredient, alpha linolenic acid (ALA), were examined for their cytotoxic, genotoxic, and gene-modulatory activities on human breast cancer cell MCF-7. Starting the MTT experiment, After 48 hours of treatment at 50 mg/ml, the methanolic extracts of CSE and ALA had a high cytotoxic effect against the MCF-7 cell line in comparison to the positive control doxorobsine (DOX). Both exhibit modest cytotoxicity against the human normal cell line (BJ1). These affects may be related to the existence of natural substances. such as fatty acids, phenolic, flavonoid, and some potential amino acids that can only affect cancer cells, not normal cells [22].Our findings are consistent with a recent study.Other studies investigated anti-proliferation activity of methanolic chia seed extract against different cancer cells as compared to other solvent extracts (aqueous, ethanol, chloroform, petroleum ether and n-hexane extracts) [23]. It was explained that the methanol extracts showed a high percentage of flavonoids and condensed tannins, and that they also had a larger phenolic content than other solvent extracts. When compared to other extraction solvents, the methanolic extract's total polyphenolic and flavonoid concentration was found to be significantly higher. [24]. similarly, it has been proposed that ALA can decrease the growth of breast malignancies [25]. Usually, abnormalities in apoptotic signaling pathways are necessary for cancer cells to divide and proliferate [26]. The methanol plant extract chia seed and alpha linolenic acid were examined for the spheroids of multicellular carcinoma. The acid phosphatase test showed that the cells treated with plant methanol extract significantly lost the shape and integrity of the spheroid's outer layer (proliferating layer). Multicellular cancer spheroids, a three-dimensional culture technique, are a more important tool for explaining the effectiveness of novel anticancer drug entities and their penetration into solid tumor masses *in vivo* than the conventional cellular monolayer. The active methanolic extract of chia seed and ALA display significant cytotoxic effect on the formed spheroids. Previous research indicated that treatment with CSE and ALA (50 μM) produced apoptosis in the outer layers of spheroids, but did not eradicate the cells due to low penetration power. In the current study, extracts had cytotoxic effects on MCF.-7spheroid [27]. To guarantee the safety of the chosen extract, a counter screening on normal cell line (BJ-1) spheroids was performed. Furthermore, in the current investigation, the [methanolic extract of CSE and active component ALA shown effective cytotoxic action against breast cancer cells in both monolayer and multilayer cancer spheroids. Other research revealed that one of the active compounds in chia seeds, rosmarinic acid, has anticancer characteristics by producing apoptosis, endoplasmic reticulum stress, G2/M cell cycle arrest, and blocking cell migration and proliferation. and reduced significantly the breast cancer (MDAMB-231) spheroid volume when treated with IC_{50} rosmarinic acid of for 48 hours compared to the untreated cells. [28]. However, the wound gap was mostly occupied by the migrating cells after 48 hours of scratching in the untreated MCF7. When compared to MCF7 cells treated with CSE, those treated with ALA exhibited a significant ($P<0.01$) decrease in migrated cells. Other studies investigated that migration inhibition of chia seed oil for 24 hours significantly inhibited migration and increased caspase-3 activation in cancer cells hepatocellular (HepG2), colorectal (HCT-116), gastric carcinoma (AGS) and endothelial cells (HUVECs) without damaging normal cells[29]. While, anther result declared that caffeic acid of chia seed (CAPE) blocks the migration and invasion process in breast cancer cells MCF-7 while caffic acid one of active component of chia seed [30]. Our result shows that the genotoxic effects of the CSE or ALA on breast cancer cells were investigated using comet

assay. For the evaluation of genotoxicity, CSE and ALA significantly induced DNA damages in MCF7 cell line and there is no significant difference between cells treated with CSE and ALA in DNA damaged cells. Another result found that chia seed oil, which is high in polyunsaturated fatty acids, significantly reduced tumor weight, indicating an increase in apoptosis with a decrease in mitosis in tumor tissues. Apoptosis is induced by enhancing caspase activity, which leads to DNA fragmentation. These beliefs are consistent with recent research in cultured human breast cancer cell lines treated with several types of omega-3 PUFA [31]. Other studies show that treatment of chia oil for 48 hours decreased DNA damage of MCF-7cell line [32]. Our results showed that CSE and ALA induced significant increase in their Bax and caspase3 genes expression compared with the untreated MCF7 cell line. Cytochrome C was increased in cells treated with ALA more than chia extract and untreated cell line. In addition, BCL2, it's showed that a significant decrease with in both chia extract and ALA in treated MCF7 cells. Other studies declared that the treatment with chia oil nanocapsules for 24 hours lead to up regulation of (total p53) TP53 promotes the expression of pro-apoptotic genes (Bax) and inhibits the production of anti-apoptotic genes (Bcl2) in the MCF-7 cell [33]. Also, [34] found that treatment of MCF-7 breast cancer cells with 54 μM myricetin (active component) of chia seed for 24 hours dramatically enhanced expression levels of apoptosis-related genes caspase-3, caspase-8, caspase-9, the Bax/Bcl-2 ratio, and the expression of p53 genes. ALA increases the production of the pro-apoptotic protein (Bax Bcl2-association protein) and reduced the level of Bcl2 (B- cell lymphoma) leading to mitochondria dysfunction. This dysfunctionis is likely mediated through the activationof caspase3, acritical enzyme in the apoptosis pathway. Moreover, other studies showed that [35] treatment of ALA for 48 hours induced apoptosis by increase in levels of Bax protein, inhibits Bcl-2 and promotes the release of cytochrome C from the mitochondria.Furthermore, activation of caspase-3 decreases DNA damage in MCF-7 cells. Studies have shown that ALA has a positive impact on a range of illnesses, including cancer. ALA inhibits proliferation, promotes apoptosis, inhibits metastasis and angiogenesis, and provides antioxidant benefits. Other investigated clear that, the development of many breast cancer cell lines in response to varying ALA levels, Changes in total cellular phospholipid fatty acids were also investigated to rule out the effects of DHA and EPA. The results suggested that ALA-mediated apoptotic activation may be limited to the BC subtype. Apoptosis was proportional to the amount of ALA absorbed into BC cells [36]. Caspases are cysteine proteases that trigger apoptosis. ALA's apoptotic activity is proportional to its ability to cause lipid peroxidation [37]. An increase in lipid peroxides may increase free radical generation, whereas reactive oxygen species (ROS) can directly activate the mitochondrial permeability transition, resulting in the loss of mitochondrial membrane potential. This leads to cytochrome c (cytochrome c) release and caspase pathway activation. ALA decreased the mRNA expression of inducible nitric oxide synthase (iNOS). lowering intracellular NO levels and suppressing lipid peroxidation by scavenging free radicals. Research shows that ALA can cause apoptosis by stabilizing HIF-1α, down regulating FASN, up regulating pro apoptotic gene Bax, and down regulating ant apoptotic gene Bcl-2. This opens up a lot of potential for ALA's clinical application [38].

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