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

Nourhan Yehia[1]; Khaled Mahmoud [2]; Nadia M. El-Beih[1]; Mai M. Zahra[1]

[1] *Zoology department, faculty of science, Ain Shams University, Cairo, Egypt.*

[2] *Pharmacognosy department, National Research Centre, Giza, Egypt*

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 chia (*Salvia hispanica* L.) seeds extract (CSE) and one of the most bioactive components is alpha linolenic acid (ALA) on MCF-7 cell line. The results showed that CSE and ALA were specifically able to inhibit proliferation with IC_{50} CSE=74.1 µg/ml and ALA=133.3 µg/ml. CSE and ALA induced morphological changes related to apoptosis, inhibition of cells adhesion, migration and reduced spheroids volumes of MCF-7 cells depending on concentration. CSE was more active on cytochrome C in cytosol, increased caspase 3 and decreased protein expression of BcL-2 in MCF-7 cells.

Keywords: *Salvia hispanica*, MCF-7, Apoptosis, ALA, apoptotic related genes.

1. Introduction

Breast (BC) cancer ranks among the highest reason of mortality and morbidity in women [1]. Breast cancer is considered as greatest prevalent malignancy in both advanced and developing nations that affects women. In Egypt, BC account for 33% of cancer cases among women, with over 22,000 new diagnoses each year. This number is expected to rise significantly due to the growing population, shifts in the demographic structure, and the adoption of a Westernized lifestyle [2,3]. 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 [4,5].

BC progression is a multi-factorial and multistage process that includes the oncogenes activation and the tumor suppressor genes inhibition. The Bcl2 protein, derived from oncogene, convenes adverse control in the cellular suicide machinery pathway and evokes a signal for the cells survival. The other protein, Bax, promotes cell death and act as apoptosis inducers. Transcriptional targets (such as Bcl2 and Bax) for p53, tumor suppressor protein which induces a programmed cell death plus arrest of cell cycle in response to damage of DNA [6,7]. 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. New drugs development and therapeutic agents has benefited significantly from the utilizing of natural resources, such as medicinal plants and herbs. Numerous physiologically active compounds found in pharmaceutical plants have the potential to be improved as therapeutic agent [8]. Phytochemicals found in medicinal plants like flavonoids, polysaccharides, triterpenoids, polyphenols, alkaloids, luteolin glycosides and phenols inhibit proliferation and trigger apoptosis of tumor cells [9]. So, the goal of this study to assess chia seeds anticancer potential beside their active component alpha linolenic acid on cancer cells of the breast, plus monitoring the mechanism of their action, with special reference to the apoptotic pathway.

2. Materials and methods

2.1. Natural products and herbal materials

Seeds of chia (*Salvia hispanica* L., family: Lamiaceae) were purchased and authenticated from an herbal-specialized company (Arafa Company, Cairo, Egypt). Pure alpha-linolenic acid (ALA, C18H30O2, 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 20 mg/ml then kept at -20°C till use.

2.2. Preparation of alcoholic extract of chia

Salvia hispanica dried seeds were grinded using a porcelain mortar and pestle till obtain a fine powder (Simax, Czech Republic). Chia seeds powder was extracted by maceration overnight in 80% methanol [10]. At 40°C, the filtered methanol was evaporating in a rotatory evaporator under vacuum until it was completely dry. The extract was then lyophilized and

*Corresponding author e-mail: khaledmmh2003@gmail.com.; (Khaled Mahmoud).

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preserved at -20ºC. Chia seeds extract (CSE) was kept at -20°C until it was used after dissolution at a stock solution of 20 mg/ml in sterile DMSO.

2.3. Conditions of cell lines and culture

Adherent human breast (MCF-7) carcinoma and normal human (BJ-1) skin cells, American Type (ATCC) Culture Collection (Manassas, VA, USA) were generously provided by Professor Stig Linder at Oncology and Pathology Department (Karolonska Institute, Stockholm, Sweden). In culture flasks, cell lines were developed using Dulbecco's Modified (DMEM) Eagle Medium, a complete culture medium, supplemented with serum of foetal bovine (10%, FBS, Gibco, Carlsbad, CA, USA), 1% L-glutamine, and 1% antibiotic/antimycotic solution contains penicillin (10000 U/ml), streptomycin (10000 µg/ml) plus amphotericin B (25 µg/ml) (all antibiotics and L-glutamine, Biowest, France). In an incubator with a water jacket for carbon dioxide, the culture flasks were maintained at 37°C, 5% CO_2, and 95% humidity (Sheldon, TC2323, Cornelius, OR, USA). Cell cultures were grown in a sterile laminar flow cabinet (class II biosafety cabinet, Baker, SG403INT, Sanford, ME, USA).

2.4. Cytotoxicity assay

In Vitro cytotoxic activity of chia seeds extract (CSE) and alpha linolenic acid (ALA) were evaluated on human breast MCF-7 tumor cell and non-cancerous (BJ-1) skin cell line by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) technique test according [11]. Fresh full DMEM medium was used to seeds MCF-7 and BJ-1 cells at 96-well plates with density (2×10^4) cells/well. After that, cells were incubated for 24 hours at same incubation conditions. The DMEM was then taken out and replaced with new medium that had not been treated with serum. Next, different concentrations of CSE and ALA (range from 1000, 500, 250, 125, 62.50, 31.25, 15.60, 7.80, 3.90, 1.95 and 0.40 µg/ml) or no treatment (as a negative control) were applied to the cells. Following 48 hours incubation, 40 µL of MTT solution (5 mg/ml, BioBasic, Markham, ON, Canada) was applied to each well after the medium had been aspirated. The plates were incubated at same incubation conditions for four further hours. To stop the reaction and dissolve the generated formazan crystals, 10% sodium dodecyl sulfate (SDS, 200µl) in deionized water (ADWIC, Egypt) was added to each well. After that, the plates were incubated for the whole night. Used 100 µg/ml of doxorubicin, which causes 90–100% cells death, was used as the positive control under similar experimental conditions. The plate was analyzed by spectroscopic analysis at 595 wavelenght using a Bio-Rad Laboratories Inc. spectrophotometer plate reader (model 3350, Hercules, CA, USA). The percentage (%) of cytotoxicity= [1- (Av_S / Av_{NC})] X 100. Where, Av_S and Av_{NC} are the averages of the sample absorbance and the -ve control, respectively. After converting the X value with X = log (x) using a non-linear regression analysis, specifically a sigmoidal dose-response with varying slop to determine the IC_{50} values.

2.5. Morphological changes assessment

Seeding cells of MCF-7 (1×10^6 cells/ml) in a 75 cm^3 tissue culture flask for 24 hours (5% CO_2, and 95% humidity) at 37°C to assess the effects of CSE or ALA on the morphological changes (%) of tumor cells (until reached 70-80% of confluence). Then, the tumor 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). Afterward 48 hours of incubation, the morphological changes of cells related to apoptosis for example cell shrinkage, condensed chromatin, apoptotic bodies and cellular debris [13, 14] was photographed at 100X magnification by using a digital camera light microscope (Olympus).

2.6. Generation of three-dimensional tumor spheroids

A uniform single spheroid of MCF-7 cells was generated as previously described by [15]. In brief, 1×10^4 cells/well of cells of MCF-7 cells were cultured onto poly- HEMA (2-hydroxyethyl methacrylate)-coated, rounded- bottom 96-well plates. Poly-HEMA is a hydrophilic polymer that forms a gel network in water. While, 95% ethanol with 6 mg/ml of poly-HEMA (Sigma Aldrich) was made as a stock solution, which was subsequently diluted 1:10 in ethanol. The diluted solution (100 µl per well) was added and allowed to dehydrated before the cells were plated. To induce spheroid development, centrifuge the plates at 1000 × g for 10 minutes. The plates were then incubated under conventional cells culture conditions at same incubation conditions for 4-5 days to allow mature spheroids (~500 µm in diameter) to form, which is suitable for certain, drug testing procedures. Before treating the tumor spheroids with the methanolic extract and compounds from chia the medium was replaced using a narrow needle attached to a pump. The needle was inserted slowly into the well and pushed down to the upper rim of the rounded-bottom plate ensuring that the spheroids were not disturbed or aspirated. The spheroids were incubated in fresh medium with or without IC_{50} concentrations of CSE or ALA at 95% humidity, 5% CO_2 and 37°C for 48 hours. Following the incubation time, images of the spheroids were taken by a digital camera attached to an Olympus light microscope. The Olympus Cell Sense Software was used to analyze spheroid diameters (d). The volume of spheroid was then determined by Spheroid volume = $4/3\pi(d/2)^3$.

2.7. Cells adhesion assay

To show the effect of CSE or ALA on the adhering properties of MCF-7 cells, cells (1×10^6 cells/ml) with control and IC_{50} concentrations of CSE or ALA were seeded into six-well tissue culture plates. The medium was removed following a 48 hours at same incubation conditions. A 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.

2.8. Assessment of cells migration

An assay for wound healing was used to study cell migration. Six-well tissue culture dishes were seeded with MCF-7 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 this gap was made, the dishes were rinsed twice with medium to get the detached cells off. The cells underwent a new incubation in either fresh media without (control) or with 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 (%) = $[(GapT_0\text{-}GapT_{48})/GapT_0]\times100$. Where, T_0 is gap (µm) immediately after scratching and T_{48} is the gap (µm) at 48 hours after scratching [16].

2.9. DNA fragmentation assay

After 48 hours of exposure to the IC_{50} concentrations of CSE or ALA as described above. MCF-7 cells were extracted from the growth flasks using trypsinization, yielding 1×10^6 cells. After being splashed twice with 1 ml of PBS having EDTA (20 mM), then these pellets were lysed using 250 µl of lysis buffer. After vortexing, the lysate was centrifuged for 30 minutes at 10,000 rpm and 4ºC after being incubated on ice for 20 to 30 minutes. The supernatant containing small DNA fragments was carefully separated half was used for gel electrophoresis [17] while the remaining supernatant and the pellet containing larger DNA fragments were used for the diphenylamine (DPA) assay [18].

2.10. Real-Time PCR

Following 48 hours of exposure to IC_{50} concentrations of CSE or ALA as mentioned previously, the cells of MCF-7 were collected from the flasks by trypsinization and counted to harvest $5 \text{ X } 10^6$ cells. Following three rounds of cells washing with 1 ml of iced PBS and centrifugation at 10,000 rpm, cells pellets were used for the measurements the expression of genes of Bax, Bcl-2, cytochrome C and caspase 3 using qPCR.

The Gene JET RNA purification kit (Thermo Fisher Scientific) was used to isolate total RNA. Then the Revert Aid First-Strand cDNA synthesis kit (Fermentas, Waltham, MA, USA) was used to obtain cDNA, as per the manufacturer's instructions. The RT-PCR analysis was performed using specific primers for the genes. To identify genes expression, the results of the selected gene's expressions were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene Table 1. The primers were supplied by Thermo Fisher Scientific. The reactions were carried out using the SensiFAST™ SYBR® Hi-ROX kit (Bioline, London, UK) according to the three phases in qPCR protecol. The Step One Plus Real-Time PCR equipment (Applied Biosystems, Foster City, CA, USA) was used to perform the reactions, and each run contain a negative control to access the specificity of primers and possible contamination.

The fractional cycle number required to achieve a quantifiable fluorescence for the amplified target gene is called a threshold cycle (Ct), and it serves to compute the target gene's relative quantification (RQ) compared to a housekeeping gene and a calibrator (control) sample as follows [19].

Table (1): Real-time polymerase chain reaction (qPCR) primer sequences

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

2.11. Statistics analysis

The study presents its results as the mean ± SE. Group differences were evaluated using Bonferroni's multiple comparison tests and one-way analysis of variance (ANOVA) in GraphPad Prism version 4.03 for Windows, established by GraphPad Software, San Diego, CA, USA. A variance was deemed statistically significant if its *P*-value was less than 0.05 [20].

3. Results

3.1. The cytotoxic effects of CSE and ALA on MCF-7 and BI-1 cell lines

The results indicated that CSE and ALA showed cytotoxic effects on the breast cancer (MCF-7) cell line in a concentration dependent manner where, the IC_{50} values were equal 74.1 µg/ml for CSE and 133.3 µg/ml for ALA after 48 hours of cells culture. In addition, non-cancerous cell line (BJ-1) with CSE and ALA showed lower cytotoxic effects (higher IC_{50} values) than the cancerous (MCF-7) breast cell line ; where , the IC_{50} of non-cancerous cells equal 176.4 µg/ml for CSE and 219.3 µg/ml for ALA after 48 hours of cells culture **Figure 1**.

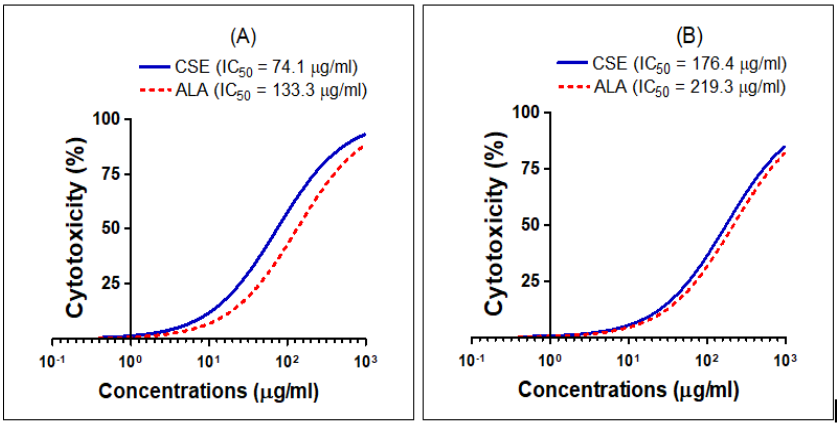

Figure (1): The cytotoxic effects of different concentrations of methanolic extract of chia extract, CSE and alpha linolenic acid, ALA on (A) MCF-7 and (B) BJ-1 cell lines after 48 hours of cells culture. IC_{50} values were determined by a non-linear regression.

3.2. Effects of IC_{50} concentrations of CSE and ALA on morphological changes, tumor spheroids (mm^3), cells adhesion ($\times10^4$) and cells migration (%) of breast (MCF-7) cancer cell line

The investigation of the cells of MCF-7 revealed that the incubation with IC_{50} concentrations of CSE, or ALA for 48 hours induced morphological changes (related to apoptosis). 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 2**.

Table (2): Effects of IC_{50} concentrations of CSE and ALA on tumor spheroid volume (mm^3), cells adhesion ($\times10^4$) and cells migration (%) of breast (MCF-7) cancer cell line after incubation time (48 hours).

Groups / Parameters	Control	CSE	ALA
Tumor spheroid volume (mm3)	0.95±0.01	0.43±0.01***	0.45±0.04***
Adherent cells (×104)	209.90±1.86	12.02±0.77***	27.56±1.55***
Cells migration (%)	16.41±2.21	8.17±0.89*	4.41±0.39**

Three separate experiments' mean ± SE is used to present the data. Significance represented by *P<0.05,**P<0.01,***P<0.001 (in comparison with control group).

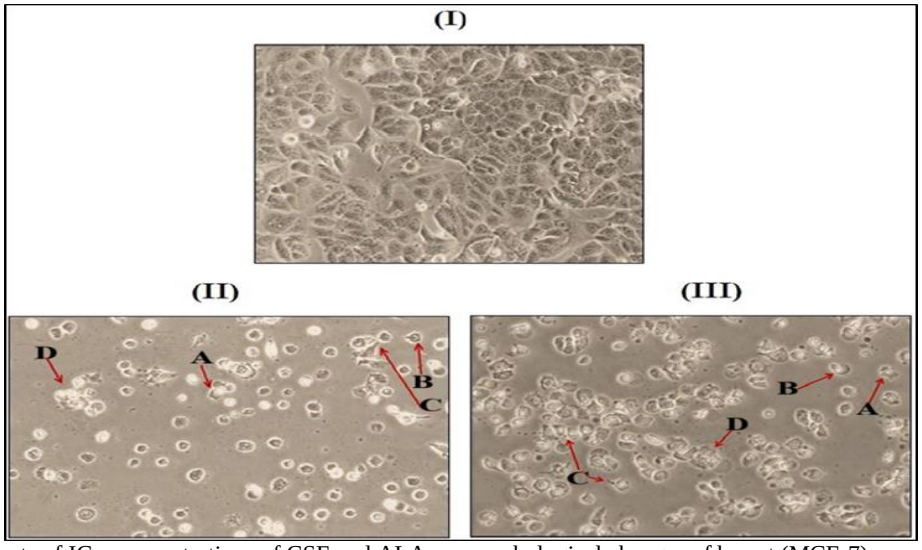

Figure (2): Effects of IC_{50} concentrations of CSE and ALA on morphological changes of breast (MCF-7) cancer cell line after incubation time (48 hours). (I) Control (II) IC_{50} CSE, (III) IC_{50} ALA. (A) Cells shrinkage, (B) Condensed chromatin, (C) Apoptotic bodies and (D) Cellular debris.

The examination of the MCF-7 spheroid cells 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. In addition, our findings indicated that treatment with IC_{50} concentrations of CSE or ALA for 48 hours showed a significant decrease in spheroids volumes, adherent cells number and inhibited their cells migration of MCF-7 cells in comparsion with the untreated cells. Also, the cells of MCF-7 treated with IC_{50} of ALA exhibited no significant change in all the previous parameters in comparsion with cells treated with IC_{50} of CSE **Table 2 and Figures 3-5**.

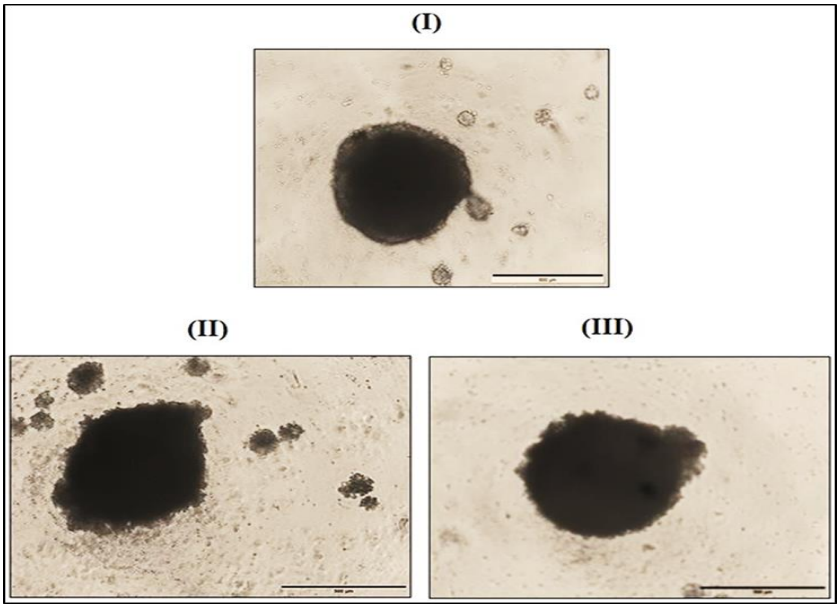

Figure (3): Effects of IC_{50} concentrations of CSE and ALA on tumor spheroid volume (mm^3) of breast (MCF-7) cancer cell line after incubation time (48 hours). Scale bar: 500 μm. (I) Control, (II) IC_{50} CSE, (III) IC_{50} ALA.

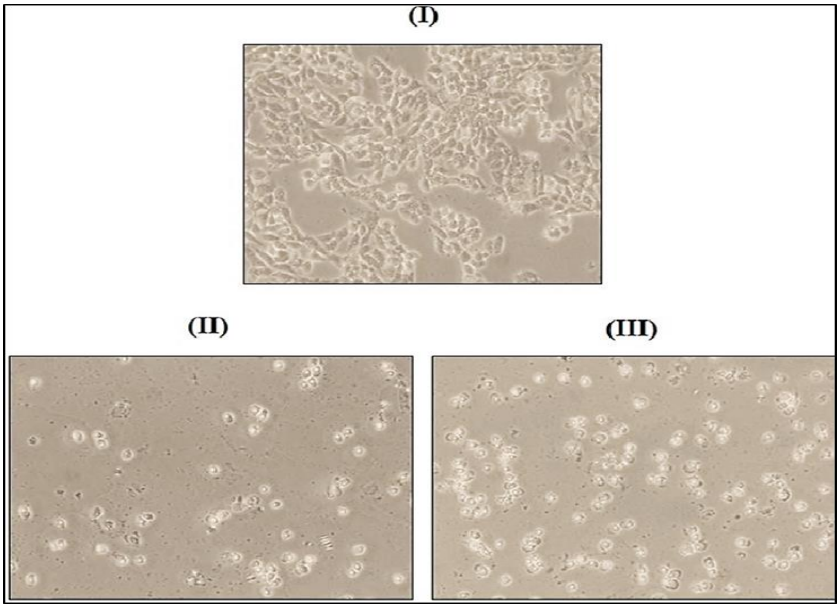

Figure (4): Effects of IC_{50} concentrations of CSE and ALA on cell adhesion ($\times 10^4$) of breast (MCF-7) cancer cell line after incubation time (48 hours). (Magnification: 40X). (I) Control, (II) IC_{50} CSE, (III) IC_{50} ALA.

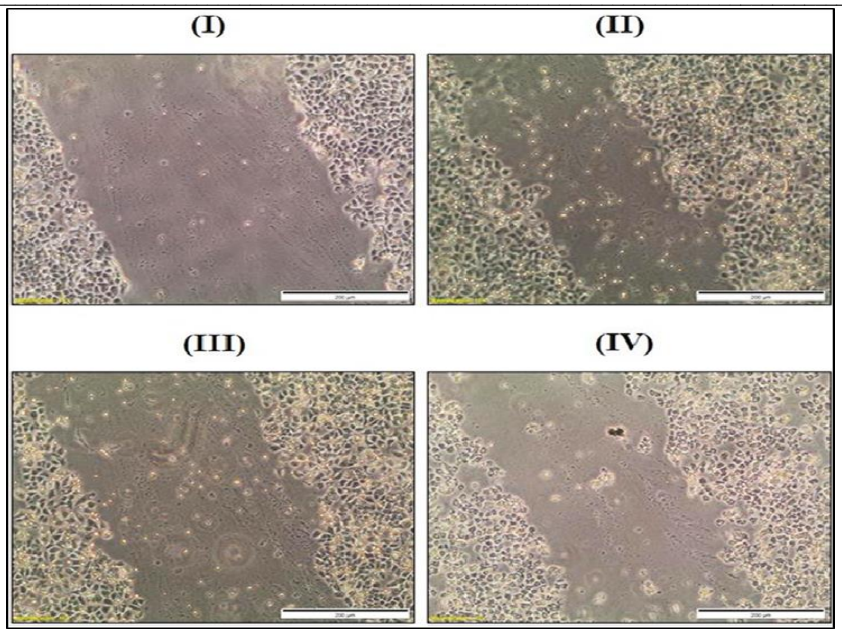

Figure (5): Effects of IC_{50} concentrations of CSE and ALA on cells migration (%) of breast (MCF-7) cancer cell line after incubation time (48 hours). Scale bar: 200 µm. **(I)** Control (untreated cells) at zero time, **(**II**)** Control after 48 hours, **(**III**)** IC_{50} CSE, **(**IV**)** IC_{50} ALA.

3.3. Effects of IC_{50} concentrations of CSE and ALA on DNA fragmentation (%) of breast (MCF-7) cancer cell line

DNA damage was evaluated by comparing profiles of DNA on agarose gel electrophoresis **Figure 7** and by using the DPA colorimetrically method measuring the amount of fragmented DNA **Figure 6**. **Figure 7** declared that the untreated breast cancer cells at lane (1) represent the least DNA fragmentation as compared with all treated groups. The DNA of treated MCF-7 cells with IC_{50} of CSE or ALA for 48 hours (lanes 2, 3) showed obvious DNA fragmentation, which appeared as a smear of fragmented DNA on gel electrophoresis. CSE and ALA significant increase in the percentage of DNA damage of breast cancer cells in comparsion with the untreated cells **Figure 6**. Additionally, the MCF-7 cells treated with IC_{50} of ALA showed a significant decrease in DNA fragmentation (%) in comparsion with cells treated with IC_{50} of CSE.

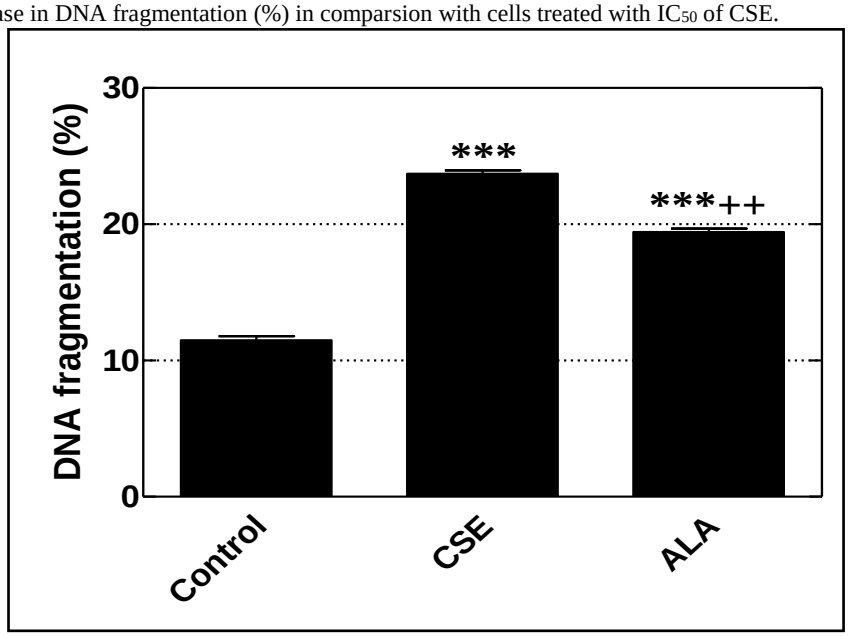

Figure (6): Effects of IC_{50} concentrations of CSE and ALA on DNA fragmentation (%) of breast (MCF-7) cancer cell line after incubation time (48 hours). Three separate experiments' mean ± SE is used to present the data. ***P<0.001 (in comparsion with the control group).^{++}P<0.01 (in comparsion with the IC_{50} of CSE).

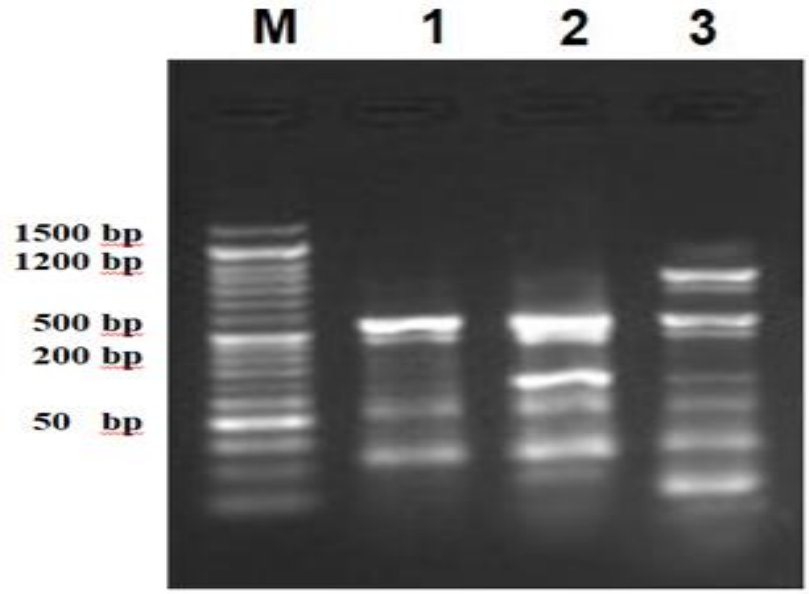

Figure (7): Agarose gel analysis of the breast (MCF-7) cancer cell line revealed DNA fragmentation. M: represents 100 bp (base pairs). Step Ladder for DNA Lane 1idicates control, Lane 2: represents CSE whereas Lane 3: represents ALA.

3.4. Effects of IC_{50} concentrations of CSE and ALA on the expression of genes Bax, Bcl-2, cytochrome c and caspase 3 of breast (MCF-7) cancer cell line

The data revealed that treatment with CSE or ALA increased significantly in the Bax, cytochrome c and caspase 3 genes expression in comparsion with the untreated MCF-7 cell line **Figure 8**. Furthermore, cancerous cells treated with CSE or ALA showed a significant decline in the Bcl-2 gene expression and Bcl-2/Bax ratio in comparsion with the untreated M C F - 7 cells **Figure 8**. Moreover, the cytochrome c and caspase 3 genes expression showed a significant decrease in the cells treated with IC_{50} of ALA in comparsion with those cells treated with IC_{50} of CSE.

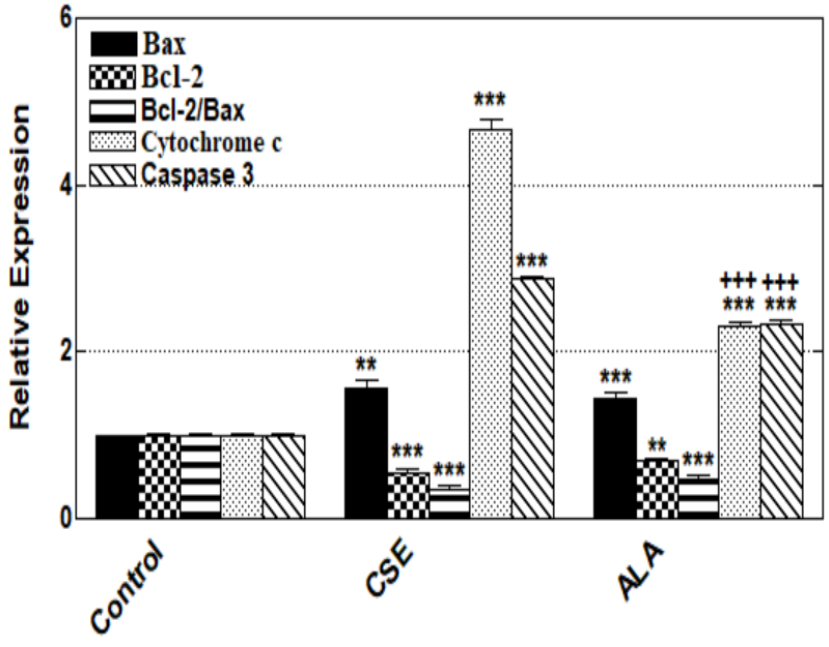

Figure (8): Effects of IC_{50} concentrations of CSE and ALA on the Bax, Bcl-2, cytochrome c and caspase 3 genes expression of breast (MCF-7) cancer cell line after incubation time (48 hours).
$^{**}P<0.01$, $^{***}P<0.001$ (in comparsion with the control group).
$^{+++}P<0.001$ (in comparsion with the IC_{50} of CSE).

4. Discussion

Finding new potent anticancer medications is difficult for both patients and researchers. Drug resistance and diverse effects development are still issues that required attention. Plant-based anticancer medications are still interested for most scientists since they are regarded to be more effective in cancer treatment [21]. This may offer a reasonable safe, very effective, and low-priced agent, including agents alongside cancer. *Salvia hispanica* L. is one of the natural products (commonly known as chia) and its active components such as tocopherols, phytosterols, carotenoids and polyphenolic chemicals, which mostly consist of derivatives of caffeic acid and flavonoids like myricetin, quercetin, and kaempferol, are plentiful in chia seeds and their oil [22]. Moreover, chia seeds provide an unusually high content of alpha linolenic acid (68%) is one of an essential omega-3 fatty acid [23]. So, the current investigation evaluate the anticancer efficacy of methanolic extract of chia (CSE) seeds and one of its bioactive components, alpha-linolenic acid (ALA) *in vitro* on the breast (MCF-7) tumor cell line and refering to their action's mechanism. In this study, the MTT assay results showed that CSE and ALA caused cytotoxicity in breast cancer cells in the manner of concentration dependent without causing a significant cytotoxicity in the non-cancerous cells. Other studies also established that the maximum antiproliferation activity of chia seeds methanolic extract against different cancer cells as compared to other solvent extracts due to the high phenolic content in the methanolic extract compared to other solvent extracts like aqueous, ethanol, chloroform, petroleum ether and n-hexane which may influence the proliferative activity of cell lines of breast cancer [24]. Other studies declared that the chlorogenic acid (CGA, the active component) in chia seeds, inhibited the viability and proliferation of breast tumor cell lines such as MDA-MB-231 and MDA-MB-453 when exposed to different concentrations of CGA over various time periods (24, 48, and 72 hours) [25]. Moreover, it was reported that ALA decreased the growth and inhibited cells proliferation in human cancer cell lines of colon and breast [26]. Based on these findings, IC_{50} concentrations of CSE or ALA for 48 hours were chosen for further investigations.

In multicellular organisms, apoptosis is essential for maintaining tissue balance and removing unnecessary or damaged cells. As a result, targeting apoptosis has become as a potential strategy in treatment of cancer. This programmed cell death begins with alterations in the cell's nuclear structure, including fragmentation, chromatin condensation and followed by membrane blebbing, cells shrinkage, and the development of apoptotic bodies [27]. Our result show that breast cancer cells treated with IC_{50} concentrations of CSE or ALA for incubation periods (48 hours) induced morphological changes related to apoptosis in the MCF-7 cells. Animal models can overcome the limitations of monolayer cultures, but they are expensive, time-consuming and often require complex procedures. More importantly, since they do not incorporate human cells or immune responses, they cannot accurately replicate human physiology complicating the translation of pre-clinical findings. Ethical perspectives about welfare of animals are also increasing highlighting the need for careful and rational management of animal model usage. Spheroids are example of intermediate (3D) three-dimensional models, have been developed a more accurately replicate the cancer microenvironment in order to solve the shortcomings of both classic models [28]. Also, the capacity of cells to adhere to the extracellular matrix or another cell is known as cells adhesion. This mechanism is vital for cells communication and regulation and it plays a crucial role in the development and maintenance of tissues. Cells adhesion plays a role in triggering signals that control cells proliferation, differentiation, migration, and survival. Alterations in cells adhesion show a significant role in progression and inhibition of cancer, making them highly valuable in the development of anticancer therapies [29]. Invasion, driven by the migration of tumor cells, is a key characteristic of malignancy. Preventing cancer cells migration, and consequently invasion is linked to a decrease in the malignancy grade [30]. Our result showed that treatment with IC_{50} concentrations of CSE or ALA reduced significantly spheroids volumes, cells adhesion and migration in MCF-7. Previous researchers found that treatment of human gastric (AGS) cancer cell line with caffeic acid (active component) of chia seeds (60 µM) induced morphological changes related to apoptosis induced a loss of cells adhesion, membrane contraction, blebbing of membrane, cytoplasmic vacuolization, cells fragmentation and the appearance of apoptotic bodies [31]. Also, ALA decrease viability of breast tumor cell lines such as MCF-7 and MDA-MB-231 through changing their morphology including membrane blebbing and reduction of cell to cell contact due to fewer formation of filopodia [32].

Our study showed that CSE and ALA induced DNA fragmentation in breast MCF-7 cancer cells, with CSE having the highest effect followed by ALA. Other reports declared that chia seeds oil which is rich in polyunsaturated fatty acids significantly reduced tumor weight, which was associated with upregulation in cancer tissues' apoptosis and a decline in their mitosis. Caspases were activated, which resulted in DNA fragmentation and apoptosis. These results are consistent with prior research that used a mixture of omega-3 polyunsaturated fatty acids to cure human cancer cell lines of the breast that were grown [33]. Furthermore, prostate cancer cell line (PC-3) treated with 800 µg/ml of ALA for 72 hours induced apoptosis, reduced cells viability, promoting DNA fragmentation by increasing expression of cyclin D1 (gene protein involved in damage and repair of DNA) [34]. Programmed cell death is a natural physiological sequence that is essential to life. Tumor suppression, tissue homeostasis, and improved infection resistance are its key functions. Throughout life, a balance between cells division and death must be maintained [35]. Furthermore, our findings demonstrated that treatment of the breast tumor cells with either IC_{50} concentrations of CSE or ALA for 48 hours induced upregulation in their Bax, cytochrome c, and caspase 3 genes expression along with a significant decrease in the expression of Bcl-2 gene and Bcl-2/Bax ratio in comparsion with the untreated breast cancer cell line. In addition, a significant decrease was noticed only in the cytochrome c and caspase 3 genes expressions of the breast cancer cells treated with ALA in comparsion with those cells treated with IC_{50} concentration of CSE. According to other research, treatment of breast cancer cells with chia oil nanocapsules for 24 hours increased the pro-apoptotic gene (Bax) expression, inhibited the production of anti-apoptotic gene (Bcl2) expression, and increased total P53 (TP53) [36]. Also, treatment of breast cancer cells (MCF-7) with 54 µM myricetin (active component) of chia seed for 24 hours dramatically enhanced the apoptosis-related genes expression (like caspases 3, 8,

9), the Bax/Bcl-2 ratio, and the P53 gene expression. Furthermore, by triggering both intrinsic and extrinsic pathways, myricetin, one of the active ingredients in chia seeds, decreased cancer cell growth. Proteins belonging to the family of Bcl-2 are primarily accountable for controlling the intrinsic (mitochondrial) apoptotic pathway [37]. Furthermore, caffeic acid (CA) an active component of chia seeds, disrupts mitochondrial membrane potential, reduces intracellular reactive oxygen species (ROS) levels and decreases mitochondrial activity in different breast cancer cells. This suggests that CA may induce apoptosis in breast cancer cells by causing mitochondrial damage [38].

Moreover, previous studies declared that ALA, a type of n-3 polyunsaturated fatty acid, promotes the suppression of proliferation, triggers apoptosis, prevents angiogenesis, metastasis and exerts antioxidant properties [39]. Moreover, further research demonstrated that treatment of ALA for 48 hours induced apoptosis by raising pro-apoptotic Bax protein and inhibits Bcl-2 levels leading to dysfunction of mitochondria. This dysfunction is likely mediated through promotes cytochrome C release and caspase 3 activation from the mitochondria [40]. Furthermore, ALA has a positive impact on a range of illnesses, including cancer. Apoptosis was proportional to the amount of ALA absorbed into cells of the breast cancer [41]. Apoptosis is triggered by cysteine proteases known as caspases. ALA's apoptotic activity is proportional to its capability of cause peroxidation of lipids [42]. An increase in lipid peroxides may boost the formation of free radical generation, while ROS can directly trigger the transition of mitochondrial permeability, which results in the potential loss of mitochondrial membrane. This leads to release of cytochrome c plus activation of caspase pathway. Alpha linoleic acid downregulating inducible nitric oxide synthase mRNA expression which also lowered levels of intracellular nitric oxide and inducing lipid peroxidation by scavenging free radicals. Researches showed that ALA can cause apoptosis by hypoxia-inducible factor-1α stabilization, fatty acid synthase downregulation, this promoting mitochondrial programmed death, and this may be another mechanism responsible for observed apoptosis [43].

5. Conclusion

Chia seeds and its component (ALA) are valuable anticancer agents that can significantly enhance intrinsic pathways of apoptosis in the breast tumor cells without affecting the normal cells, as proved in the current *in vitro* study. In general, the highest anticancer activity was achieved by CSE followed by ALA, providing these compounds may become promising anticancer agents in treating BC patients in the near future.

6. Ethical approval

To ensure ethical cell lines usage, the research protocol was approved by Research Ethics Committee, Ain Shams University, Faculty of Science, Zoology Department. As approved by **code: ASU-SCI/ZOOL/2025/1/1** on date 6/1/2025.

7. Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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