

CYTOTOXIC ACTIVITY OF BASIL SEEDS (*Ocimum basilicum* L) EXTRACTS ON SOME BREAST CANCER CELL LINES (*IN VITRO*)

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ABSTRACT

In this study, four types of basil seed extracts were used, being cold aqueous extract, hot aqueous extract, petroleum ether extract, and methanolic extract. Nine concentrations (10000, 5000, 2500, 1250, 625, 312.56, 156.25, 78.125, 39.0, 0.0) µg/ml were used to study the extracts cytotoxicity against two breast cancer cell lines MCF7 and AMJ13 beside REF cell line as normal cells. The methanol extract showed the highest inhibition rate of 64.4% on MCF7 cell line at concentration of 1250 µg/ml and 42.4% on AMJ13 cell line at a concentration of 39.0 µg/ml with significant difference as compared to control sample during the 72hr exposure period. While the petroleum ether extract showed a lower inhibition rate of 16.02% at a concentration of 78.1 µg/ml on MCF7 cell line and 35.3 % at a concentration of 312.5 µg/ml on AMJ13 cells during a 72hr exposure period, compared with the two aqueous extracts which showed a slight effect compared to the control. The cold aqueous extract showed the slight effect of 24.4% at a concentration of 5000 µg/ml on AMJ13 cell line and no toxicity was shown on MCF7 cells. All extracts showed no effect on REF normal cells. In all effective concentrations the basil seed extracts caused a damage to the DNA that cannot be repaired, and thus the cells are heading to programmed death. This effect on the genetic material was studied using the comet assay and mitotic index.

Key words: MCF7, AMJ13, comet assay, mitotic index, MTT assay

عايده وعوده

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الفعالية السمية لمستخلصات بذور الريحان (*Ocimum basilicum* L.) على بعض الخطوط الخلوية لسرطان الثدي خارج

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المستخلص

في هذه الدراسة تم استخدام أربعة أنواع من مستخلصات بذور الريحان، المتمثلة بالمستخلص المائي البارد، والمستخلص المائي الساخن، ومستخلص بتروليم ايثر، والمستخلص الميثانولي وباستخدام تسعة تراكيز (10000، 5000، 2500، 1250، 625، 312.56، 156.25، 78.125، 39.0، 0.0) مايكروغرام / مل لدراسة السمية الخلوية للمستخلصات ضد خطين من خلايا سرطان الثدي (MCF7,AMJ13) وREF كخط الخلايا الطبيعي اظهر المستخلص الميثانولي اعلى معدل تثبيط بواقع قتل 64.4 % عند استعماله بتركيز 1250 مايكروغرام/مل على خط خلايا MCF7 و 42.2 % بتركيز 39.0 مايكروغرام / على خط خلايا AMJ13 مقارنة مع معامل السيطرة. في حين اظهر مستخلص الإيثر البترولي معدل تثبيط أقل بنسبة 16.02 % عند تركيز 78.1 مايكروغرام / مل على خط خلايا MCF7 و 35.3 % بتركيز 312.5 مايكروغرام/ مل على خط خلايا AMJ13 خلال فترة تعرض لمدة 72 ساعة، مقارنة بالمستخلصات المائية التي أظهرت تأثيراً طفيفاً مقارنة بالسيطرة. أظهر المستخلص المائي البارد تأثيراً طفيفاً بنسبة 24.4% بتركيز 5000 مايكروغرام/ مل على خط خلايا AMJ13 ولم يظهر أي سمية على خلايا MCF7. لم تظهر جميع المستخلصات أي تأثير على خط خلايا REF الطبيعية. ادت مستخلصات بذور الريحان في جميع التراكيز الفعالة الى احداث الضرر بالحامض النووي DNA بحيث اصبحت الخلية غير قادره على الاصلاح، مما قادت الى الخلايا الى الموت المبرمج. تمت دراسة هذا التأثير على المادة الوراثية باستخدام مقايصة المذنب ومعامل الانقسام الخلوي

الكلمات المفتاحية: MCF7, AMJ13 ، تقنية المذنب، معامل الأنقسام الخلوي، اختبار MTT assay

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INTRODUCTION

Basil (*Ocimum basilicum*) is a most important aromatic plant. The preventive and therapeutic medical (20) utilization of this plant is an age-long practice across the globe (3). Herbs and plant metabolites/extracts are gaining wide attention owing to their multiple beneficial health effects, medicinal values, and nutraceutical properties, particularly in the present era of emerging drug resistance and untold side effects of chemical drugs (21). Currently a lot of drugs (from herbal medicines) were entered to the cosmetic industry and international markets that takes more attention (to the green cosmetic raw materials) that extracted from plants sources (22)(11). The *Ocimum basilicum* L. commonly called as Basil or Sweet Basil, can be used in fresh and dried conditions. Many pharmacological effects of *Ocimum basilicum* L. in several diseases with potent antioxidant, anti-aging, anti-inflammatory, immune-stimulating properties, anticancer, antiviral, and antimicrobial properties (15). Basil was also known in traditional medicine as an antifungal medicine, which used for the treatment of many infections including urinary tract and gastrointestinal infections. It was also used for many disease symptoms including diarrhea, conjunctivitis, bronchitis, headaches and fever (6). The phytochemical analysis of *Ocimum basilicum* L. showed that the plant contained terpenoids, alkaloids, phenolics, flavonoids, tannins, saponin, reducing sugars, cardiac glycosides, steroids glycosides, and essential oil, these compounds was providing effective against certain chronic diseases (10, 25). To the present time, the most humans died due to cancer diseases. The cancer incidence has increased globally and especially in Iraq, it has increased owing to several factors related to environmental pollution associated with several years of conflicts (1). Breast malignant tumors are the second leading cause of mortality among Iraqi women (1). The anti-cancer activity of *Ocimum basilicum* essential oil with cytotoxic effect has been investigated by some researchers (7, 31). Therefore, the objective of this study was to investigate the cytotoxic effects of many extracts of *Ocimum basilicum* (four extracts) on breast cancer cell lines compared with a normal cell line.

MATERIALS AND METHODS

The current study was carried out in the laboratories of Iraqi center for Cancer and Medical Genetics Research /AL-Mustansiriyah University, and laboratories of Food Science department\ College of Agricultural Engineering Science \ Baghdad University during 2021/2022./Baghdad- Iraq.

Extracts preparation

The *O.basilicum* seeds (locally known as (Reyhan) were obtained from local markets (Baghdad). The plant seed was identified and authenticated by the National herb of ministry of Agriculture of Iraq. Four extracts were used in this study.

Soxhlet extraction

The *O. basilicum* seeds powder were prepared using mechanical grinder. The powder extract (25 gram) were prepared using two different solvents (petroleum ether extract, and methanol). Then the soxhlet extract was used for extraction process, and it take eight hour to complete defatting. The separated parts (of *O. basilicum* seeds) were dried at room temperature (25 °C) according to (2). The two extracts were ready to use in next experiments.

Aqueous extraction

The aqueous extracts were prepared according to (22) cold and hot water extracts were prepared. The first extract was prepared using hot water (aqueous hot extract), 0.1 gram of basil seed were added to 29 ml distal water in ratio of (30:1) v/v, after shaking in water bath (Mamert, Germany) for 20 min at different temperature (30, 50 °C). The hydrated basil seed blended (Pensonic, Malays) for 30 sec. The extract was filtered through a muslin cloth. The separated parts were dried in oven (Mamert Universal, Germany) at 45 °C to be ready to use in next experiments. The second aqueous extract (aqueous cold extract) was prepared using cold water according to the modified method of (22), 0.1 gram of basil seed were dissolved in 29 ml distal water in ratio of (30:1), then shaking with magnetic stirrer for 20 min. The hydrated basil seed was blended (Panasonic, Malays) for 30 sec. The extract was filtered through a muslin cloth. The separated parts were dried in oven (Mamert Universal, Germany) at 45 °C to be ready to use in next experiments. 0.1 g of the powder of each extracts were used for

cytotoxicity assay by dissolving in 10 ml Phosphate buffer saline PBS, and sterilized by mile-pore filtered (0.22 μm .) to be ready for used in the next experiments.

Reagents for chemical tests

According to (2), the chemical test reagents used to identify the active (secondary metabolites) components in the plant extract. The following reagents were used to test each extracts as follow:

Saponins Test (Foam Test) (2).

Phenols and tannins Test (Ferric chloride test) (2).

Flavonoids Test using ethyl acetate and ammonium chloride (2).

Terpenoids Test using the Salkowski technique with chloroform and concentrated H_2SO_4 (5).

Cell line and maintenance of cell cultures

Two human breast cancer cell lines were used in this study that prepared and storage in the cell bank laboratory/ experimental therapy department/ Iraqi center for cancer and medical genetics research. These cell lines including the Iraqi breast cancer cell line AMJ13 that was established from the primary tumor of a 70-year-old Iraqi woman with a histological diagnosis of infiltrating ductal carcinoma, it's an elongated multipolar epithelial-like cell shape (9). And MCF7 cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman, it's an epithelial cancer cell line derived from breast adenocarcinoma (18). And the Iraqi rat embryonic fibroblast REF cell line established from the experimental therapy department/Iraqi center for cancer and medical genetics research, its use as a normal control model in this study. Both AMJ13 and REF cell Lines have grown in RPMI media (US biological, USA) with 10% fetal bovine serum (FBS) (Capricorn- Scientific Germany) and supplemented with 100 $\mu\text{g}/\text{mL}$ streptomycin and 100 u/mL units penicillin, While MCF7 was grown by using MEM media US biological, USA with 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, 10% fetal bovine serum (FBS) (Capricorn- Scientific, Germany) (1).

Cell line preparation for cytotoxicity study:

According to Frishney (17) growth media (MEM and RPMI) were decanted off. Trypsin-

versene enzyme T.V. (Two to three ml) was added to the culture and rocked the flask gently. After approximately 2-3 minute the most of the cells had detached from the flask. Cell growth medium were added to form a cell suspension to be ready to use. Then, the cell with growth medium (as cell suspension) were added (200 μl) to the sterile 96- well microliter plate reader (Santa Cruz Biotechnology, USA). The plates were sealed with a self-adhesive film, and incubated at 37 $^{\circ}\text{C}$.

MTT assay

The MTT ASSAY KIT(ELabscience) was used to test the cytotoxicity of *O. basilicum* extracts on three cell lines (AMJ13, MCF7, and REF) with modification from (22). Cell suspension were added in 96-well plates (Santa Cruz Biotechnology, USA). After 24h the cells became a monolayer then it was exposed to the dilutions of extracts which is (10000, 5000, 2500, 1250, 625, 312.5, 156.257, 78.0625, 39.1 $\mu\text{g}/\text{ml}$) then incubated at 37 $^{\circ}\text{C}$ for 72h (the exposure time). After 72h, MTT ASSAY was used as staining kit. So (50 μl) of MTT solution (3- (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] were added to each well of the 96-well plates, then incubated at 37 $^{\circ}\text{C}$ for 4 h . Finally 150 μl of Dimethyl Sulfooxide (DMSO) was added to each well for dissolving the crystals of formazan so it would be ready for screening at a wavelength 580 with a Fluorometer (BMG LABTECH, Germany).

Single cell gel electrophoresis (comet assay)

The cell lines MCF7 and AMJ13 where is exposed to the methanolic extract of basil seeds at the concentrations (The IC50 of concentrations 1250, 39 $\mu\text{g}/\text{ml}$ of the methanol and ethanol extract respectively), then the cell suspension was taken directly in 0.75% low melting point agarose (US Biological, USA) dissolved in phosphate buffer saline (PBS). It was cast on to microscope slides and coated with 0.5% normal melting agarose (US Biological, USA). The cells were then lysed for 1hr at 4 $^{\circ}\text{C}$ in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10). After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution ((300 mM NaOH, 1mM EDTA, pH>13). Electrophoresis was

conducted at 4°C for 30 min at electric field strength 0.73 V/cm (30mA). The slides were then neutralized with nutilization buffer (0.4 M Tris, pH 7.5) and stained with 100 µl of ethidium bromides (2 ug/ml) (Sigma Chemicals, USA) and covered with cover slips. The slides were examined at 200 x magnification fluorescence microscope (Micros MCX 500, Austria) connected to a CCD camera (Infinity Capturer, Micros, Austria) which connected to a computer-based image analysis system, and images were analyzed using Comet Assay IV software (Perceptive, England) according to (29) with some modification. images were randomly selected from each sample DNA damage were measured as the mean comet tail DNA of cancer cell. The measurements that were recorded Olive tail Moment defined as the product of the tail length and the fraction of total DNA in the tail. Tail Moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail). Tail length defined as the distance of DNA migration from the center of the nuclear core and it is used to measure the value of DNA damage (13 ,29, 30 ,33).

Table 1. Qualitative analysis of active compound of *O.basilicum* seed extracts

Phytochemicals to be detected		petroleum ether extract	methanolic extract	Aqueous hot extract	Aqueous cold extract
Saponins	Wagner's test Ammoniumchloride	-	+	+	+
Flavonoids	+ Ethyl acetat	+	+	-	-
Phenols	Ferric chloride test	-	+	+	+
Tannins	ferric chloride Concentrated H2SO4	-	+	+	+
Terpenoids	+ chloroform	+	+	+	+

+ positive
- negative

Cytotoxicity effect of extracts on cancer and normal cell line: The effect of *O.basilicum* extracts on Breast cancer cell line (MCF7), Breast cancer cell line (AMJ13), and Normal cell line (REF), with all concentrations (39.0, 79.1, 156.25, 312.5, 625, 1250, 2500, 5000, 10000 µg/ml) and exposure period 72 h. were studied . The cytotoxicity of the methanolic

Mitotic Index (MI): First of all the metaphase cell were obtained from MCF7 and AMJ13 cell line that treated with IC50 of basil extraction (1250,39.1µg) for 72h. change media were performed before 4h then colcemid (as preventing spindle fiber formation) added (20 µ/ml) for 25 min followed by hypotonic treatment (KCL0.07sn) for 45 min. A fixation procedure with methanol: glacial acetic acid(3:1v:v) was preformed freshly .the with Giemsa stain (as neuclear staining) (7). Finally calculation of M.I were performed according to equation .

Cell division coefficient (MI) = number of dividing cells / total number of counted cells × 100

Statistical analysis

Experiments data were analyzed using Graph Pad Prism Programs V. (7.0) and SAS Statistical analysis system (2012). The differenecees between the control and experimental samples were calculated statistically using T- test. (A P value ≤ 0.05) was considered statistically significant.

RESLUTS AND DISCUSSION

Table 1 elucidate the qualitative analysis of *O. basilicum* L. seeds extract active compound . The results showed presence of phenoles, tannins, flavonoids terpenoids and saponin in methanol extracts for basil seed (Table 1).

extract on the MCF7 cells was dependent on the concentration of the extract, the inhibition rate was increased significantly with highest effect at a concentration of (1250 µ/ml) with inhibition rate of 64.4 % as showed in figure (1-A) compared with control.

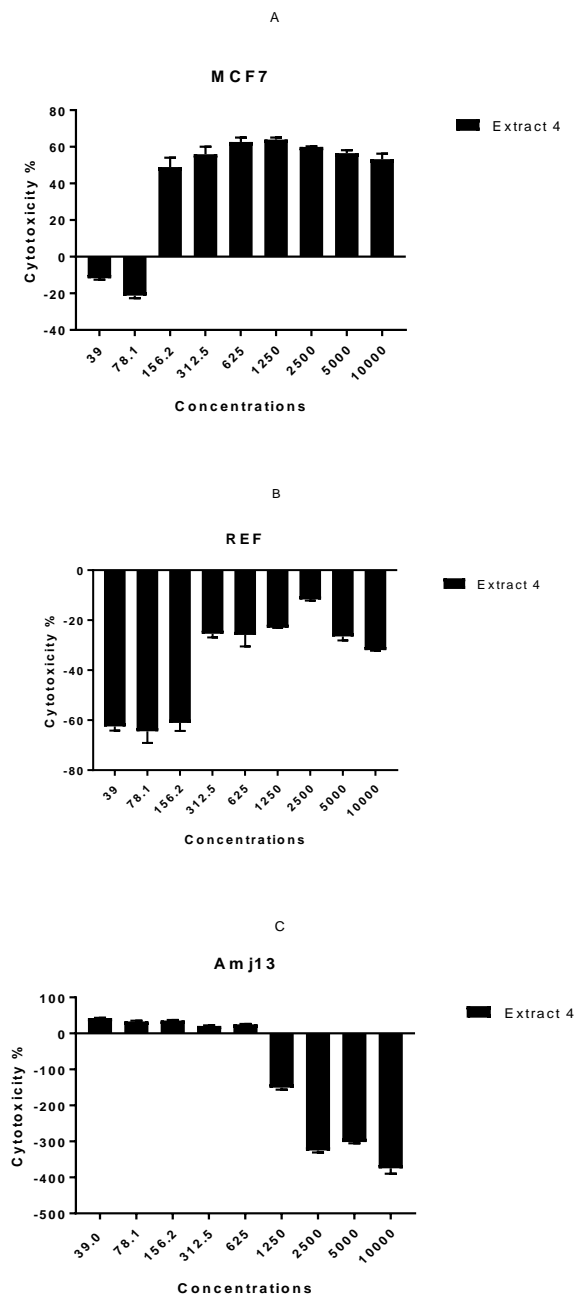


Figure 1. Effect of methanol extract of *O.basilicum* seed on A(MCF7), C(AMJ13) and B(REF) normal cell line after 72hr
 While the results showed slight effect on AMJ13 with the highest inhibition rate as 42.2% at a concentration of 39.0 µ/ml and with increased the cell growth (proliferation rate) at the highest concentrations (625-10000 µ/ml) (figure 1-C). Compared with no effect on the normal cells REF as shows in Figure (1-B). The cytotoxicity effect of the Petroleum ether extract on the MCF7, showed a slight inhibition (16.02%) at concentration of 78.1 µg/ml (figure 2-A) compared with control. While the results showed more effect on AMJ13 with the slightly inhibition rate (35.3

%) at a concentration of 312.5 µg/ml.(figure 2-C) Compared with no effect on the normal cells REF as showed in figure (2-B).

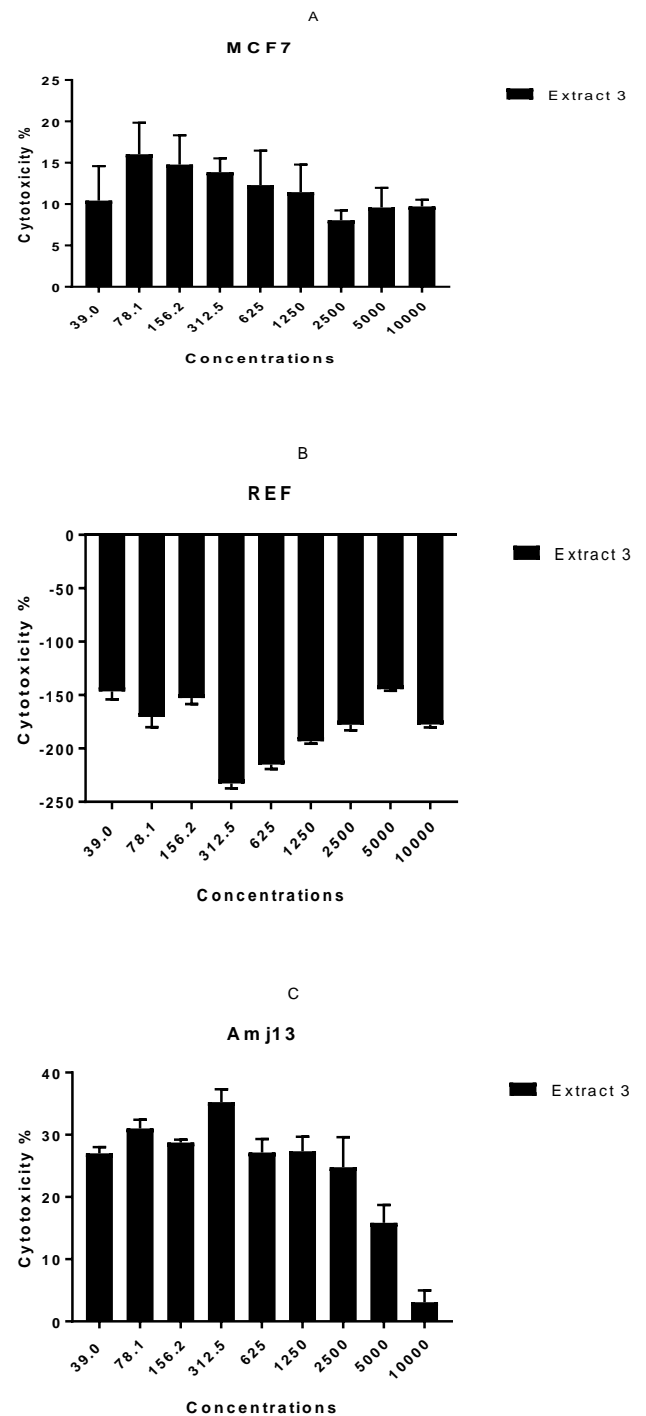


Figure 2. Effect of petroleum ether extract of *O.basilicum* seed on cancer cell line A(MCF7), C (AMJ13),and normal cell line B(REF) after 72h

The results of aqueous hot extract showed that there was no effect of hot aqueous basil extract on breast cancer MCF7 and AMJ13 (figure 3-A and C), its increased the proliferation rate in all studied concentrations. The hot aqueous

extract also had no effect on normal cells REF as shows in Figure (3-B).

effect on normal cells REF as shows in Figure (4-B).

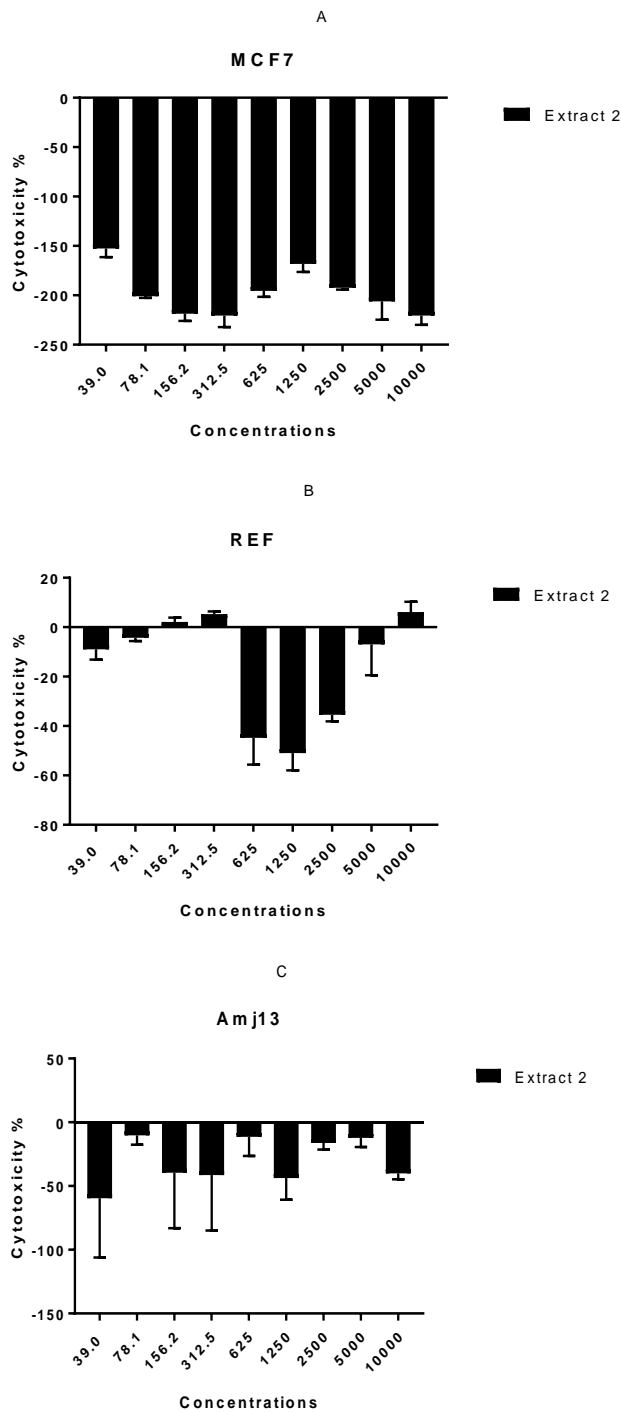


Figure 3. Effect of hot aqueous extract of *O. basilicum* seed on A (MCF7), C (AMJ13), and normal cell line B (REF) after 72h

The results of aqueous cold extract showed that slight effect on AMJ13 with proliferation rate as 24.24% (figure 4-C) compared with control it increased the proliferation rate in all studied concentrations. and there was no effect of cold aqueous basil extract on breast cancer MCF7 (figure 4-A), The extract also had no

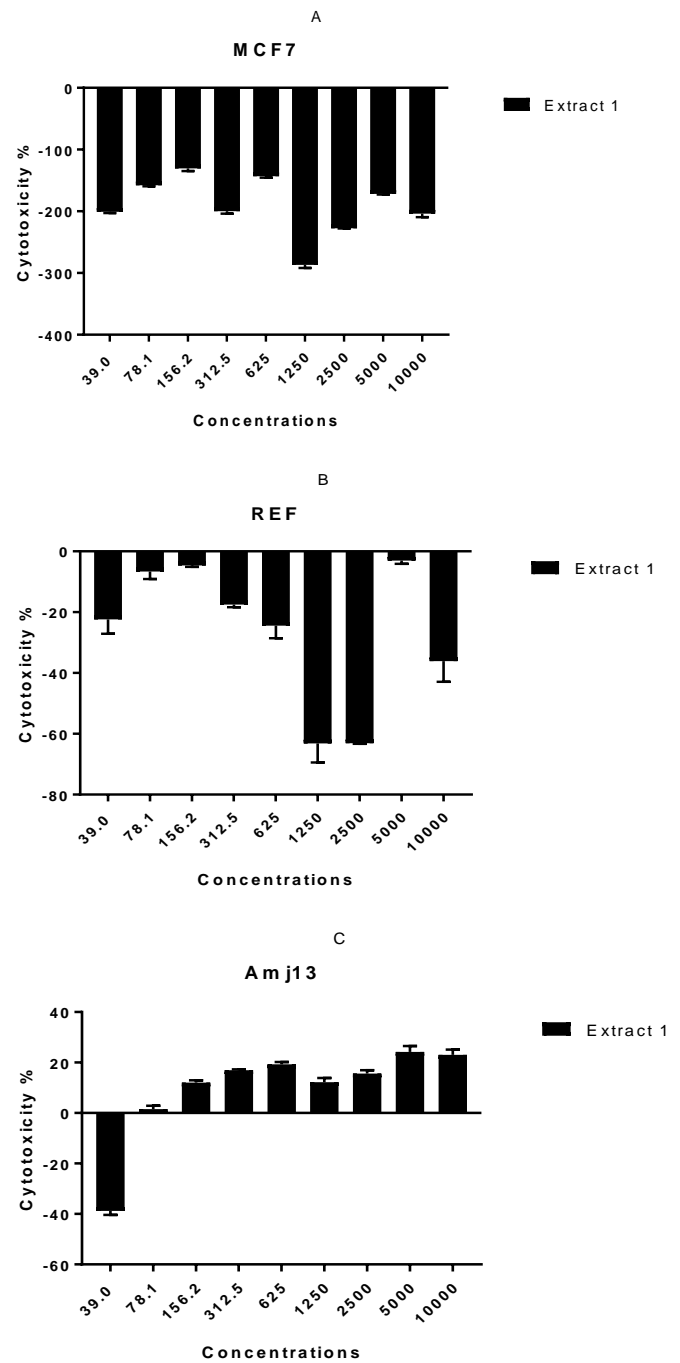


Figure 4. Effect of cold extract of *O. basilicum* seed on A (MCF7), C (AMJ13), and normal cell B (REF) after 72h

Single cell gel electrophoresis (comet assay): As the breast cancer cell lines MCF7 and AMJ13 are exposed to the methanolic extract of *O. basilicum* seeds at the IC50 concentrations. There were no effect or damage in DNA of normal cell REF as showed in figures (5 and 6).

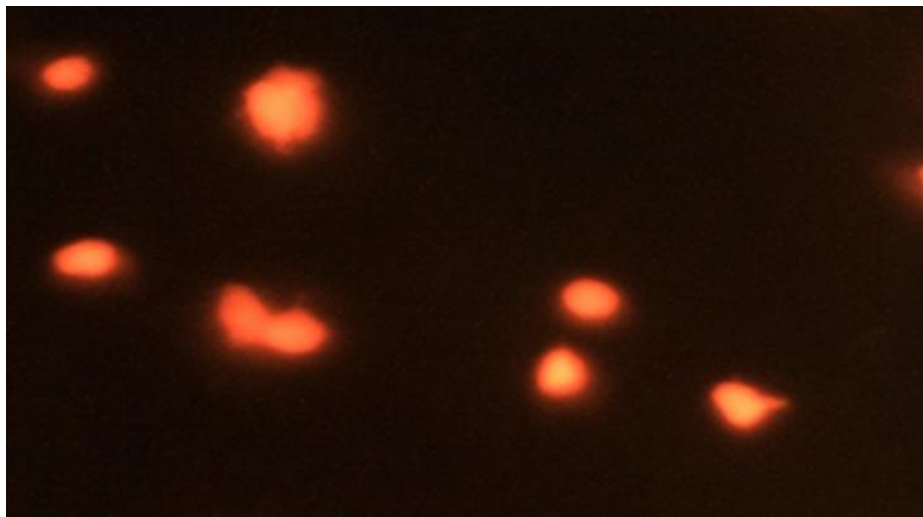


Figure 5. Comet assay no effect of methanolic extract of basil on normal cell REF

While there was server damage in MCF7 after it exposed to extracts compared with mild

damage in control (without exposure) as shows in Figure (6)

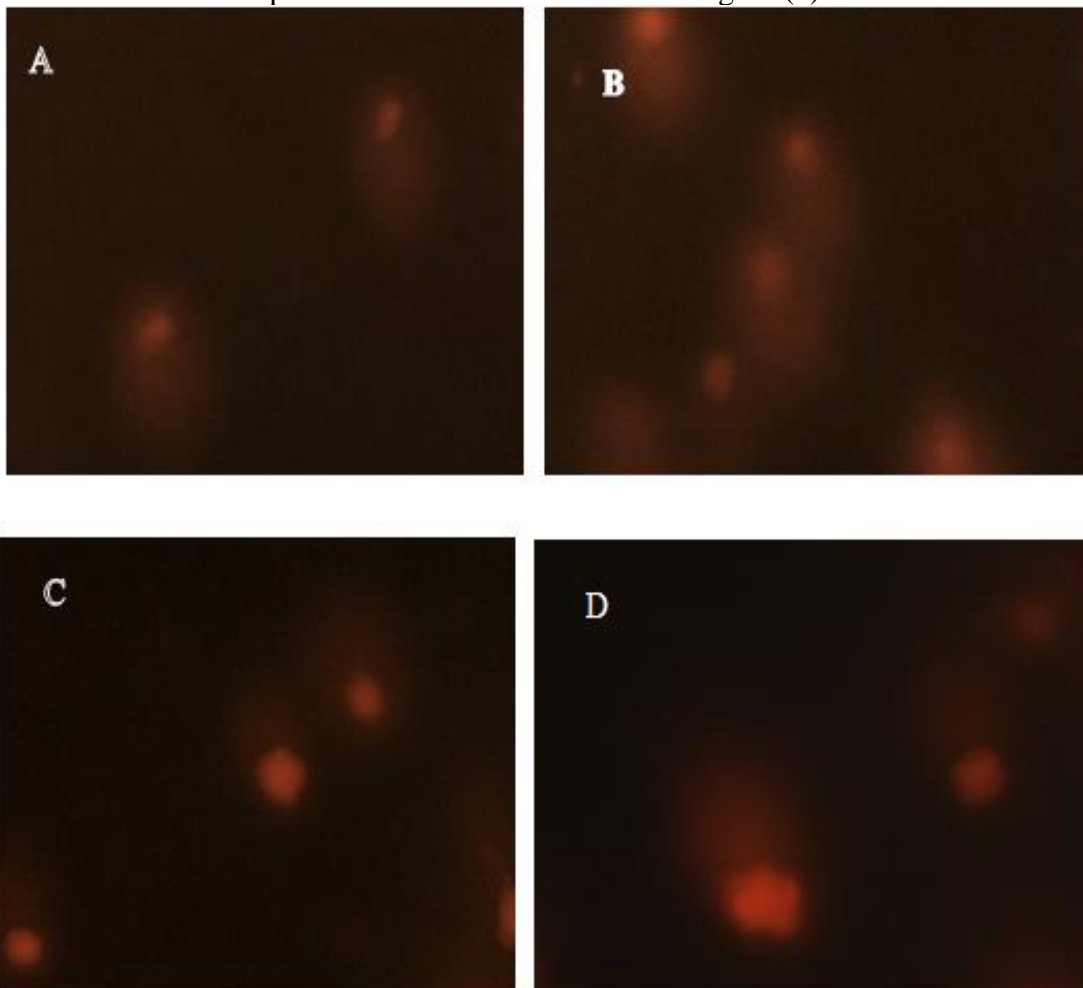


Figure 6. Comet assay effect of methanolic extract of *O. basilicum* seed on breast cancer cell

MCF7, before and after exposed ,(A) and (B) as exposed to extracts (C) and (D) as control

Also, the results showed that there was a sever DNA damage in AMJ13 when exposed to

extract compare with mild damage in control (without exposure) as shown in figure (7).

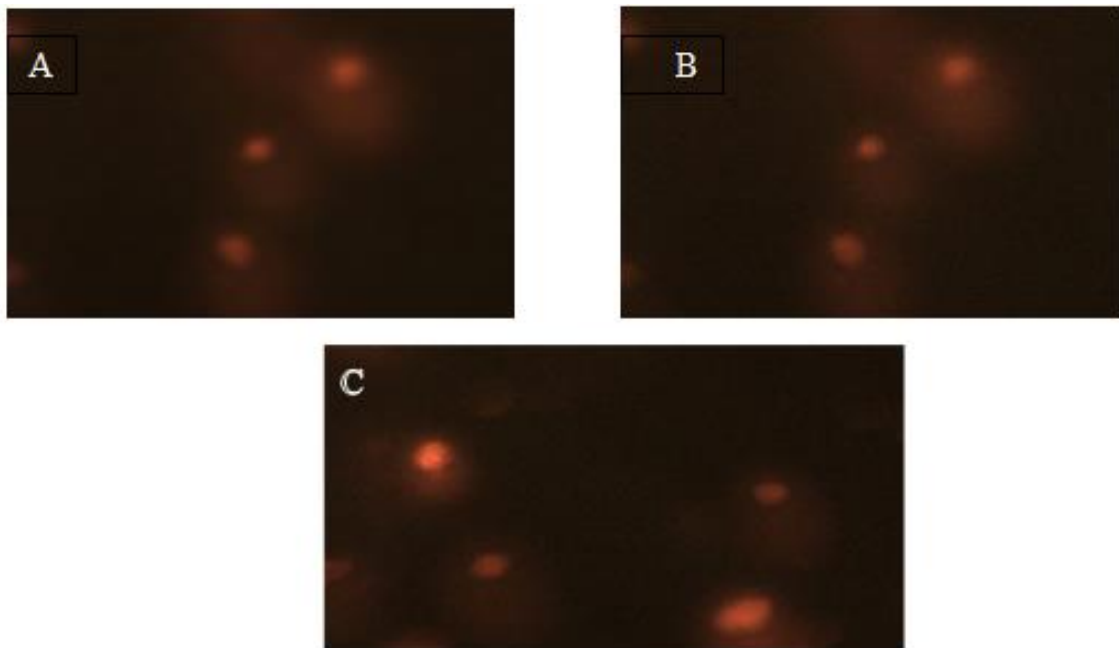


Figure 7. Comet assay effect of methanolic extract of *O.basilicum* seed on breast cancer cell line AMJ13 before and after exposure (A) and (B) as exposed to extract (C) as control

The result demonstrated that there were significant differences in the effects of basil and dilutions on the DNA of each of comet assay as showed in figure (5, 6,7) MCF7 and

AM3 cell line compared with control as shown in Table (2) these effect proved according to the parameter

Table 2. Comparison of the studied treatment of basil seed methanolic extract on cancer cell lines MCF7 ,AMJ13 and normal cell line REF according to the comet test

<u>means±standerd error</u>			
(DNA in Tail)	Olive Moment	Comet	Treatmen
a 4,54±30,32	c 92 .7000±6742,6	ab ,68 ,17 ± 340,30	amj+ extrac
a 4,88±41,87	bc 37592,3±113922,0	ab 158,75±513,42	mcf7+exctract
a7,12 ±38,38	a 15838,6±216267,17	a 20,27±584,66	mcf7
b 2,69±17,13	b 20519,4±142347,17	ab 30,69±449,25	amj13
c 0, 65±3,01	bc 5368,5±79857,9	b11,43±269,55	Control(REF)
**12.64	*6655.70	**26.83	LSD value
The averages with different letters within the same column differ significantly between them			
.(P≤0.01) ** , (p≤0.05)*			

Mitotic Index (MI)

The division coefficient was calculated using the following equation.

Cell division coefficient (MI) = (number of dividing cells / total number of counted cells × 100

The methanol basil extract resulted in reducing the cell division coefficient between dividing and non-dividing cells as showed in figure (8). Also, a decrease in the MI of the IC50 concentrations of the untreated cells from 0.6 to 0.2 for the MCF7-treated cells and from

0.12 for AMJ13 un treated cell to 0.4 for treated cell as shown in table (3).

Table 3. Mitotic index value of *O. basilicum* seeds on breast cancer cell lines (MCF7 and AMJ13

Cell line	With treatment	Without treatment
MCF7	0.2	0.6
AMJ13	0.4	1.2

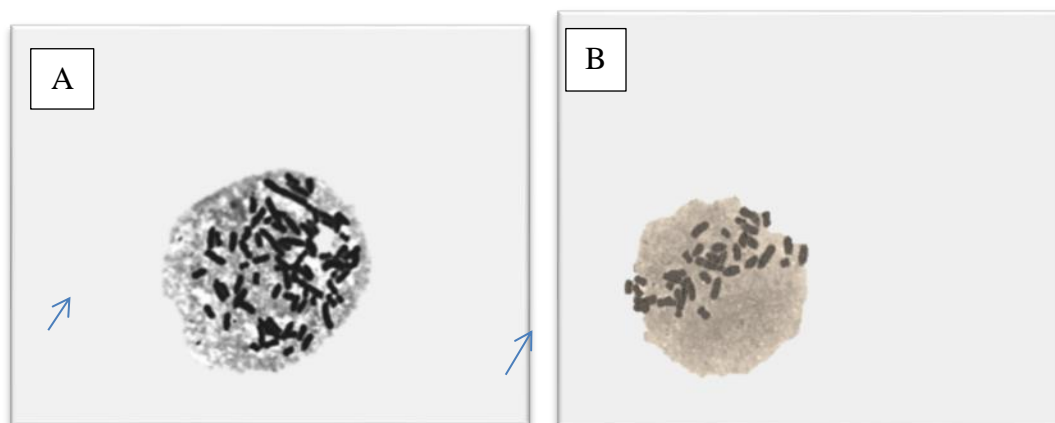


Figure 8. Metaphase for cell line A(AMJ13) ,B(MCF7) under magnification100X

Secondary metabolism compounds in general and plant secondary metabolites in particular have attracted the attention of scientists, researchers and drug production companies due to the abundance of these compounds in nature and many activities in the therapeutic field. Among these activities is the toxic (lethal) effectiveness of cancer cells, as these compounds have mechanisms which inhibit the ability of cancerous cells to grow (26) This prompted us to evaluate *O. basilicum* against human cancer cell lines. Our study support earlier findings regarding the cytotoxic effect of *O. basilicum* against murine leukemic cells (23) and its chemo-preventive effects on carcinogen-induced papilloma-genesis in mice and in in another study. The methanol extract of *O. basilicum* were tested against liver (HEPG2) and colon carcinoma cell lines (HCT116), the results showed strong efficient cytotoxic activity of the methanol extract of *O. basilicum* against the tested human cell lines (4), in this study the methanolic extract showed cytotoxicity effect against cell line MCF7 in concentration 1250 μ g/ml, It is approximately a previous study in which methanol extract for the basil showed cytotoxicity effect against cell line MCF7. Most carcinomas normally show a greater degree of DNA damage with extensive comet tails. The comet assay is one of the most promising genotoxicity tests developed to measure and analyze DNA damage in single cells (29). The tes was used as a parameter for assessing the DNA damage index at the effective concentration the cells presented the highest damage index compared with positive control, that mean the extract increased the apoptosis for cell (14) to investigation the

overall genotoxicity that produced by the abstract of basil alkaline comet assay was used these result demonstrated that crude extract of basil increased significantly DNA damage and genotoxic effect compared with control (14). The findings of the present study suggest that the proper use of herb products is safe and may provide some beneficial effects and more study must be continued (30)(25). Finally, there was an effect of the methanol basil extract in reducing the cell division coefficient between dividing and non-dividing cells (33, 8) Due to the effect of methanolic basil extract on the cytotoxicity assay of concentrations 1250 μ g/ml on MCF7 and 39.0 μ g/ml on AMJ13, these concentrations were tested cell division were studied by Mitotic index (MI). Where a decrease in the MI of the mentioned concentrations of the untreated cells from 0.6 to 0.2 for the MCF7-treated cells and from 0.12 for AMJ13 un treated cell to 0.4 for treated cell. This came as confirmation of the toxicity of the methanol extract of basil, as the decrease of MI indicates that there is a clear toxic effect either through inhibition of the cell life cycle or loss of ability to grow in those cancerous lines in comparison with MI (27). Another studies indicate to the effective role of methanolic extract of some plants as some researchers concluded that it causes chromosomal aberrations thereby and decreasing the mitotic index with increase in dose concentration in longer treatment times (18) . So the basil plant containing of flavonoids and phenolic compounds .These compounds act as antioxidant (12, 32) by scavenging free radicals and their proposed protective role in tumor development and it leads to opoptotic.

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