Cytotoxicity Effect of Ethanolic Olive Leaves Extract against Prostate Cancer Cell Line

التأثير السمي للمستخلص الكحولي لأوراق نبات الزيتون ضد خط خلايا سرطان البروستات

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Abstract:

This work aimed to study the cytotoxic effect of Ethanolic Olive Leaf extract isolated from local *oleo eurepea* plant on affected living prostate cancer cells towards apoptosis by using high content screening assay (HCS). To achieve this goal, we examine and *in vitro* evaluate the cytotoxic effect of the Ethanolic Olive leaves extract (OLE) at different concentrations (100, 50, 25, 12.5, 6.25 µg/ml) in a triplicate dilutions in one culture models (prostate cancer cells PC3) and exposure time of treatment (24 hrs) by high content screening. The study found that olive leaves extract at (50 and100 µg/ml) when incubated with these cells, olive leaves extract mediated lead to significant changes(171%,126%) respectively in mitochondrial membrane potential (MMP) and significant increasing (43.61%,70.15%) of releasing cytochrome C into cystol, also olive leaves extract at 100 µg/ml were found effective and appeared to cause the significant induction(74.77%) of nucleus size and morphology and also significant result (81.71%) in cell membrane permeability and viable cell count (significant value $P<0.05$) suggesting that olive leaves extract appeared as a potential PC3 inhibitor when compared to doxorubicin(20µM) as positive control while other doses marked not any significant effect.

Keywords: Olive leaves, Ethanolic extract, PC3, cellomic multi parameter, cytotoxicity

الخلاصة:

هدف العمل الحالي الى دراسة التاثير السمي للمستخلص الكحولي لأوراق نبات الزيتون الماخوذة من نبات(*Oleo eurepa*) المحلي على خط الخلايا الحية لسرطان البروستات ومن خلال الموت المبرمج لهذه الخلايا وباستخدام فحص المحتوى العالي (HCS) ولتحقيق هذا الهدف تم فحص وتقيم التاثير السمي للمستخلص الكحولي لأوراق الزيتون وبتراكيز مختلفة (100,50,25,12.5,6.25) مايكروغرام / مل وعلى نوع واحد من المزرعة الخلوية (خط الخلايا لسرطان البروستات) ولفترة تعرض 24 ساعة وبمعدل ثلاث قراءات ومن خلال فحص المحتوى العالي وجدت الدراسة ان المستخلص الكحولي لأوراق الزيتون وبتركيزي (50و100 مايكروغرام/مل)عند معاملته للخلايا ادى الى حصول تغييرات معنوية احصائيا(171%،126%) على التوالي في فعالية غشاء المايتوكوندريا والى زيادة نفاذية cytochrome C وبأزدياد معنوي 4.61 % و 70.15%. كذلك بينت الدراسة ان هذا المستخلص وبتركيز 100 مايكروغرام/ مل كان ذو تاثير معنوي (74.77%) على حجم النواه وحصول تغيرات مظهرية ايضا، وكما كان سببا في زيادة معنوية (81.71%) لنفاذية الغشاء الخلوي للخلايا بالاضافه الى التغيير المعنوي الحاصل في عدد الخلايا الحية عند معاملتهم بالمستخلص. وبالتالي اقترحت الدراسة اعتبار المستخلص الكحولي لأوراق نبات الزيتون مثبط فعال لخط الخلايا الحية لسرطان البروستات مقارنة بعقارdoxorubicin(20مايكرومولر)باعتباره سيطرة موجبة اضافة للخلايا الغيرمعاملة باعتبارها سيطرة سالبة.

Introduction:

Prostate cancer as known as carcinoma of the prostate is the progress of cancer in prostate, a gland in the male reproductive system (1). Prostate cancer cells transport from prostate to other parts of the body specifically bones and lymph nodes (2). The early stage of prostate cancer cells are androgen depend that can be eliminated by surgery or anti androgen therapy. However in the later stage malignant prostate cancer cells appear a progressive behavior and androgen independency that become intensive to hormone ablation therapy (3).Factors that expand the risk of prostate cancer include old age, family history of the disease and race. About 99% of cases occur in those over the age of 50 years. Other factors, that may be involved are diet high in processed meat, red meat, or milk products or low in certain vegetables.(4).Prostate cancer is the second most public men cancer worldwide and the particauly common cancer in 84 countries (5).It's a major health concern spectively development countries with greater proportion of elderly men in general population(6).Its characterize in 24% of all death cancer and classified as the fifth common of all cancer(7).The survival percentage for this disease during the last decade 5 years arise from 73.4% in 1999-2001 to 83.4% in 2005-2007(8). Hence kinds of chemotherapeutic agents have been tested to treat patients with prostate cancer at various stages. The results of these attempts have been not promising, and therefore researchers are working hard to find new anticancer drugs as therapeutic regimens against prostate cancer (9). Medical plants are considered to be one of the hopeful options for providing new bioactive compounds for cancer treatment (10).Olive tree *Olea europea* is a species consisting to the family Oleaceae, native to the Mediterranean basin, Asia and parts of Africa (11). The antioxidant capacity of olive leaf contributes to many health benefits. The *in vitro* antioxidant action of olive has documented and linked to such benefits as chemo protection, anti-inflammatory action, prevention of atherosclerotic plaque formation (12) and anti-carcinogenic properties that lead to the prevention of some cancers and finally situation of thyroid (13). Epidemiological and laboratory evidence also record that the differences in incidence of cancer, in general and prostate cancer in particular (14), may be correlated with the presence of certain polyphenols, especially flavones in the diets of this community (15). Hence, the aim of this study is to examine the therapeutic effect of ethanolic olive leaf crude extract and cellular mechanism underling the anti-cancer activity on human prostate cancer.

Materials and Methods:

Plant preparations and extraction:

Fresh green Olive leaves were collected from gardens of Baghdad province/Iraq and classified as *olea europaea* L. by the Prof. Dr. Ali AL-Mossawy, the Biology department, college of science, Baghdad University. Fifty gram of leaves is soaked with 70% ethanol (1:10 w/v) and covered at 20 ^{o}C for 24 hrs than mixture was filtered through whatman No.1 filter paper. The remainder was extracted again to obtain large amount all extracts are combined and lyophilized at 45 ^{o}C to be used in subsequent tests (16). The powdered extract was melted in ethanol 30%, and then sterilized by filtration (Millipore, pore size; 0.22 µm) to be test.

Cell culture:

PC-3 cells were obtain from American Type Culture Collection (ATCC), and maintained in RPMI (Gbico, Carlsbad, CA), supplied with 10% FCS (Sigma), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma). The cells were cultured in an atmosphere of 5% CO_2 at 37Ċ. All cells used in this study were between passages 5 and 15. PC3 cells incubated with: positive control (Doxorubicin 20 µM) and different concentrations of ethanolic olive leave extract (6.25, 12.5, 25, 50, and 100 µg/ml). Comparison between with-and without-treatment and positive control values for cells were made in order to assess the anti- cancer potential of OLE (17).

Cytotoxicity assay:

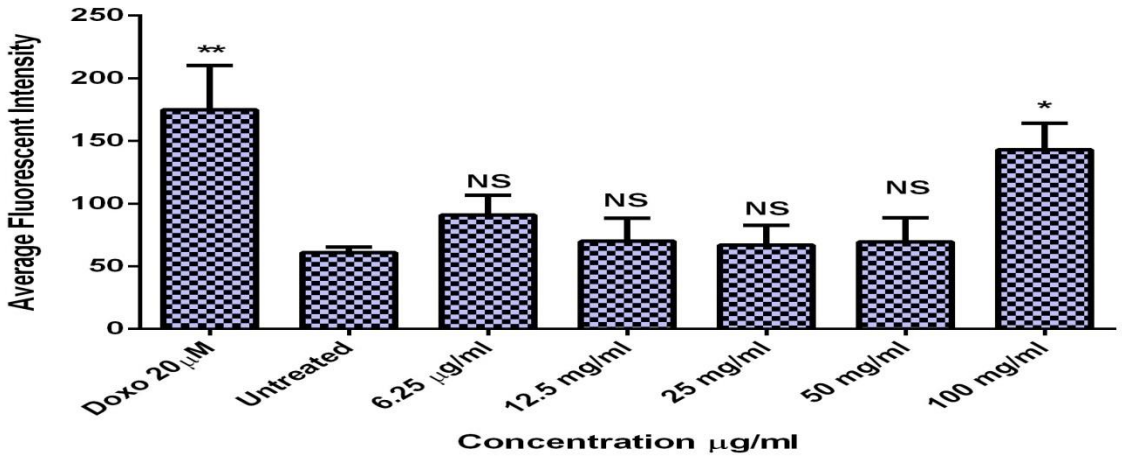
Cytotoxicity assay was done by using high content screening (HCS) kits, these kit enable simultaneous measurements in the same cell of independent parameter that include valid cell count, total nuclear intensity, mitochondrial membrane permeability, cytochrome C release, and changes in cell membrane permeability. After 24 hours of incubation, MMP dye and the cell permeability dye were added to live cells and incubated for 30 minutes at37 ºC. Cells were fixed, permeabilized, blocked with 1X blocking buffer before probing with primary cytochrome C primary antibody and secondary Dye Light conjugate anti-mouse for 1 hour each. Hoechst was added into the staining solution to stain nucleus. Plate with stained cells was analyzed using the Array scan high content screening (HCS) system (Cellomics, PA, USA). The Array Scan HCS is computerized automated fluroscene imagining microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. In each well, 1000 cells were analyzed. Images were acquired for each fluorescence channel, using suitable filters. Images and data regarding intensity and texture of the fluorescence of the cell population within each cell, as well as the average fluroscence of the cell population within the well were stored in Microsoft SQL database for easy retrival. Data were captured, extracted and analyzed with Array Scan II Data Acquisition and data Viewer version 3.0 (cellmics) (18).

Statistical Analysis:

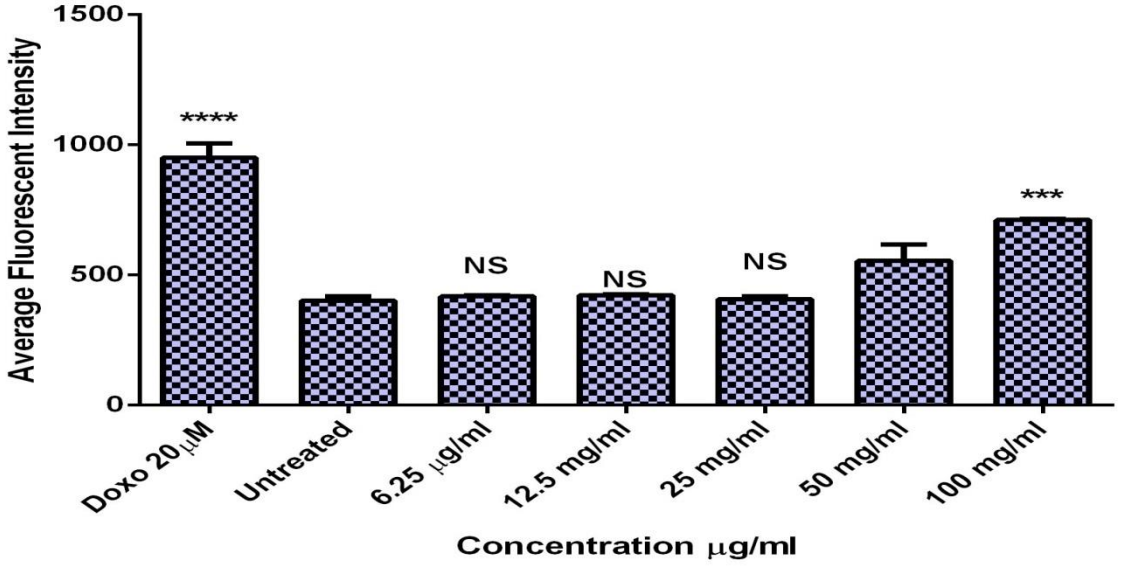
An analysis of variance (one way ANOVA) was performed using Prism statistical software package (Graph Pad Software, USA).A difference at $P<0.05$ was respected statically significant.

Results :

Figure (1) and (2) respectively showed the incubation of Prostate Cancer cells (PC3) with differend concentrations of OLE (100, 50, 25, 12.5, 6.25 µg/ml) for 24 hrs to show the cytotoxic effect on cell membrane permeability, nucleus size and morphology change. Results represented that, the highest concentration (100 µg/ml) appeared to have significant effect ($p<0.05$) on cell membrane permeability and nucleus size and morphology change comparing with positive control (Doxorubicin 20µg M) and untreated cell as a negtive control.While other cocentrotions (50, 25, 12.5, and 6.25 µg/ml) were not siginficantly effect.

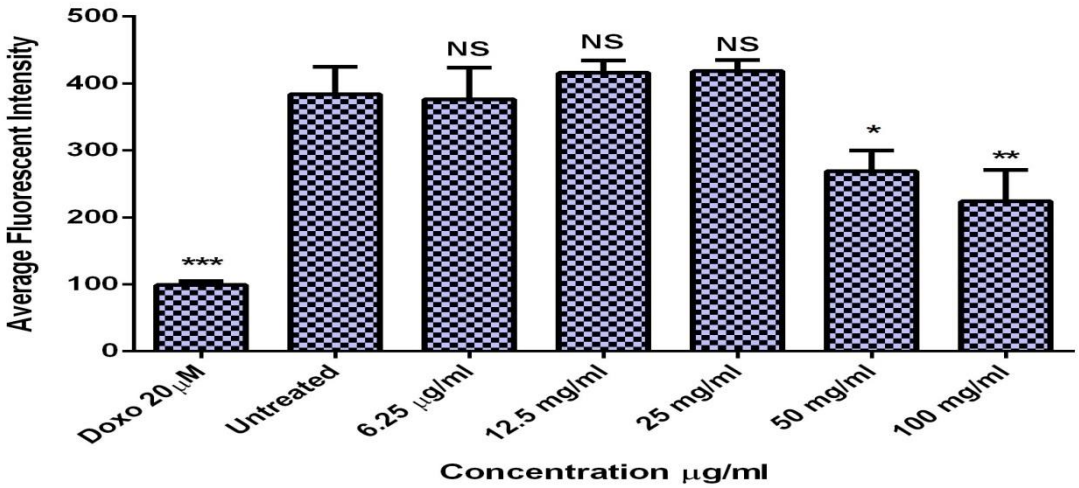


Figure(1):Effect of different concentrations of olive leaves extract on cell membrane pearmebility using PC3 cells after 24 hours of incubation at 37 ºC and evaluated on the Array Scan HCS Reader.

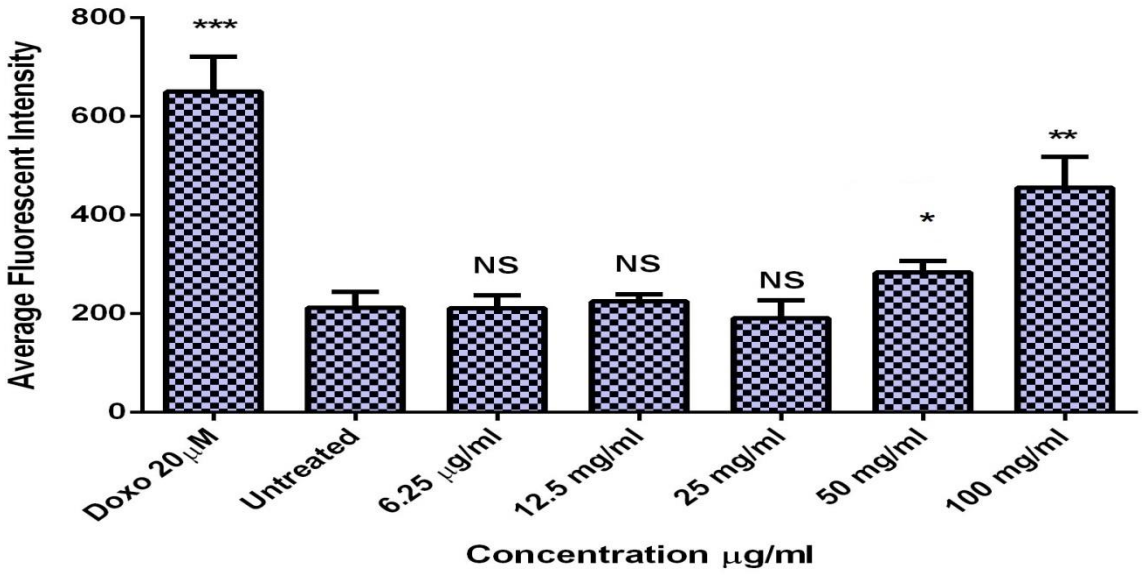


Figure(2):Effect of different concentrations of olive leaves extract on nucleus size and morphology change using PC3 cells after 24 hours of incubation at 37 ºC and evaluated on the Array Scan HCS Reader.

Results of figure (3) and (4) revealed that changes in mitochondrial membrane potential and cytochrome C realizing of PC3 cells effected significantly ($P<0.05$) by two doses of OLE (50 and 100 µg/ml) after 24 hrs of incubation, while other doses (6.25, 12.5, 25 µg/ml) were considered the dose in-depend significantly ($P>0.05$).

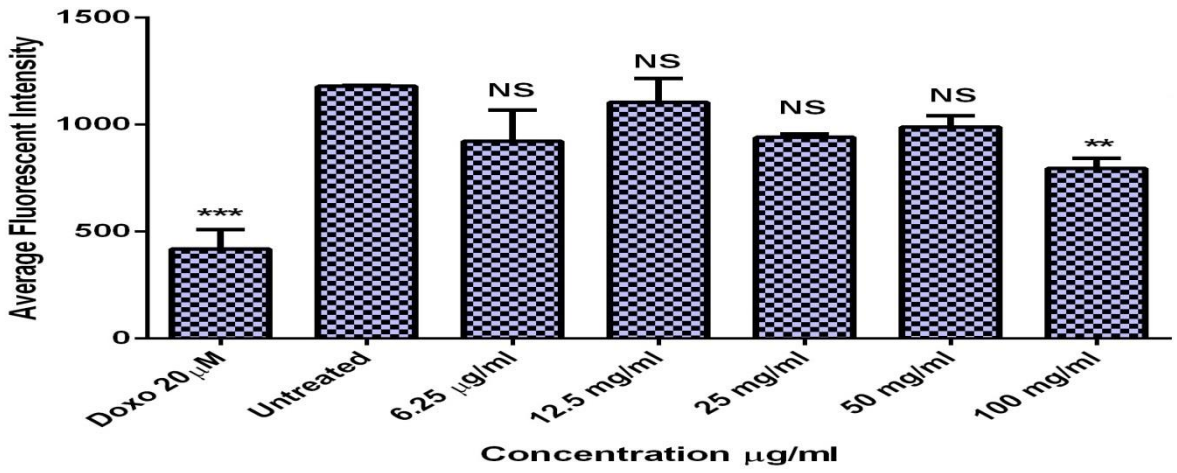


Figure(3):Effect of different concentrations of olive leaves extract on mitocondrial membrane potential changes using PC3 cells after 24 hours of incubation at 37 ºC and evaluated on the Array Scan HCS Reader.



Figure(4): Effect of different concentrations of olive leaves extract on Cytochrome C realizing using PC3 cells after 24 hours of incubation at 37 ºC and evaluated on the Array Scan HCS Reader.

In figure (5) we examined the OLE concentration on (OLE incubated with PC3 cell) and the result revealed that OLE was decrease significantly of PC3 cell viable cell count when treated with (100 µg/ml) of OLE. This was reveled that does was respected depend significntly(P<0.05) when comparing with positive conrol and untreated cell.



Figure(5): Effect of different concentrations of olive leaves extract on viable cell count using PC3 cells after 24 hours of incubation at 37 ºC and evaluated on the Array Scan .

Discussion:

High content screening analysis provided quantitative information on changes in commonly used cellular parameters including cell membrane permeability, nucleus size and morphology change, mitochondrial membrane potential, cytochrome C, and viable cell count. It has been reported that changes in cell membrane permeability are often associated with an ongoing toxic or apoptotic response and the cell loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity (19), which is represented by alteration of translocation of phosphoatidyl serine from the inner to the outer leaflet of the plasma membrane (20). In this study, we utilize this is as a key parameter for the estimation of the cell- extract interaction. Other researcher showed that the intensity of MCF-7 cell line increased significantly cell permeability when treated with 25, 50 and 100 µg/ml of purified lignin (21). Another marker of apoptosis is condensation and fragmentations of nucleus. The results of this study demonstrate that the highest dose (100µg/ ml) was responsible of morphological changes of PC3 cells by staining the cells with Hoechst 33342 dye and comparing with positive and negative control leading to significantly increase of nuclear intensity corresponding to apoptotic changes following OLE treatment. One study indicated that dry olive leaves extract was more effective in reducing DNA damage in the pretreatment protective role against L-thyroxine effect (22).On other hand the morphological hallmark of apoptosis in nucleus was seen by DNA condensation nuclear morphology and intensity when A549 cells treated with the dose depended panduratin A at 5µg/ml (23). Two main pathways lead to apoptotic namely intrinsic pathway and extrinsic pathway binding to death receptor such as Fas (CD 95) leading to activate caspase-8(24).Induction of these proteins lead to permeabilization of mitochondrial membrane which that lead to reasling of cytochrome C (25).Changes in the mitochondrial trans membrane potential in PC3 cells treated with OLE were quantified by flow cytometry with mitochondrial membrane potential dye (MMP) and the result appeared after 24 hrs of incubation with (50,100

µg/ml) decreased significantly of mitochondrial membrane potential in PC3 cells with de-polarized mitochondria. As mitochondria- mediate cell death signaling event, opening of the mitochondrial permeability transition pore causes the release of cytochrome c into cytosol. When cells treated with different concentrations of OLE (6.25, 12.5, 25, 50, 100 µg/ml), we remarked that the doses dependably cause cytochrome C release comparing to positive control were (50 µg/ml) and (100 µg/ml). By supposing that the changes in cell viability are directly related to toxic effect of OLE at 100µg /ml concentration, the significant decrease in cell viability down cell count when compared to cell count at 20 µg/ ml doxorubicin as positive control. It has been postulated that Olive oil can induce significant levels of apoptosis in various cancer cells and decreased cell viability, inhibited cell proliferation and induced cell apoptosis in MCF-7 human breast cancer cell (26).Other studies showed that olive leaves crude extract were found to inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary bladder carcinoma (T24) and bovine brain capillary endothelial (BBCE) (27, 28).

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