Study the apoptotic effect of N_butanol extract of Ur tica dioica on T24 bladder cancer cell line

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Abstract
Background: Cancer is wide spread disease nowadays affected large number of human .Bladder cancer affected tissue of the bladder and can be metastases to other part of the body .There are many risk factor for bladder cancer include smoking ,family history and radiation .Doxorubicine is one of anticancer agent that used in treatment of Bladder cancer .Urtica dioica is a family of plant known for their medicinal activity from folk medicine .Urtica dioica is a member of urticaeas and it is known for it is anticancer activity .One of most mechanism of anticancer agent is to induce apoptosis in cell.

Methods: in the present study we investigate the apoptosis effect of N_ butanol extract of urtica dioica against T24 bladder cancer cell line.

Results: The result show increase in the apoptotic effect on the cell by increasing the concentration of extract.

Conclusion: The N_ butanol extract of urtica dioica show significant apoptotic effect on T24 bladder cancer cell line

INTRODUCTION
Cancer is a main health trouble affects adults and young people, men, women and children. It is the second most important reason of fatality worldwide following cardiovascular diseases. In 2018, the number of cancer deaths was 9.6 million, with a regular of one of every six deaths in the world. 70% of these deaths were recorded in developing countries. Bladder cancer is type of cancer that can arise from tissue of bladder . Beside it can arise from the bladder it can spread to other part of body. There are many risk factor for bladder cancer : smoking ,family history, prior radiation therapy,frequent bladder infections and exposure to certain chemicals. The most common type is transitional cell carcinoma. Other types include squamous cell carcinoma and adenocarcinoma. Sign and symptoms of bladder cancer typically causes blood in the urine, which might be visible or visible by microscope only. Blood in the urine is the most common symptom in bladder cancer and is painless. Visible blood in the urine may be of only short duration, and a urine test may be required to confirm nonvisible blood. 80–90% of patient have bladder cancer firstly presented with visible blood in urine. Treatment of Bladder cancer. Non-muscle invasive Bladder cancer: Non-muscle invasive bladder cancer can be “shaved off” using an electrocautery device attached to a cystoscope, which in that case is called a resectoscope. The procedure is called transurethral resection of bladder tumor (TURBT) and serves primarily for pathologial staging. In case of non-muscle invasive bladder cancer the TURBT is in itself the treatment, but in case of muscle invasive cancer, the procedure is insufficient for final treatment. Chemotherapy Given a single instillation of chemotherapy to the bladder after TURBT has exposed profit in decrease the recurrent of bladder cancer by 35% in non-muscle invasive bladder cancer. Mitomycin, Cepirubicin, Pirarubicin and Gemcitabine is Medications used for this purpose. After TURBT, chemotherapy should be given in few hours. Because after few hours the remaining tumor cell adhere and cover strongly by extracellular matrix and decrease the efficacy of chemotherapy. Immunotherapy Muscle invasive Bladder cancer Multimodal therapy Now adays the two most used chemotherapy regimens for neoadjuvant chemotherapy are Methotrexate, Vinblastine, Adriamycin, Cisplatin (MVAC)and gemcitabine with cisplatin (GC). Other regime include dose dense MVAC(DDMVAC)and Cisplatin, Methotrexate and Vinblastine (CMV).Although, optimal regime has not been established, the preferred regime for neoadjuvant therapy is MVAC. Doxorubicin is an anthracycline, discovered from Streptomyces peucetius in 1960, classified into natural product like daunorubicin, and synthetic like epirubicin, and idarubicin. They are highly effective anti-neoplastic agents. Phytotherapy Even with the considerable developments of recent medicine, cancer remain a Serious problem and main reason of fatality international and experimental has shown unwanted health complications related with cancer treatments. Therefore, there is a must to build up a successful treatments to defeat the disadvantages of the Prevailing treatment strategies. Researchers resorted to therapeutic plants to discover their healing capabilities against cancer as other diseases. Phytochemicals as a cancer treatment Experimentally, plant isolated phytochemicals have been shown to induce specific anticancer activities such as apoptosis, decreasing cell proliferation. Apoptosis There are 37.2 trillion cells in human body, this number of cells is keeping up by controlling two important biological processes, cell division and cell death in which there is an equal balance between...
cell division and cell death. This death occurs to cells that are no longer functional or needed so they suicide in planned way\textsuperscript{13}, therefore this death considered one of the types of "programmed cell death" and more specifically named with "apoptosis"\textsuperscript{14}. Apoptosis is the main organize means of cell death that occurs when DNA damage can’t be repaired\textsuperscript{14,15}. UrticaseaThe family of Urticasea is one of the most herbal families known with its therapeutic bioactivity against different diseases. Urticasea family consist of common herbs that characterized with stinging hairs and dark green leaves\textsuperscript{16}. General description of \textit{Urtica dioica}\textit{Urtica dioica} is a dioecious, herbaceous, perennial plant, 1 to 2 m tall during summer and passing in the winter to the ground. One of the most valuable techniques used for analyzing medicinal plants component is high performance liquid chromatography (HPLC)\textsuperscript{17}. Which is used to detect the component of N\textsubscript{butanol} extract of \textit{urtica dioica}.

**MATERIAL AND METHODS**

**Plant collection**

\textit{Urtica dioica} was collected during March – May 2018 from Jisr Dayala _ Baghdad province, Iraq. The leaves was collected, dried in shed, rendered to coarse powder. Preliminary examination for the active constituents will be done for each fraction using different chemical reagents. Extraction by maceration with ethanol then fractionation according to active constituents. Isolation of different constituents will be done using HPLC technique. Extraction method Aerial part of the plant were powdered and weight 350 grams of the powdered then soaked in 2500ml (1:7) 80 % ethanol, with occasional shaking, at room temperature. After 3 days, the ethanol soluble materials were filtered off, repeat the process 3 times for 9 days. The filtrate was evaporated to dryness under vacuum using rotary evaporator. A dark greenish residue was obtained. The residue was suspended in 500ml water and partitioned successively with n-hexane, chloroform, ethyl acetate, and n-butanol until reach clear layer for each fraction. The N\textsubscript{butanol} fraction filtered, and evaporated to dryness.\textsuperscript{18} Doxorubicin stock solution10 mg of DOX dissolved in 100 μl dimethyl sulfoxide (DMSO) to form stock solution, then diluted with RPMI to preferred concentrations ranging from 1.25μM to 80μM, this stock used as a positive control\textsuperscript{19}. The N\textsubscript{butanol} extract of \textit{urtica dioica} (NBEUD) stock solution was made by dissolve 4 mg of dried NBEUD in 50 μl dimethyl sulfoxide (DMSO) to form stock solution, then diluted with RPMI to preferred concentrations ranging from 20μg and 40μg.

**Apoptosis assay**

Annexins are a family of calcium-dependent phospholipid-binding proteins that preferentially bind phosphatidyserine (PS). Under normal physiologic conditions, PS is predominantly located in the inner leaflet of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution across the phospholipid bilayer and is translocated to the extracellular membrane leaflet marking cells as targets of phagocytosis. Once on the outer surface of the membrane, PS can be detected by fluorescently labeled Annexin V in a calcium-dependent manner\textsuperscript{20}. Flow cytometry The following protocol was performed\textsuperscript{21}. Cells were treated for 48 hours with 1.25 μM DOX, 20 ,40μg of NBEUD, combination of 1.25 μM Doxorubicine with 20 μg NBEUD and combination of 1.25 μM Doxorubicine with 40 μg of NBEUD. Each flask was trypsinized and centrifuge 1000 g for 3 minute.3.Cells well washed with PBS and centrifuged 1000 g for 3 minute.4.Cell were suspended in 2 ml of medium .5.Cell were stained with Annexine v and propidium iodide using abcam kit (ab 14085) through manufacture instruction .6.The extend of apoptosis and necrosis in cell was detected by flow cytometry (BD FACs caliber| BD biosciences,San Jose ,CA ,USA)and rate data were analyzed by flow software (7.6.1 version)

**Statistical analysis**

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). Three-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means. P < 0.05 is considered statistically significant. Also the CompuSyn program was used to estimate the combination index (CI) and to identify the type of the drug interaction\textsuperscript{14,11}.

**RESULT**

HPLC analysis show that NBEUD contain chlorogenic acid which is polyphenolic compound and Rutin which is flavonoid compound. Figure (1,2)

![Figure 1](image1.png)

**Figure 1.** Chlorogenic acid found in HPLC analysis at 14.8 minute

![Figure 2](image2.png)

**Figure 2.** Rutin found in HPLC analysis at 27 concentration 1.25μM induce 25.73%±1.43 figure (4), while in combination of NBEUD 20μg with 1.25 μM DOX show apoptotic effect 25.27%±5.17 figure (6). In the concentration of 40μg of NBEUD show early apoptosis 7.87%±2.23 figure (7) while in the combination with DOX show 10.31%±1.46
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Late apoptosis
Treating bladder cancer cell line T24 with NBEUD, DOX and combination revealed that in the late apoptosis 20µg of NBEUD induce 3.48±0.83 figure(5), while in the DOX alone at concentration 1.25µM induce 3.18±0.55 figure (4), while

Apoptosis
Treating bladder cancer cell line T24 with NBEUD, DOX and combination revealed that apoptosis of 20µg of NBEUD induce 8.59±0.67 figure(5), while in the DOX alone at concentration 1.25µM induce 28.91±1.50 figure (4), while in combination of NBEUD 20µg with 1.25 µM DOX show

**Figure 3.** Early apoptosis late apoptosis and apoptosis in control cell of T24 bladder

in combination of NBEUD 20µg with 1.25 µM DOX show apoptotic effect 6.72±0.44 figure (6).In the concentration of 40µg of NBEUD show late apoptosis 2.17±1.42 figure (7) while in the combination with DOX show 2.75±0.47 figure (8) , figure (10) show late apoptosis.

**Figure 4.** Early apoptosis, late apoptosis and apoptosis after 48 hours of treatment of of NBEUD, DOX 1.25 µM apoptotic effect 31.99±4.73 figure (6).In the concentration of 40µg/ml of NBEUD show apoptosis 10.04±0.83 figure (7) , while in the combination with DOX show 13.06±1.32 apoptosis figure (8) , figure (11) show apoptosis.
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Figure 5. Early apoptosis, late apoptosis and apoptosis after 48 hours of treatment of T24 bladder cancer cell line NBEUD 20 µg/ml.

Figure 6. Early apoptosis, late apoptosis and apoptosis after 48 hours of treatment of T24 bladder cancer cell line NBEUD 20 µg and DOX 1.25 µM.
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**Figure 7.** Early apoptosis, late apoptosis and apoptosis after 48 hours of treatment of T24 bladder cancer cell line NBEUD 40 µg.

**Figure 8.** Early apoptosis, late apoptosis and apoptosis after 48 hours of treatment of T24 bladder cancer cell line NBEUD 40 µg and DOX 1.25 µM.
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Figure 9. Early apoptosis of control and treatment cell of T24 bladder cancer cell line after 48 hours of exposure to NBEUD20 µg, NBEUD40 µg, and 20 µg, 40 µg with 1.25 µM DOX.

Figure 10. Late apoptosis of control and treatment cell of T24 bladder cancer cell line after 48 hours of exposure to NBEUD20 µg, NBEUD40 µg, and 20 µg, 40 µg with 1.25 µM DOX.

DISCUSSION

Apoptosis, is a cell suicide program, characterized by DNA fragmentation, formation of apoptotic bodies and nuclear condensation leading to single cell death without affecting the surrounding tissue (22). Cytotoxic agents may induce apoptosis by initiating death signaling pathways in target cells by activation of death receptor, DNA damage, disturbance in mitochondrial function and ROS damage (23). Phosphotidylserine (PS) (procoagulant phospholipids) located in the membrane of cells. During apoptosis, the cell exposed PS to the cell surface and exhibits procoagulant and proinflammtory activities of the dying cells (24).

Doxorubicin, induced apoptosis by inhibiting TOP2α leading to damage of DNA, formation of free radical hydrogen peroxide (H₂O₂) by oxidized quinine to semi-quinine moiety leading to lipid peroxidation, DNA damage and caused apoptosis. These mechanisms affect not only tumor cells but also normal cells (cardiac cells) and cause cardiotoxicity (25). The balance between pro-apoptotic proteins (Bax) and anti-apoptotic proteins (Bcl2) is essential for progression of cancer, recent studies reported that any change in balance between these proteins responsible for survival or death in human breast cancer, so increase the expression level of anti-apoptotic proteins leading to survival in many types of cancer cells (26). Brigida et al (2019) reported that urtica dioica can induce apoptosis in human prostate carcinoma in LNCaP cell line by increased the activities of caspase 3 and 9 following 24 hour treatment (27).

Figure 11. Apoptosis of control and treatment cell of T24 bladder cancer cell line after 48 hours of exposure to NBEUD20 µg, NBEUD40 µg, and 20 µg, 40 µg with 1.25 µM DOX.

Arkene et al (2014) reported that Urtica dioica selectively killed NSCLC cells, by promoting ER-mediated apoptosis (28). Santos et al (2011) reported that Rutin to induce DNA strand breaks in GL-15 cells and induce apoptosis (29). Rutin exerts its anticancer effect by inducing apoptosis by arresting the G0/G1 phase in the cell cycle. This cell cycle arrest can be attributed by acting upon Bcl-2 family proteins which regulates all major types of cell death (30). Kazuo et al (2017) reported that BAX and BCL2 gene expression in A549 cells was enhanced and reduced, respectively, by Chlorogenic acid. In addition, Chlorogenic acid increased CASP3 gene expression levels. The increase in BAX gene expression in A549 cells in response to chlorogenic acid treatment was likely associated with ROS (31). JAI-SING et al (2012) reported that Chlorogenic acid induced cytoxic effects occur through induction of apoptosis by the disruption of the mitochondrial membrane potential (reduction of ΔΨm), ROS production, activation of caspase 3, 9 induction of apoptosis (32). Srabanti et al (2010) reported that Chlorogenic acid induced ROS generation in K562 cells was accompanied by disruption of the mitochondrial membrane potential and release of cytochrome c and SMAC from mitochondria to the cytosol. Thus, ROS act as upstream signaling molecules to initiate Chlorogenic acid mediated cell death (33). Many studies reported that ROS are key signaling molecules in mammalian cells, accumulation of ROS leading to mitochondrial dysfunction and induction of apoptosis (34).

The present study showed that NBEUD induced apoptosis on bladder cancer cell line T24 in dose dependent manner when compared with control cell. Table (3.5), show that treating bladder cancer cell line T24 with NBEUD, DOX and combination revealed that apoptosis of 20 µg of NBEUD induce 8.59±0.67 while in the DOX alone at concentration 1.25µM induce 28.91±1.50 while in combination of NBEUD 20 µg with 1.25 µM DOX show apoptotic effect 31.99±4.73. In the concentration of 40µg/ml of NBEUD show apoptosis 10.04±0.83 while in the combination with DOX show 13.06±1.32 apoptosis table (3.5), figure (3.32).


Cancer occurs as the result of a disturbance in the homeostatic balance between cell growth and cell death. Overexpression of antiapoptotic genes and underexpression
of proapoptotic genes can result in the lack of cell death that is characteristic of cancer. Apoptosis was the major reason of cell death induced by antitumor drugs and radiosensitization drugs. The B cell lymphoma/leukemia-2 gene (BCL2) and the Bcl2-associated X protein gene (BAX) are an oncogene and a cancer suppressor gene, respectively. In many pathological studies, an unbalanced BCL2/BAX ratio (BCL2/BAX > 1) has been recognized as a signature of the acquisition of apoptosis resistance in cancer cells [24,25]. Hongyan, et al (2013) show that rutin could induce LAN-5 apoptosis and decrease BCL2 expression and BCL2/BAX ratio. This evidence indicates that the Autin has regulatory role in the BCL2/BAX balance of tumor cells [24].

REFERENCES
1. (https://www.cancer.gov)
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