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ANTIMICROBIAL AND ANTICANCER ACTIVITIES OF EXTRACTS FROM *URGINEA MARITIMA* FRUITS

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Abstract

Background: Increasing antibiotic resistance among human pathogenic microorganisms and the failure of conventional cancer therapies attracting great attention among scientists in the field of herbal medicine to develop natural antimicrobial and anticancer drugs. Thus, the antimicrobial and anticancer activities from fruits of the medicinal plant *Urginea maritima* (L.) Baker that unexplored previously were investigated in this study.
Materials and Methods: Fruits of *U. maritima* plant were collected, dried, ground, and extracted by hot water, ethanol, methanol, and acetone. The fruit extracts were examined for their potential as antimicrobial and anticancer agents using the agar well diffusion method and MTT assay, respectively. The gene expression of some cancer-related gene markers was determined by RT-PCR.
Results: All fruit extracts of *U. maritima* exhibited antibacterial activity against *S. aureus* and *E. coli*. Methanol and ethanol extracts exhibited anticandidal activity. Ethanol and acetone extracts displayed non-hemolytic activity and selective cytotoxicity against breast cancer MCF7 cells with IC_{50} values that considered as active treatments. Concerning DNA fragmentation and gene expression after treatment of MCF7 cells with the most promising acetone extract, induction of apoptosis was proposed. The expression of cancer-related gene *TNF* after 6 hours, tumor suppressor genes (*p53* and *BRCA1*), and immune response genes (*IL-2* and *IL-6*) was induced. The expression of anti-apoptotic gene *Bcl2* in treated MCF7 cells was reduced.
Conclusion: Fruit extracts of *U. maritima* exhibited antimicrobial and anticancer activities. This result may lead to the use of these extracts for treatment of some infectious diseases and certain types of cancer.

Keywords: *Urginea maritime*; antibacterial; antifungal; anticancer; gene expression

Abbreviations: ATCC, American type culture collection; Bcl2, B-cell lymphoma 2; BRCA, Breast Cancer; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL, Interleukin; MCF7, Michigan cancer foundation-7; TNF, Tumor necrosis factor.

Introduction

Increasing antibiotic resistance among human pathogenic bacteria and fungi is becoming a serious challenge and a cause for global concern. Emergence of multidrug-resistant pathogenic strains of bacteria and fungi has caused a high rate of morbidity and mortality among patients. Therefore, there is a need to explore new sources for discovering novel classes of active secondary metabolites that have biological activities against multidrug-resistant bacteria and fungi. Therefore, the potential antimicrobial activities of extracts derived from plant origin were investigated in the current study.

Cancer is a serious problem and a life-threatening illness. Cancer cells grow and spread without control throughout the body. Metastasis of cancer cells is considered as the major cause of cancer related mortality (DeSantis et al., 2011). New diagnosed cases are expected to rise by about 70% over the next two decades to reach twenty-two million cases (WHO, 2012). Cancer is considered as the second leading cause of death after cardiovascular disease worldwide (Turgay et al., 2005). In Jordan, cancer is the second most frequent cause of death after heart disease. It is the cause of death of more than 10,000 Jordanians from 1996 till now (Ministry of Health, Jordan Cancer Registry, 2013). Enormous efforts are invested to cope with cancer problem, but limited success has been achieved with most of the therapeutic strategies (Abu-Dahab and Afifi, 2007). Therefore, one of the current trend in cancer therapy is to find a

cure from natural resources. Recently, there are various conventional therapies to fight cancer, including surgery, chemotherapy, immunotherapy, and radiotherapy (American Cancer Society, 2016). Unfortunately, there are some considerable limitations to use them due to the efficiency, availability, high cost, and its undesired side effects (Xu et al., 2009). Moreover, many cancer treatment protocols lack safety (Rebecca, 2004). Thus, there is an urgent need to find a safe and active anticancer agent. Three groups of genes; proto-oncogenes, tumor suppressor genes, DNA repair and immune enhancing genes (Schneider, 2012) control cancer cells. The study of these genes when using anticancer drugs, which up-regulate or down-regulate cell-controlling genes, has become very important to determine the mechanism of killing in cancer cells.

The efforts in this study are oriented with hope toward finding new antimicrobial and anticancer agents from extracts of plant origin. Nature provides us with many products classified as secondary metabolites that originate from plants and have been used since ancient times to find new treatments to cancer. Therefore, this study is initiated to explore the potential anticancer activity of the selected medicinal plant *Urginea maritima* (L.) Baker (sea squill). The plant *U. maritima* is classified as a herb of the Liliaceae family and it is indigenous to the Mediterranean region including Jordan (Bozcuk et al., 2011). It has been well-known as a medicinal herb since early times, this plant is known to exert diuretic, cardiotonic properties, treat edema and has been used as a cough syrup (Bozcuk et al., 2011). On the other hand, the medicinal and poisonous activities of this plant refer to the presence of active phytochemicals. Analyses of *U. maritima* lead to the identification of many active compounds, such as cardiac steroids, anthocyanins, flavonoids, polysaccharides, and calcium oxalate (Al-Tardeh et al., 2006). Conversely, the acute toxicity of these compounds have been an evidence concerning the efficiency and possession of this to target and attack cancer cells (Mijatovic et al., 2007). Furthermore, *in vitro* and *in vivo* epidemiological data suggested that *U. maritima* derived cardiac steroids mediated anticancer activities through a regulated number of cellular processes such as proliferation, apoptosis and cell cycle arrest in various types of cancer cell lines including human leukemia, breast cancer cells (MCF7 and MDA-MB2311), prostate, melanoma, pancreatic, lung, and colon cancer cells (Daniela et al., 2003). It was reported that the bulb and leaf extracts of *U. maritima* have some potential antifungal activity (Abou-Jawdah et al., 2004; Khan and Abourashed, 2010). However, the antimicrobial and anticancer biological activities of this medicinal plant are still largely unexplored. A literature survey on the *U. maritima* species indicates that no previous study that examines the antimicrobial and anticancer activities of extracts from fruits of *U. maritima*.

In this study, screening of *U. maritima* fruit extracts was conducted against two test bacteria (*Staphylococcus aureus* and *Escherichia coli*), one fungus (*Candida albicans*), and breast cancer MCF7 cells to determine the antimicrobial and anticancer activities of fruit extracts, and to study the gene expression of some cancer related gene markers including tumor suppressor genes (*p53* and *BRCA1*), antitumoral gene (*TNF*), anti-apoptotic gene (*Bcl2*), and immune response genes (*IL-2* and *IL-6*) by reveres transcription-polymerase chain reaction (RT-PCR).

Materials and Methods

Plant collection

The plant *U. maritima* was collected in March 2014 from Princess Tasneem bint Ghazi Technological Research Station in Al-Salt (Jordan), with coordinates: 32°05'N 35°40'E. The collected plant was identified by a taxonomist specialist Mr. Refad Khawaldeh at Jordan Royal Botanical Garden where a voucher specimen (Sharab 073/2014) is conserved for future reference.

Preparation of plant extracts

The fruits of *U. maritima* was collected and washed with distilled water, dried at room temperature and then grind using blender. Each 10 g powdered plant material was extracted by refluxing with 100 ml of different solvents (hot water, ethanol, methanol, and acetone) for two weeks at room temperature with shaking at 150 rpm. All samples were filtered through white cheesecloth paper and Whitman filter papers, size 11.0 cm. Thereafter, solvents were evaporated until dryness at room temperature. The crude extracts were dissolved in 0.05% dimethyl sulphoxide (DMSO) to a final stock concentration of 30 mg/ml. All extracts were purified by filtration through 0.22 μm filter units and kept at -20 °C until use.

Antimicrobial activity

Three test microorganisms, including; *Staphylococcus aureus* ATCC 25923 (Gram-positive bacterium), *Escherichia coli* ATCC 25922 (Gram-negative bacterium), and *Candida albicans* ATCC 10231 (Fungus) were used in this study.

Test bacteria and test fungus were grown in nutrient broth (NB) at 37°C for 24 h and in sabouraund dextrose broth (SDB) at 28°C for 48 h, respectively. Bacterial and fungal cultures were serially diluted and adjusted to achieve $2x10^6$ colony forming units (CFU/ml) for bacteria and $2x10^5$ spore/ml for fungi (Ceylan et al., 2008).

The antibacterial activity of plant extracts was screened in triplicates against test bacteria by using the agar well diffusion according to the method of Perez et al. (1990) using nutrient agar (NA) plates and incubation at 37°C for 24 h. The antifungal activity of plant extracts was performed in triplicates against test fungus in the same manner as

described for bacteria but by using sabouraund dextrose agar (SDA) plates and incubation at 28°C for 48 h. The antimicrobial activity was evaluated by measuring the inhibition zone diameter in mm around the wells.

Hemolytic activity

Human erythrocytes were freshly prepared and used to determine the hemolytic activity of *U. maritima* plant crude extracts. Hemolytic activity was tested on blood agar medium according to manufacturer's instructions (Oxoid, UK), supplemented with 5% normal human erythrocytes. A 50 µl of each crude extract was inoculated into each well (5 mm i.d.) prepared on blood agar plates. The type of hemolysis was determined after incubation of plates at 37°C for 48 h (Carrillo et al., 1996).

Mammalian cells and culture conditions

Two mammalian cell lines, including; normal cells (MCF10A; non-tumorigenic human breast epithelial cell line) and cancer cells (MCF7; human breast adenocarcinoma cell line) were kindly supplied from Faculty of Medicine/University of Jordan and used in this study. The human breast cancer cell line MCF7 was used to investigate the anticancer activity of plant crude extracts. Whereas, MCF10A cells were used to determine the selectivity of anticancer activity produced against MCF7 cells.

The adherent human breast cancer cell line MCF7 was grown in Dulbecco's modified Eagle's medium (DMEM), pH 7.4, supplemented with 10% feotal bovine serum (FBS), 40 µg/ml gentamicin, 50 µM 2-mercaptoethanol, 10 mM N-2-hydroxyethylpiperzine-N-2-ethane sulfonic acid (HEPES), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and it was harvested at ~70% confluence and subcultured every 48 h at 37^{o}C in a humidified 5% CO_2 incubator (Freshney, 2005). To harvest the adherent MCF7 cells, growth medium was removed and cells were washed with PBS. To produce a cellular suspension, a cell dissociation solution made of trypsin-EDTA (1X) was added and incubated at 37^{o}C for 5 min in a humidified 5% CO_2 incubator. Trypsinized cells were reseeded in fresh medium at ~10^5 cells/ml and incubated at 37^{o}C in a humidified 5% CO_2 incubator.

The adherent MCF10A cells were grown in DMEM/F12 media, pH 7.4, supplemented with 5% horse serum, 20 ng/ml of epidermal growth factor (EGF), 10 µg/ml insulin, 100 µg/ml hydrocortisone, 10 ng/ml cholera toxin, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were harvested, trypsinized, and reseeded in the same manner as for MCF7 cells.

Cytotoxicity screening assay

For screening assay, 100 µl of each non-hemolytic *U. maritima* fruit crude extract (400 µg/ml constant concentration) was added in fresh DMEM medium, mixed thoroughly by pipetting and inoculated into adherent MCF7 cells supplemented by new 100 µl medium and were loaded into each well of 96 well micro test plates. Cells were plated at a density 2x10^4 cells/well, counted by hemocytometer. The cultures were incubated at 37^{o}C in a humidified 5% CO_2 incubator for 72 h. At the end of incubation time, the viability of cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay (Heiss et al., 1997).

To determine the selectivity of cytotoxicity (killing cancerous cells specifically), the cytotoxic *U. maritima* plant crude extract was performed in the same manner for normal MCF10A cells but using DMEM/F12 medium.

***In vitro* cytotoxicity assay**

To measure the level of cell viability, the MTT test method was used according to Pledgie-Tracy et al. (2007). Briefly, cells (MCF7 and MCF10A) were cultured in 96-well plates (2x10^4 cells/well) containing 100 µl medium and were incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. After that, 100 µl fresh medium was added to the cells in the plates with another 100 µl medium containing different concentrations of *U. maritima* plant extracts (12.5, 25, 50, 100, 200, and 400 µg/ml). The *U. maritima* fruit extracts were added in triplicates and incubated for another 72 h. Cancer cells (untreated with extracts) were treated with different concentrations of the positive control Doxorubicin (12.5, 25, 50, 100, 200, and 400 µg/ml). Diluted *U. maritima* fruit extracts were freshly prepared in DMEM medium prior to each experiment. The metabolic activity of each well was determined by the MTT assay and compared to those of untreated cells (negative control). Negative control (all vehicle components except plant extract) was run in triplicates parallel to each assay. At the end of incubation, media was removed from each well and MTT dye solution (5 mg/ml) was dissolved in PBS and added as 20 µl/well in the plates. Plates were incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. After all, 100 µl of DMSO was added to each well, mixed thoroughly to dissolve the dye crystals, and immediately incubated at room temperature in the dark for 15 min. The absorbance was measured using microplate reader (TECAN, Austria) at 450 nm with a reference wavelength of 630 nm (Heiss et al., 1997).

Determination of median inhibitory concentration

The 50% inhibitory concentration (IC_{50}) was determined by comparing the average of mortality values of six crude concentrations (12.5, 25, 50, 100, 200, and 400 µg/ml) with that produced from negative control as well as with results of the positive control Doxorubicin. The IC_{50} values, regression equations, and correlation coefficients (R^2) were determined by using log-probit analysis (MS Excell, Microsoft Co., 2010). Each treatment was achieved in triplicate.

Mechanism of action determination

DNA fragmentation was performed according to Wang et al. (2004). MCF7 cells ($2x10^6$ cells/well) were exposed to the IC_{50} of non-hemolytic *U. maritima* fruit extracts for 6 h and 24 h. After incubation, cells were washed with PBS buffer, harvested by 1X trypsin, and pelleted by centrifugation. Cell pellets were subsequently resuspended in 600 µl of lysis buffer, and DNA was extracted using Promega Genomic DNA purification kit according to manufacturer's instructions (Promega, USA). Then 10 µl of DNA solution was transferred to 1.2% agarose gel and electrophoresis was carried out at 80 V for 2 h with 0.5X Tris-borate-EDTA (TBE) buffer as running buffer. DNA in the gel stained with ethiduim bromide was visualized under UV illuminator and the degree of apoptotic DNA fragmentation was determined by comparison with negative and positive controls.

RNA Extraction

The non-hemolytic extract from *U. maritima* fruits that exhibited a promising selective cytotoxicity with the best IC_{50} value was used to treat MCF7 cells separately for 6 and 24 h with the IC_{50} and the quadruple IC_{50}. About $5x10^6$ cells that were grown in DMEM medium at pH 7.4 and supplemented with 10% FBS were treated with plant extract in concentrations equivalent to IC_{50} and $4xIC_{50}$. The cells were washed with PBS buffer, harvested by using 1X trypsin, and then centrifuged to pellet cells. RNA was extracted from pellet cells using the RNA extraction Mini Kit according to manufacturer's instructions (Affymetrix, USA). Moreover, RNA was extracted from MCF7 treated with $1xIC_{50}$ and $4xIC_{50}$ of Doxorubicin drug (Abcam, UK). On the other hand, RNA was extracted from untreated MCF7 breast cancer cells (negative control) grown in DMEM and 10% FBS. The quality and quantity of total RNA was assessed by Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., MA, USA) and the purity of the RNA was assessed by the ratio of A260/A280. RNA samples were kept at -80ºC until use.

cDNA synthesis by reverse transcription PCR

The RNA samples were reversely transcribed to their complementary DNA (cDNA). The RT reaction consisted of 1 µg of total RNA, oligo (dT) primer (Promega, USA), and Improm-II Reverse Transcriptase (Promega, USA) in a total volume of 20 µl according to manufactures' instructions using the thermocycler, lifePro PCR System (BIOER, China). A negative control using Diethylpyrocarbonate (DEPC) treated water without RNA sample was used to check if there was any contamination. After that, the cDNA samples were kept at -20ºC until use. The quality and quantity of total cDNA samples were assessed by Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., MA, USA).

Gene expression analysis

The cDNA generated from RNA that extracted from the treated and untreated MCF7 cells was used as template to examine the expression level of six different genes (*P53*, *Bcl2*, TNF-α, IL-2, IL-6, and *BCRA1*). The gene *GAPDH* was employed as a housekeeping gene. The primers of these genes (Table1) were designed in this study using Primer3 design program (Koressaar and Remm, 2007; Untergasser et al., 2012) and using the available sequences for these genes in GenBank (Benson et al., 1999).

The PCR reaction consisted of 10 µl of 2_X i-MAX II Mix (Intron, Korea), 2 µl of the cDNA (50 ng/µl), 1µl of 10 pM/µl of each primer (Table 1), 1 µl of 25mM $MgCl_2$, and 5 µl of RNase free water in a total of 20 µl. Samples were spin before loading in the rotor's wells. The PCR program was run as follows: initial denaturation at 95 °C for 5 min.; 35 cycles of 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72 °C for 1.30 min, and final extension at 72 °C for 10 min. The thermocycler, lifePro PCR System (BIOER, China) was used. The PCR products were visualized using 2% agarose gel electrophoresis and electrophoresis was carried out at 100 V for 1.5 h with 0.5X TBE buffer as running buffer with 7.5 µl / 150 ml agarose gel RedSafe (Intron, Korea) nucleic acid staining solution.

Table 1: Oligo-nucleotide primer sequences designed in this study by Primer3 software

Gene	Sequence accession No.	Primer sequence	Annealing temperature (^{o}C)	Amplicon size (bp[a])
p53	AB082923.1	UM1F: 5'-GGCTCTGACTGTACCACCAT-3'	59.09	141
		UM1R: 5'-CACCTCAAAGCTGTTCCGTC-3'	59.13	
Bcl2	XM_006722523.1	UM2F: 5'-TTCTTTGAGTTCGGTGGGGT-3'	59.16	178
		UM2R: 5'-GCTGAAACTCCCTTAGCCCT-3'	59.38	
TNF	M26331.1	UM3F: 5'-GTCAACCTCCTCTCTGCCAT-3'	59.09	188
		UM3R: 5'-CCAAAGTAGACCTGCCCAGA-3'	59.02	
IL-2	S77834.1	UM4F: 5'-AACTCACCAGGATGCTCACA-3'	58.94	159
		UM4R: 5'-TGCTGATTAAGTCCCTGGGT-3'	58.33	
IL-6	JQ250825.1	UM5F: 5'-CCCTGACCCAACCACAAATG-3'	59.03	142
		UM5R: 5'-CTACATTTGCCGAAGAGCCC-3'	58.98	
BRCA1	NM_007294.3	UM6F: 5'-TGCAGCATTTGAAAACCCCA-3'	58.87	186
		UM6R: 5'-TGGCGCTTTGAAACCTTGAA-3'	58.89	
GAPDH	NM_002046.5	UM7F: 5'-AGGTCGGAGTCAACGGATTT-3'	59.03	168
		UM7R: 5'-TGACGGTGCCATGGAATTTG-3'	59.11	

[a]bp: Base pair

Results

Antimicrobial Activity

It was found that methanol and ethanol extracts of *U. maritima* fruits exhibited anticandidal activity and antibacterial activity against *S. aureus* and *E. coli* (Table 2). Whereas, aqueous and acetone extracts of *U. maritima* did not exhibit anticandidal activity but exhibited antibacterial activity against *S. aureus* and *E. coli*.

Table 2: Antimicrobial activity of extracts from *Urginea maritima* against human pathogenic microorganisms

Plant species	Plant part	Solvent	Inhibition Zone (mm)[a]		
			Staphylococcus aureus **ATCC 25923**	***Escherichia coli*** **ATCC 25922**	***Candida albicans*** **ATCC 10231**
Urginea maritima	Fruit	Water	21.33±1.67	25.67±1.86	0
		Ethanol	18.00±1.15	20.33±1.20	0
		Methanol	18.33±0.67	19.33±1.20	14.67±1.67
		Acetone	28.67±2.58	17.00±0.58	15.67±0.88

[a]Inhibition zone diameters are expressed as Means±SE.

Hemolytic and Anticancer Activities

The crude extracts of *U. maritima* fruits were tested for their hemolytic activity against human erythrocytes (Table 3). It was found that ethanol and acetone extracts exhibited non-hemolytic activity. Whereas, Aqueous and methanol extracts exhibited α-hemolysis.

Non-hemolytic *U. maritima* fruit extracts (ethanol and acetone extracts) was screened for their cytotoxicity against noncancerous MCF10A cells and breast cancer MCF7 cells (Table 3). The MTT assay was used to determine the degree of cytotoxicity of each crude extract. It was found that ethanol and acetone extracts of *U. maritima* exhibited selective cytotoxicity against breast cancer MCF7 cells (Table 3), killing MCF7 cells specifically. Both extracts exhibited low cytotoxicity on noncancerous MCF10A cells. Whereas, ethanol and acetone extracts produced high (+++) and very high (++++) cytotoxicities against cancerous MCF7 cells, respectively.

Table 3: Erythrocidal activity and selective cytotoxicity screening of non-hemolytic *Urginea maritima* fruit extracts against human noncancerous MCF10A cells and breast cancer MCF7 cells.

Property	Human cells	Solvent			
		Water	Ethanol	Methanol	Acetone
Type of Hemolysis[a]	**Erythrocytes**	α	γ	α	γ
Cytotoxicity degree[b]	**MCF10A**	-	+ (29.78)	-	+ (22.01)
	MCF7	-	+++ (88.96)	-	++++ (99.53)
Selectivity[c]		-	+	-	+

[a]Type of hemolysis: α; partial hemolysis, γ; no hemolysis. [b]The degree of cytotoxicity of nonhemolytic extracts was graded on the basis of the relative value of absorbance to the vehicle: ++++, very high (<0.1); +++, high (0.1 to <0.4); ++, moderate (0.4 to <0.7); +, low (0.7 to <0.9); ±, very low (0.9 to <0.95); -, non-toxic (≥0.95). The inhibition percentage was given in parentheses. [c]Selective cytotoxicity of a plant extract is when the plant crude extract has at most low cytotoxicity against MCF10A cells and has at least moderate cytotoxicity against breast cancer MCF7 cells.

As shown in Table 4, the IC_{50} values for the non-hemolytic ethanol extract of *U. maritima* against breast cancer MCF7 cells was 11.01 µg/ml. Whereas, IC_{50} values for the non-hemolytic acetone extract of *U. maritima* against breast cancer MCF7 cells was 6.01 µg/ml. While, Doxorubicin drug (positive control) exhibited cytotoxic effect with higher IC_{50} value (52.35 µg/ml).

Table 4: The median inhibitory concentration of non-hemolytic selectively cytotoxic *Urginea maritima* plant crude extracts against breast cancer MCF7 cells.

Plant	Plant part	Solvent	IC_{50}[a] (µg/ml)	R^{2}[b]	Regression equation[c]
Urginea maritima	Fruit	Ethanol	11.01	0.93	y = 48.59ln(x) + 47.993
		Acetone	6.01	0.91	y = 37.502ln(x) + 59.355
Doxorubicin	-	-	52.35	0.85	y = 48.562ln(x) + 23.692

[a]IC_{50}: the median inhibitory concentration. [b]R^2: correlation coefficient. [c]Y; inhibition percentage, X; inhibitory concentration at Y (X = IC_Y).

DNA fragmentation

DNA fragmentation assay was performed to detect DNA laddering (apoptotic feature) in MCF7 cells after treatment with *U. maritima* fruit extracts that exhibited selective cytotoxicity. As shown in Figure 1, DNA fragmentation was observed in MCF7 cells treated with non-hemolytic selectively cytotoxic ethanol and acetone extracts from *U. maritima* after 6 h and 24 h of incubation; the DNA patterns were consistent with nuclear fragmentation that occurs during apoptotic cell death. Furthermore, when comparing the DNA fragmentation results of MCF7 cells treated with *U. maritima* fruit extracts with the drug doxorubicin (positive control) and with negative control (untreated cells), *U. maritima* plant extracts tested in this study showed potential induction of apoptosis in breast cancer MCF7 cells (Figure 1).

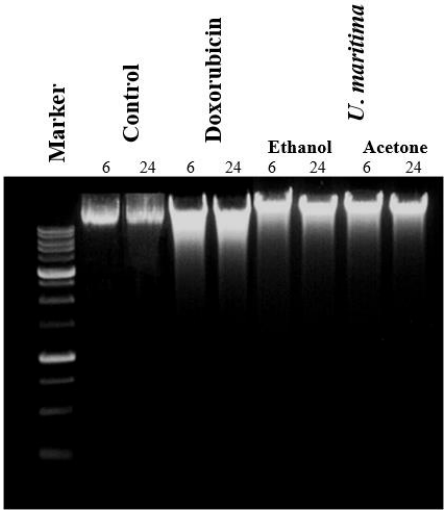

Figure 1: Agarose gel electrophoresis (1.2%) of DNA extracted from MCF7 cells treated with IC_{50} of non-hemolytic ethanol and acetone extracts of *Urginea maritima* fruits after 6 h and 24 h incubation. Marker; 1Kb molecular weight marker. Control; untreated MCF7 cells (negative control) after 6 h and 24 h. Doxorubicin; Doxorubicin treated cells (positive control) after 6 h and 24 h incubation.

Gene expression

The molecular mechanism induced by the most promising acetone extract of *U. maritima* fruits in inhibiting MCF7 breast cancer cells were investigated after treatment with equivalent volume to IC_{50} and to $4xIC_{50}$ concentrations for 6 h and 24 h. Analysis of gene expression of several apoptosis related genes (*p53*, *Bcl2*, *TNF*, and *BRCA1*) and immune response genes (*IL-2* and *IL-6*) as well as the house keeping gene *GAPDH* was achieved by RT-PCR technique (Figure 2).

Results presented in Figure 2A show the expression levels of different genes in treated cells with $1xIC_{50}$ and $4xIC_{50}$ concentrations of *U. maritima* fruit acetone extract at 6 h and 24 h. In comparison to cells treated with Doxorubicin (Figure 2B) and the untreated cells (Figure 2C), the expression level of *p53* increased in MCF7 cells subjected to acetone extract of *U. maritima* fruits and this expression induced with increasing the concentration of the extract and the incubation time (Figure 2A). Likewise, the expression of *IL-2* gene was higher in extract-treated and Doxorubicin-treated cells, and the expression induced with increase of the concentration and the incubation time (Figure 2). The expression of *IL-6* gene followed almost the same trend of *IL-2* expression. Furthermore, *BRCA1* gene was observed up-regulated in cells treated with $1xIC_{50}$ and $4xIC_{50}$ fruit extract at both incubation periods compared to negative control (Figure 2A and 2C). Likewise, the same result regarding the expression of *BRCA1* gene was obtained when cells treated with Doxorubicin drug at $1xIC_{50}$ and $4xIC_{50}$ but with slightly lower expression level than that observed in treated cells with acetone extract of *U. maritima* (Figure 2B). Compared to the negative control, the expression of *Bcl2* gene reduced in MCF7 cells treated with $1xIC_{50}$ acetone extract and in cells treated with $4xIC_{50}$ Doxorubicin and this expression decreased as the incubation time increased (Figure 2). The expression of *TNF* gene increased after 6 h and almost the same with moderate expression level in the untreated control cells and cells treated with acetone extract (Figure 2A and 2C). The expression of *TNF* gene was higher in the cells treated with $1xIC_{50}$ and $4xIC_{50}$ of Doxorubicin at 6 h compared to 24 h (Figure 2B). The expression level of the house keeping gene *GADPH* was showing the same trend in all treatments.

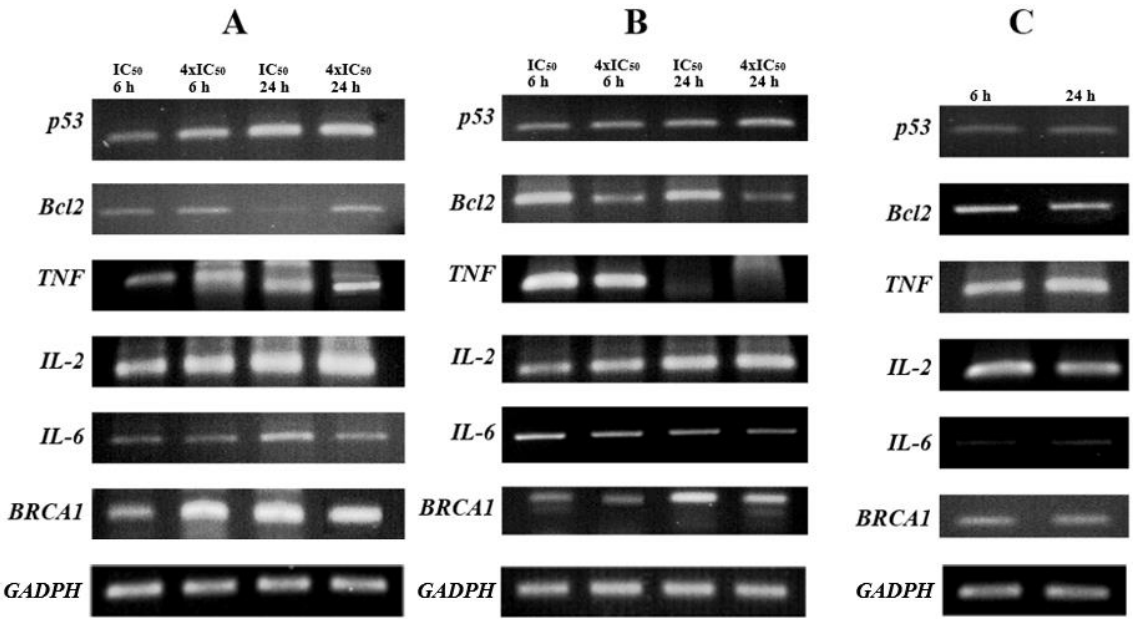

Figure 2: RT-PCR analysis of the expression of *p53*, *Bcl2*, *TNF*, *IL-2*, *IL-6*, and *BRCA1* genes as well as the house keeping *GADPH* gene in breast cancer MCF7 cells treated with $1xIC_{50}$ and $4xIC_{50}$ after 6 h and 24 h of incubation. **A**, *Urginea maritima* fruits acetone extract; **B**, Doxorubicin drug (positive control); **C**, Untreated breast cancer cells (negative control).

Discussion

Microbial resistance to conventional antibiotics has become a major healthcare problem (Alanis, 2005). There are considerable alternative sources of natural antimicrobials derived from medicinal plants with different mode of actions. Medicinal plants are rich in secondary metabolites of antimicrobial properties (Tiwari and Singh, 2004, Lewis and Ausubel, 2006). Many clinically useful antibiotics are derived from plants (Gilani and Atta-ur-Rahman, 2005). Therefore, there is an urgent need to discover new antimicrobial agents from unexplored plants which may produce secondary metabolites that can control serious bacterial and fungal infections.

Cancer is the second cause of death in the world and in Jordan after cardiovascular diseases (Tarawneh et al., 2010; WHO, 2012). Breast cancer is the second most common cancer in the world and the most frequent cancer among women (WHO, 2012). In Jordan, breast cancer was the most common among Jordanian females, with 36.5% of all cancers in women (Ministry of Health, Jordan Cancer Registry, 2013). Thus, there is an urgent need to explore natural

sources of anticancer agents such as plant secondary metabolites. Plants are used in the treatment of human diseases from earlier time. Therefore, a scientific interest for phytotherapy was increased in several medical fields including oncology. The use of plants in medicine has affected the identification of natural compounds for example cocaine, morphine, vinblastine, taxolo, and codeine (Balunas and Kinghorn, 2005). In this study, solid-liquid extraction was used to cover all range of phenolic compounds in *U. maritima* plant materials by using polar protic solvents (hot water, ethanol, and methanol) and polar aprotic solvent (acetone).

It was found that aqueous, ethanol, methanol, and acetone extracts of *U. maritima* fruits exhibited high antibacterial activity against test bacteria (Gram-positive bacterium *S. aureus* and Gram-negative bacterium *E. coli*). Methanol and acetone extracts from fruits of *U. maritima* exhibited promising antifungal activity against *C. albicans*. This is the first report on antimicrobial activity of fruit extracts prepared from *U. maritima*. On the other hand, it has been reported that the bulbs extract of *U. maritima* has some potential antifungal activity (Khan and Abourashed, 2010) and the leaves extract exhibited moderate to low antifungal activity (Abou-Jawdah et al., 2004).

The nonhemolytic *U. maritima* ethanol and acetone extracts screened for their selective cytotoxicity against noncancerous MCF10A and cancerous MCF7 cells. It was found that ethanol and acetone extracts of *U. maritima* fruit exhibited selective *in vitro* cytotoxicity against breast cancer cells by discrimination between cancer MCF7 cells and normal MCF10A cells, killing the former cells specifically. The *U. maritima* fruit ethanol and acetone extracts showed no hemolysis against human erythrocytes (Table 3). Thus, the anticancer activity in such plant extracts was not attributable to the induced hemolysis. Ethanol and acetone extracts of *U. maritima* fruits exhibited promising anticancer activity against breast cancer cells with the lowest IC_{50} value of acetone extract (6.01 µg/ml). According to the American National Cancer Institute (NCI), any crude extract possessing an IC_{50} less than 20 µg/ml is considered as active treatment against the tested cancer cells (Chen et al., 1988). Therefore, ethanol and acetone extracts of *U. maritima* fruits which had IC_{50} less than 20 µg/ml can be considered as an active treatment against breast cancer cells. This result may be related to the acute toxicity of this plant to target cancer cells (Mijatovic et al., 2007). This finding may lead to the use of these extracts for medicinal and pharmaceutical purposes including treatment of certain types of cancer specially breast cancer. There are no previous studies that demonstrate the potential anticancer activity for *U. maritima* fruit extracts. However, it was reported that the viability of neuroblastoma SH-SY5Y cells are selectively inhibited by aqueous extract of cardiotonic steroids from *U. maritima* bulb (Elghuol et al., 2016).

In order to investigate whether apoptosis may have played a role in mediating the cell death of MCF7 cells after treatment with non-hemolytic *U. maritima* plant extracts, DNA fragmentation analysis of cells treated with the active extracts revealed that those extracts had a significant effect on the DNA when compared with the positive control Doxorubicin (Figure 1). This result suggests a possible induction of apoptosis after treatment of MCF7 cells with active plant extracts. Doxorubicin is an antibiotic with cytotoxic action which is conducted by inhibition of Topoisomerase II that act as DNA intercalator to prevent synthesis of nucleic acid and induce apoptosis (Wang et al., 2004). Interestingly, moderate DNA degradation in treated cells after 6 h and 24 h had been observed (Figure 1) compared with that in untreated cells (negative control) and Doxorubicin drug (positive control). Therefore, the DNA patterns produced in this study were similar to that presented by Gao et al. (2002) who reported that resveratrol from plant extract induced DNA fragmentation in 32Dp210 leukemic cells. On the other hand, the obtained DNA fragmentation result in this study is contrary to the finding of Tanih and Ndip (2013) who reported that apoptosis induction was detected by conversion of intense DNA band to faint band with no DNA fragmentation.

It was reported that internucleosomal DNA fragmentation is not essential for apoptotic cell death, and that some necrotic cell death is accompanied by internucleosomal DNA fragmentation, suggesting the possibility that this fragmentation may not be sufficient as an indicator of apoptotic cell death (Cohen et al., 1992; Schulze-Osthoff et al., 1994). Moreover, active induction of apoptosis in cancer cells when treated by plant extracts is an attractive way to cure many types of cancers. However, cancers have various strategies to escape from the recognition and elimination by the surveillance of host immune system. These strategies include altered expression of genes and proteins involved in cell survival and death (Croce, 2008). Among them, one of common survival strategy of cancer cells is to escape from apoptosis by deregulation of apoptotic genes, or hyper-activation of anti-apoptotic genes (Fesik, 2005). For these reasons, induction of apoptosis in breast cancer MCF7 cells by the most active *U. maritima* fruit extract (acetone extract) was evaluated in this study at molecular level by analysis of gene expression of several apoptosis related genes (*p53*, *Bcl2*, *TNF*, and *BRCA1*) and immune response genes (*IL-2* and *IL-6*) as well as the house keeping gene *GAPDH* by RT-PCR technique to make sure that the mechanism of killing was occurred due to induction of apoptosis in MCF7 cells.

The expression level of the tumor suppressor gene *p53* in cells treated with acetone extract of *U. maritima* fruits was observed and compared with the negative control (untreated cells) and with the positive control (Doxorubicin). Figure 2 illustrated that the expression of *p53* was induced in MCF7 cancer cells treated with acetone extract. Under normal growth conditions, *p53* is a short-lived protein and is expressed at low levels within the cell. In response to various cellular stresses, including exposure to DNA-damaging agents, irradiation, hypoxia, and nucleotide depletion, *p53* is rapidly induced and functions as a transcriptional activator (Glaspy, 2002). In response to anticancer agents, damaged DNA triggers ATM kinase activity which catalyzes phosphorylation of *p53* protein. Accumulation of phosphorylated *p53* proteins in DNA-damaged cells activates two types of events cell cycle arrest and apoptosis induced (Jin et al., 2014). The p53 protein is a transcription factor that activates specific genes, including *p21*. The p21 protein, a member of the Cdk inhibitor family, blocks activation of the Cdk-cyclin complex, leading to cell cycle arrest too. Furthermore, p21 protein activates gene-encoding proteins that trigger cell apoptotic processes through binding and

inactivation of *Bcl2*, an apoptotic inhibitor (Jin et al., 2014). Moreover, the results are consistent with Alshehri and Elsayed (2012) who found that *p53* was induced after treatment of MCF7 cells with *Cichorium endivia* phenolic extract.

The anti-apoptotic gene *Bcl2* was down regulated in the treated cancer cells with both Doxorubicin and acetone extract of *U. maritima* fruits. The Bcl2 oncoprotein suppresses or delays the induction of apoptosis in prostate, skin, lymphoid tissues, and mammary gland (Yang et al., 2004). The obtained results are in agreement with that obtained by Alshehri and Elsayed (2012) who demonstrated reduction in expression of *Bcl2* in MCF7 cells treated with *C. endivia* phenolic extracts. Down regulation of Bcl2 after treatment of MCF7 cells by Doxorubicin could be attributed to the fact that Doxorubicin up regulate the expression of Bax protein and down regulate the expression of antiapoptotic Bcl2 proteins (Leung and Wang, 1999).

Treatment of MCF7 cells with acetone extract of *U. maritima* fruits and Doxorubicin drug lead to induction of expression of *TNF* gene after 6 h. However, the expression of *TNF* was reduced after 24 h (Figure 2). Recent studies (Szelényi, 2001; Brabers and Nottet, 2006) proved that the NF-κB factors, such as TNF, also serve as connecting links between inflammation and cancer. The TNF protein is released mainly from macrophages and regulates immune cells. Its dysregulation and overproduction lead to cancer and other diseases. The TNF protein also plays a role in the activation of NF-κB by binding to a TNF receptor present on the cell surface that in turn triggers a pathway that leads to the activation of apoptosis (Szelényi, 2001; Brabers and Nottet, 2006). Thus, the result of this study showed that apoptosis was induced at early stage (at 6 h) since *TNF* expression was reduced.

The expression of *IL-2* was so high in cells treated with *U. maritima* acetone extract and Doxorubicin when compared with that in the untreated control cells at 6 h and 24 h, in order (Figure 2). Interleukin-2 is a lymphokine that synthesized and secreted primarily by T helper lymphocytes that have been activated (Otsuki et al., 2010). Cytokines like IL-2 evaluated for possible use as immune stimulator in cancer patients, so IL-2 elicits as strong antitumor response in laboratory animals (Kleinsmith, 2006). At present, IL-2 is an approved treatment for advance cancers disease, such as advanced kidney cancer and melanoma (Kleinsmith, 2006). As well as, IL-2 is also being used experimentally to stimulate antitumor lymphocytes that are isolated from a patient's tumor site and grown in the laboratory prior to being injected back into the bloodstream (Kleinsmith, 2006). Therefore, induced expression of *IL-2* in this study suggests an enhanced immune response against cancer.

The expression of *IL-6* was almost the same in cells treated with IC_{50} and $4xIC_{50}$ concentrations of *U. maritima* extracts and Doxorubicin. Interleukin 6 is an interleukin that react as both a pro-inflammatory cytokine and an anti-inflammatory cytokine. In humans, IL-6 is secreted by T cells and macrophages to stimulate immune response, especially during infection and tissue damage leading to inflammation (Kleinsmith, 2006). The involvement of IL-6 in cancer has been quite controversial. It has two opposite faces in the tumor microenvironment; IL-6 has roles in both tumor-promoting and -suppressive activities by mobilizing T cell responses (Fisher et al., 2014). Therefore, the increased expression of *IL-6* in MCF7 cells might function as an antitumor factor.

Finally, *BRCA1* tumor suppressor gene was up regulated in cells treated with *U. maritima* acetone extract (Figures 2). The same result was obtained when cells treated with Doxorubicin drug. Recent study (Roy et al., 2012) revealed that the protein encoded by this gene is involved in repairing DNA damage, especially double strand damage. The results of this study agreed with the obtained result by Bosviel et al. (2012) who showed that soy phytoestrogens increase the expression of *BRCA1* tumor suppressor gene.

Conclusions

This is the first study to illustrate that fruit extracts of *U. maritima* exhibited antimicrobial and anticancer activities. Fruit extracts are considered to be a promising source of antimicrobials. The highest cytotoxicity against MCF7 cells was produced from acetone extract and possessed IC_{50} less than 20 µg/ml that can be considered as active treatment against breast cancer cells. Therefore, fruits of *U. maritima* are a hopeful source of biologically active metabolites that may be used in the development of future drugs for treatment of infectious diseases that caused by human bacterial and fungal pathogens as well as for treatment and/or improvement of current cancer therapies. Further studies concerning evaluation of *in vivo* cytotoxicity and determination of the chemical structure of the bioactive metabolites in fruit extracts are required to be used in medicinal and pharmaceutical applications.

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