ANTI-CANCER EFFECTS OF Zygophyllum album AND Suaeda palaestina EXTRACTS ON HUMAN LIVER CANCER CELL LINES

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Traditional medicines derived mainly from wild plants, still play major roles in the management of various diseases (Karunanayake and Tennekoon, 1993; Narayana and Dobriyal, 2000). Medicinal plants are currently used as raw materials for extraction of active ingredients which are used in the synthesis of several drugs including treatment of cancer disease (Rasool, 2012). In addition, the World Health Organization (WHO) has reported that about 80% of the world population relies on conventional medicine for their primary health care (Latif et al., 2014). Out of a total of 250,000 plant species present on earth, approximately one thousand showed anticancer activities (Bibi et al., 2012).

Cancer is one of the most dangerous diseases in humans and presently there is a considerable amount of new anticancer agents derived from natural products like medicinal plants (Sharma, et al., 2011). Globally, about 1 in 6 deaths is due to cancer, whereas cancer is the second leading cause of death worldwide, as well as it is responsible for an evaluated 9.6 million deaths in 2018 (WHO, 2018). The potential of using medicinal plants as anticancer drugs was recognized in 1950’s by U.S National Cancer Institute (NCI). Since 1950, major contributions have been made for finding out naturally existing anticancer drugs (Sharma et al., 2011). Moreover, about 60% of the actually used anticancer drugs have been extracted from natural products, mostly of plant origin (Latif et al., 2014).

Zygophyllum album is a halophyte shrubby plant belonging to Zygophyllaceae family Täckholm and Boulos, (1977) which includes about 27 genera and 285 species frequently restricted to arid and semiarid areas (Beier et al., 2003). Indeed, many plants belonging to this genus have anti-inflammatory, molluscicidal, and expectorant activities (Hassanean et al., 1993). Furthermore, Ksouri et al. (2013) reported that dichloromethane fraction of the halophytic species; Zygophyllum album had cytotoxic activity on cells viability particularly lung (A549) and colon (DLD-1) cancer cell lines. These finding resorted that these halophytic species have appreciable antitumour activity in the less polar extracts. Belmimoun et al. (2017) reported that Z. album had antioxidant, anti-inflammatory,
and antimicrobial activities. Besides, Z. *album* decreased significantly the rate of DNA fragmentation and the incidence of micronucleated polychromatic erythrocytes compared with doxorubicin treated group in mice (Ezz El-Din *et al.*, 2018).

*Suaeda palaestina* (*S. palaestina*) is a halophyte shrubby plant belonging to the Chenopodiaceae family, which is an annual herbaceous plant particularly abundant in the Mediterranean salt marshes. Hypoglycaemic and hypolipidaemic activities have been reported in this family. Patra *et al.* (2011) stated that *S. maritima* is a rich source of natural antioxidant with moderate antimicrobial activities. On the other hand, the dichloromethane extract of *Suaeda fruticosa* showed the highest anticancer activity against human lung carcinoma (A549) and colon adenocarcinoma cell lines (DLD-1, Caco-2 and HT-29) with specificity against DLD-1 (IC50 = 10±1 μg/ml). These findings demonstrated the remarkable potentiality of this edible halophyte as valuable source of antioxidants which exhibit original and interesting anti-inflammatory and anticancer capacities (Oueslati *et al.*, 2012).

The cyclin B1–CDK1 complex is a key regulator of mitotic entry (Nigg, 2001). A large number of proteins are phosphorylated by the cyclin B1–CDK1 complex prior to mitotic entry, which initiates the mitotic events, including nuclear envelope breakdown, centrosome separation, spindle assembly, and chromosome condensation (Heald and McKeon, 1990; Peter *et al.*, 1990; Nigg *et al.*, 1996). Inactivation of CDK1 kinase activity by degradation of cyclin B1 is required for both mitotic exit and DNA replication in the following cell cycle progression. Multiple layers of regulation of the mitotic events are linked to the control of the activity of the cyclin B1-CDK1 complex in a number of situations to make cells enter mitosis, arrest at G2-phase, or skip mitosis (Nakayama and Yamaguchi, 2013).

Apoptosis is a crucial pathway for regulating homeostasis, responding to DNA damage and controlling cell proliferation. Apoptotic cells including cancerous cells undergo a natural process of dying (Mahani *et al.*, 2015). The induction of apoptosis in cancer cells is one of the most helpful mechanisms for the development of drugs used in the treatment of cancer (Hu and Kavanagh, 2003). Apoptosis plays a crucial role in getting rid of the hyperproliferating mutated cells from the system.

Medicinal plants induce the process of apoptosis which is involved in different tumor suppressor genes including *p53* (Brady and Attardi, 2010). It also plays a critical role in the regulation of cell cycle arrest in the G2/M phase and is involved in DNA repair during activation of ribonucleotide reductase (Levine, 1997). Apoptosis could be stimulated by *p53* gene through down regulation of *Bcl-2* gene, and activation of caspases (Haupt and Haupt, 2004). It has been known that *p53* contributed to the transcriptional acti-
vation of large numbers of target genes (Mirzayans et al., 2012).

The present study aims to assess the effects of dichloromethane crude extracts of the halophyte *Z. album* (Zygophyllaceae) and *S. palaestina* (Chenopodiaceae), on in vitro human cancer cell lines growth. The possible underlying antiproliferation mechanisms were investigated, by studying the effects of *Z. album* and *S. palaestina* on cell cycle arrest, regulatory and apoptosis-related genes of the HepG2 cell line.

**MATERIALS AND METHODS**

1. **Plant collection and extract preparation**

Fresh whole plants were collected from South Sinai, and Borg El-Arab, Alexandria (Egypt), respectively, in March 2015 by Desert Research Center (DRC) Egypt. These halophytes were identified at the Flora and Phyto-Taxonomy Research Section, Horticultural Research Institute, Agricultural Research Center, Giza, Egypt. The plant materials were dried at ambient temperature and stored in a dry place prior to use. The plant samples were washed well with water, dried at room temperature in the dark, and then ground in an electric grinder to give a coarse powder. Shoot powders (50 g) were extracted by dichloromethane solvent. The powders were soaked in 150 ml solvent at room temperature for 48 h. The plant extracts were collected drop wise and filtered using Whatman No. 1 filter paper. The residues were soaked in 150 ml of solvent for 24 h and were filtered again. The extracts were then dried and finally placed in glass vials and stored at -20°C and the extracts were re-suspended in 1% dimethyl sulphoxide (DMSO) before testing (Moustafa et al., 2014).

2. **Cell lines**

Human hepatocellular carcinoma cancer cell line (HepG2) obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA, that was used in the present study. The cells, treated with half-maximal inhibitory concentration (IC50), were 27.74 μg/ml for *Z. album*, and 30.76 μg/ml for *S. palaestina* according to a pervious study (El-Attar et al., 2019).

3. **Cell cycle analysis by propidium iodide (PI) staining using flow cytometry**

To assess effect crude extract of *Z. album* and *S. palaestina* on the cancerous cell division in HepG2, the cells were treated with plant extracts and were digested with warm Trypsin-EDTA + warm Phosphate-buffered saline (PBS)-EDTA (0.25%) (500 μl + 500 μl) with incubation for 10 minutes at 37°C. The mixture was centrifuged at 450 rpm for 5 min, and then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cell pellet was re-suspended in 500 μl warm PBS, centrifuged and the supernatant was removed. A volume of 150 μl PBS + 350 μl ice-cold absolute ethanol (final cons. of EtOH is 70%) was added, mixed with a pipette, then vortexed
several times, and incubated at 4°C for 1 hour to fix the cells. To remove ethanol, the mixture was centrifuged at 350 rpm for 10 minutes and then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cell pellet was re-suspend in 500 μl warm PBS, centrifuged and the supernatant was removed. The pellet was re-suspended in 100 μl PBS and was stored at 4°C for up to 4 days. In darkness, the cells were stained with 100 μl of PI solution + 50 μl RNase A solution (100 μg/ml), and incubated in darkness for 30-60 min. The stained cells were read in Attune flow cytometer (Applied Bio-system, US).

4. Determination of the expression levels of regulatory and apoptosis-inducing genes

Total RNA was isolated from HepG2 cells using Gene JET RNA Purification Kit (Thermo Scientific, #K0731, USA) according to the manufacturer’s protocol. Total RNA (5 μg) was reverse transcribed using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA. The cDNA was used as a template to determine the relative expression of the apoptosis-related genes using StepOnePlus real time PCR system (Applied Biosystem, USA). The primers were designed by Primer 5.0 software (Table 1). The housekeeping gene β-actin was used as a reference to calculate fold change in target gene expression. A 25-μl PCR mix was prepared by adding 12.5 μl of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 μl of cDNA template, 1 μl forward primer, 1 μl reverse primer, and 8.5 μl of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40-45 cycles of amplification of DNA (denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec). At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, and relative gene expression was determined using $2^{-\Delta\Delta CT}$ method.

RESULTS AND DISCUSSION

1. Effect of Z. album and S. palaestina crude extract on cell cycle arrest utilizing Flow Cytometry

In a previous study (El-Attar et al., 2019) the results at the in vitro level, indicated that Zygophyllum album and Suaeda palaestina extracts induced anti-proliferative effects and high cytotoxicity on human cancer cell lines and no cytotoxic activity on normal cell lines. The half-maximal inhibitory concentration (IC50) observed in the human hepatocellular carcinoma (HepG2) was 27.74 μg/ml in Z. album, while IC50 in S. palaestina, it was 30.76 μg/ml. After realizing the high inhibition rate of HepG2 on cell viability by Z. album and S. palaestina, we tempted to assess possible cytotoxicity effects of both the tested extracts on cell cycle arrest using flow cytometry based on cell cycle
checkpoints. Comparison of 27.74 μg/ml of Z. album treated cells to the control group has shown that the cell cycle distribution of HepG2 cells was indeed affected (Fig. 1). The G0/G1 phase showed a reduction from 66% to 34%, and also the S-phase from 20% to 19% in the control and Z. album treated cells, respectively. On the other hand, the percentage of HepG2 cells at G2/M phase highly increased for Z. album treatment to be about 3.35 folds when compared to the control, as G2/M phase increased from 14% to 47%. The cell cycle distribution of HepG2 cells treated with 30.76 μg/ml of S. palaestina, was also affected. The G0/G1 phase decreased from 66% in control to 31% treated cells and the S-phase was reduced from 20% to 18% (Fig. 2). The percentage of G2/M phase in S. palaestina treatment to be about 3.6 folds when compared to the control in the same phase, as G2/M increased from 14% to 51%. These results showed significant accumulation of treated HepG2 cells in the G2/M phase, and confirmed that Z. album and S. palaestina have profound cytotoxic effects via induction of G2/M phase arrest of the cell cycle.

Many investigators have shown that different cytotoxic agents such as radiation and some medicinal plants can induce G2/M phase accumulation; G2/M arrest is followed by cell death. Multiple cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest (Bonelli et al., 1996; Sleiman and Stewart, 2000; Wu et al., 2006; Gismondi et al., 2013). Al-Sensoy et al. (2018) found that Atriplex halimus extract resulted in G2/M arrest and apoptosis in treated human hepatocellular carcinoma cell line (HepG2). Moringa oleifera, a medicinal plant, was found to induce G2/M arrest by increasing the expressions of genes related to G2/M such as p53, and p21. Furthermore, it stimulated apoptosis via decreased anti-apoptotic Bcl-2 expression, while it increased the expressions of caspases 3 and 7 (Gismondi et al., 2013; Adebayo et al., 2017).

2. Determination of the expression levels of cell cycle regulatory gene and apoptosis-regulatory genes.

2.1. Effect of Z. album and S. palaestina treatments on relative expression of Cyclin B1 and CDK1 regulatory genes in HepG2 cells

The expression levels of cyclin B1 and CDK1 genes in HepG2 were determined by quantitative real time-PCR. Figs (3 and 4) and Tables (2 and 3) showed that, compared to the control (untreated) HepG2 cells, the expression levels of cyclin B1 and CDK1 were down-regulated at a concentration equivalent to their IC50, Z. album (27.74 μg/ml) and S. palaestina (30.76 μg/ml) for 24 h. The results indicated that both Z. album and S. palaestina triggered G2/M arrest involved transcriptional suppression.

The cell cycle is mediated by the activation of a highly conserved protein kinase family, the cyclin-dependent kinases (CDKs) (Stewart et al., 2003). Cyclins can activate CDKs by forming complexes.
with Cdk5, and these cyclin/CDK complexes are cell cycle regulators. Among them, the cyclin B1/CDK1 complex, in which B-type cyclins associate with CDK1, is considered as one of the primary regulators of transition from the G2 to M phase. This complex was originally discovered and defined as the maturation-promoting factor or M phase-promoting factor (MPF) (Shangguan et al., 2014). The cell cycle arrests at the G2 phase and mitosis cannot occur without activation of the cyclin B1/CDK1 complex. In the present study, flow cytometric analysis showed that *Z. album* and *S. palaestina* caused G2/M phase cell cycle arrest, suggesting the occurrence of sequential events of cell cycle arrest followed by apoptosis.

### 2.2. Determination of the expression levels of apoptosis-regulatory genes

In order to determine the expression level of pro-apoptotic and anti-apoptotic genes in treated cells, the mRNA levels of *P53*, *caspase-3* and *Bcl-2* were evaluated by qRT-PCR. After 24 h treatment with IC50 concentrations of the *Z. album* and *S. palaestina*, *P53* expression was upregulated in HepG2 cells, with 1.78-fold and 2.87-fold, respectively, (compared to untreated control). In addition, *caspase-3* expression was upregulated in HepG2 cells, with 2.08-fold after *Z. album* treatment and 5.46-fold after *S. palaestina* treatment. On the other hand, the expression level of *Bcl-2* mRNA was markedly down-regulated in HepG2 cell lines after treatment by *Z. album* (0.24-fold) and *S. palaestina* (0.55-fold) (Tables 4 and 5 and Figs 5 and 6).

These results revealed that *Z. album* and *S. palaestina* enhanced the activity of pro-apoptotic *P53*, tumour suppressor gene; which is critically involved in cell cycle regulation, DNA repair, and programmed cell death. In addition, they stimulated the activity of *caspase-3*, which evidently serves as a marker of programmed cell death. On the other hand, anti-apoptotic *Bcl-2*, which contributes to cancer formation and progression by promoting the survival of altered cells, decreased at a concentration equivalent to their IC50. All the above indicating that both plants extract triggered the progression of apoptosis to circumvent the possible occurrence of carcinogenic effects.

Evaluation of *P53*, *caspase-3*, and *Bcl-2* expression is a common approach used for the analysis of apoptosis upon treatment with related compounds. This was in partial agreement with the results reported by Al-Senosy et al. (2018) and Mitupatum et al. (2016).

Finally, based on the aforementioned results, the crude extracts of *Zygophyllum album* or *Suaeda palaestina* proved to have anti-proliferative effects and induced cell cycle arrest at the G2/M phase and apoptosis in human cancer cell lines. These data suggest that both extracts could be considered as new effective and promising herbal therapeutic agent for human cancer treatment.
SUMMARY

The present study aims to assess the anticancer activity of the extracts of the two medicinal plants Zygophyllum album and Suaeda palaestina. In a previous study, human liver cancer cell lines (HepG2) showed the cytotoxicity of the two plants by MTT assay. The half-maximal inhibitory concentration (IC50) observed in the HepG2 was 27.74 μg/ml in Z. album, while IC50 of S. palaestina was 30.76 μg/ml. According to IC50 of both plants, the flow cytometric analysis was investigated and indicated that the treatment of HepG2 cells with Z. album and S. palaestina led to substantial elevation of G2/M phase cell cycle arrest. The real time-PCR was used to measure the mRNA levels of cyclin B1 and cyclin dependent kinase (CDK1) genes. Both genes showed down-regulation of expression levels in HepG2 with Z. album and S. palaestina compared to the untreated cells. The apoptotic mechanism activated by both plant extracts resulted in up-regulations of p53 and Caspase 3 and the down-regulation of Bcl-2 on mRNA levels on HepG2 after treatment with both extracts. In conclusion, these results suggest that Z. album and S. palaestina could be considered as candidate species being a good natural source of anticancer agents.

REFERENCES


Oueslati, S., R. Ksouri, H. Falleh, A. Pichette, C. Abdelly and J. Legault (2012). Phenolic content, antioxidant, anti-inflammatory and anticancer activities of the edible halo-


Table (1): Forward and reverse primers sequence for *Bcl-2*, *caspase 3*, *p53*, *CycB1*, *CDK1*, and *β-actin* genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′ ------ 3′)</th>
<th>Reverse primer (5′ ------ 3′)</th>
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<tr>
<td>Bcl2</td>
<td>AGGAAGTGAAACATTTCCGGTGAC</td>
<td>GCTCAGTTCCAGGACCAGGC</td>
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<tr>
<td>Caspase 3</td>
<td>TTAATAAAGGTATCCATGGAGAACT</td>
<td>TTAGTGATAAAAATAGAGTTCTTTTGAG</td>
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<tr>
<td>p53</td>
<td>TAACAGTTTCTGATGGGCACC</td>
<td>AGGACAGGCACAAACACGCACC</td>
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<tr>
<td>CycB1</td>
<td>CTCTGCTCTGGTGAGAGGA</td>
<td>CTGATCCAGAATAACACCTGA</td>
</tr>
<tr>
<td>CDK1</td>
<td>TTTCAGAGCTTTGGGCACCT</td>
<td>CCATTTTGCCAGAAAATTCCGT</td>
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<tr>
<td>β-actin</td>
<td>CACCAGCTGGAGCAGCAT</td>
<td>ACAGCCTGGATAGCAACG</td>
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Table (2): Relative expression of *cycB1* and *CDK1* genes in HepG2 cells following treatments with *Z. album* using qRT-PCR.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fold change mean</th>
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<tbody>
<tr>
<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.04</td>
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<tr>
<td><em>cycB1</em> gene</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.05</td>
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<tr>
<td><em>CDK1</em> gene</td>
<td>0.11 ± 0.01</td>
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Table (3): Relative expression of *cycB1* and *CDK1* genes in HepG2 cells following treatments with *S. palaestina* using qRT-PCR.

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<td>Control (untreated) HepG2 cells</td>
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<td><em>cycB1</em> gene</td>
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<td>Control (untreated) HepG2 cells</td>
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<tr>
<td><em>CDK1</em> gene</td>
<td>0.36 ± 0.04</td>
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Table (4): Relative expression levels of *P53*, *Bcl-2*, and *Caspase 3* genes in HepG2 cells following treatments with *Z. album*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fold change mean</th>
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<tbody>
<tr>
<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td><em>P53</em> gene</td>
<td>1.78 ± 0.09</td>
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<tr>
<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.06</td>
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<tr>
<td><em>Bcl-2</em> gene</td>
<td>0.24 ± 0.02</td>
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<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td><em>Caspase 3</em> gene</td>
<td>2.08 ± 0.14</td>
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</table>
Table (5): Relative expression levels of $P53$, $Bcl-2$, and $Caspase 3$ genes in HepG2 cells following treatments with $S.\ palaestina$.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fold change mean</th>
</tr>
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<tbody>
<tr>
<td>Control (untreated) HepG2 cells</td>
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<tr>
<td>$P53$ gene</td>
<td>2.87 ± 0.12</td>
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<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.06</td>
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<tr>
<td>$Bcl-2$ gene</td>
<td>0.55 ± 0.04</td>
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<tr>
<td>Control (untreated) HepG2 cells</td>
<td>1.00 ± 0.09</td>
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<tr>
<td>$Caspase 3$ gene</td>
<td>5.46 ± 0.32</td>
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Fig. (1): Effect of $Z.\ album$ on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with $Z.\ album$ at the concentration of 27.74 $\mu$g/ml in order to check the cell cycle distribution, then analyzed by flow cytometry.
Fig. (2): Effect of *S. palaestina* on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with *S. palaestina* at the concentration of 30.76 µg/mL in order to check the cell cycle distribution, then analyzed by flow cytometry.

Fig. (3): Effect of *Z. album* on cell cycle regulatory genes at G2/M phase after exposure to 27.74 µg/ml, mRNA expression of *cycB1* and *CDK1* genes were assessed by quantitative RT-PCR. *P < 0.05, compared to the control group.*
Fig. (4): Effect of *S. palaestina* on cell cycle regulatory genes at G2/M phase after exposure to 30.76 μg/ml, mRNA expression of *cycB1* and *CDK1* was assessed by quantitative RT-PCR. *P < 0.05, compared to the control group.

Fig. (5): Effect of *Z. album* on apoptosis-related genes after exposure to 30.76 μg/ml, mRNA expression of *P53, Bcl2, and Caspase 3* genes were assessed by quantitative RT-PCR. *P < 0.05, compared to the control group.

Fig. (6): Effect of *S. palaestina* on apoptosis-related genes after exposure to 30.76 μg/ml, mRNA expression levels of *p53, Bcl2, and Caspase 3* was assessed by quantitative RT-PCR. *P < 0.05, compared to the control group.