The Anticancer Activity of Artemisia Judaica Crude Extract in Human Hepatocellular Carcinoma HepG2 Cells by Induction of Apoptosis and Cell Cycle Arrest

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ABSTRACT

Objective: The present study is focused on the anticancer activity of the methanolic extract of the medicinal plant, Artemisia Judaica, on Human hepatocellular carcinoma (HepG2) and normal liver (THLE2) cell lines, as well as the mechanisms involved were also investigated.

Methods: The collected aerial parts of the plant were extracted by maceration with methanol and the crude extract was collected and stored until use. Cytotoxicity and cell proliferation, cell cycle analysis and the expression level of apoptosis-related genes such as p21, Cyclin B1, Cyclin-dependent kinase 1 (CDK1), p53, Bcl-2 and Bax in HepG2 cell lines were estimated using the MTT colourimetric, flow cytometry and quantitative real-time PCR (qRT-PCR) assays, respectively.

Results: The results showed that Artemisia Judaica extract (IC50 = 33.76μg/ml) displayed strong cytotoxicity and antiproliferative effect in HepG2 cancer cells. On the other hand, this extract exhibited no cytotoxic activity on the liver normal cell line (THLE2). Flow cytometric analysis of propidium iodide staining revealed that the treatment of HepG2 cells with Artemisia Judaica led to an increase in G2/M phase cell cycle arrest. The qRT-PCR assay revealed that both cyclin B1 (Cyclin B1) and cyclin-dependent kinase (CDK1) genes, as well Bcl-2 showed down-regulation expression levels in HepG2 treated with Artemisia Judaica compared to the untreated cell line. Furthermore, the apoptotic mechanism activated by the plant extract resulted in up-regulation of p53, P21 and Bax at mRNA level on HepG2 cell line.

Conclusion: These results suggest that Artemisia Judaica could be a promising candidate species as a natural source of anticancer molecules.

Key Words: Artemisia Judaica, Antiproliferation, Apoptosis, Anticancer, HepG2 cell line

INTRODUCTION

Cancer is one of the major causes of death worldwide. It is characterized by genetic alterations of normal cells which become malignant cells. Those are characterized by uncontrolled cell growth, immortality, invasiveness, and the ability to form distant metastasis. It was found that natural products may interfere with the carcinogenesis process by altering the behaviour of tumour cell and targeting cancer cells signalling pathways.¹

Among different cancer types, liver cancer is the 4th most common cause of death from cancer worldwide, the incidence of human hepatocellular carcinoma (HCC) is increasing particularly in males in some countries. Hepatitis B and C viruses (HBV and HCV) and dietary aflatoxin intake remain the major causative factors of HCC, where different modes of cancer therapy for HCC have been tried such as surgery, chemotherapy, radiotherapy, as well, tremendous works have been done at the molecular level.² The use of natural products and supplements of medicinal plants has very increased over the past three decades with more than 80% of people worldwide depend on them for some part of primary healthcare.³ The diet enriched with naturally occurring substances significantly reduces the risk for some cancers. Consequently, many drugs used for the
treatment of cancer have been discovered from medicinal plants.\(^4\)

Biologically active components of the medicinal plants target tumour cells by different mechanisms, resulting in angiogenesis, inhibition of carcinogenesis, cell cycle arrest, oxidative stress, autophagy or differentiation and apoptosis.\(^5\)\(^-\)\(^9\) *Artemisia* was found to possess sesquiterpene lactones and other derived phytochemicals as active components. Sesquiterpene lactones were used for their therapeutic and other properties.\(^10\) Recently, monoterpens, sesquiterpenes, sesquiterpene lactones, flavonoids, coumarins, sterols, polycyclic ethers have been isolated from *Artemisia* species.\(^11\) Some *Artemisia* species have shown that it possesses medicinal properties such as anti-bacterial and anti-cancer effects.\(^12\) Few species of *Artemisia* such as *Artemisia Judaica*, showed promising anticancer activities against the proliferation of cancer cell lines.\(^13\) The main effect of its essential oils is attributed to the main constituents, the thujone, in this species,\(^14\) enriched with fraction has potential anticancer activities.\(^15\)

*A. Judaica* (Arabic name, Shih Balady) is a perennial fragment shrub that is abundant in North Africa and Middle Eastern countries.\(^16\) As well in Saudi Arabia, Yemen and Egypt.\(^17\)\(^-\)\(^19\) This plant grows abundantly in different parts of the Arabian peninsula such as Saudi Arabia and Yemen,\(^17\)\(^18\) and has been used in traditional Egyptian medicine for the treatment of gastrointestinal disorders.\(^19\) Besides, the *Artemisia* species have been used in Iranian traditional medicine as an anti-infectious, anti-bacterial, gastric tonic, digestive and stomachic.\(^20\) Isolated compounds from *A. Judaica* have exhibited antiviral, antibacterial, antifungal, and cytoprotective effects.\(^21\)\(^-\)\(^23\) and for the treatment of hepatitis, cancer and menstrual-related disorders.\(^24\) The composition of *A. Judaica* includes artemisinic acid, sesquiterpene lactones, methyl wormwood, artemisinic alcohol, eucalyptol, *Artemisia* ketone, camphor, caryophyllene, piperitone and essential oil.\(^25\)\(^-\)\(^26\) Besides, diverse chemical components, such as flavonoids, coumarins, sterols, polycyclic ethers, monoterpenes, polyphenols, sesquiterpenes and sesquiterpene lactones, have been found in plants from the *Artemisia genus*.\(^22\)\(^-\)\(^24\)

Sesquiterpene lactone is also a potent apoptotic inducer in cancer cells via multiple pathways. It is readily depleted intracellular glutathione (GSH), disrupts cellular redox balance, depletes intracellular thiols,\(^27\) triggers an intracellular reactive oxygen species (ROS),\(^28\) decreases anti-apoptotic Bcl-2 protein expression and induce apoptosis.\(^29\) Studies of the molecular mechanisms have shown that caspase and p53-independent activation, downregulation of Bcl-2, generation of ROS, reduction of mitochondrial membrane potential, increased amount of Bax protein, arrest of the cell cycle at G2/M-phase, suppression of the Notch 1, and inhibition of NF-κB are important mechanisms for the cytotoxic effects of *Artemisia*.\(^30\) As well, flow cytometric analysis for cell cycle revealed increment of G2/M phase cell cycle arrest after treatment of HepG2 cells with *A. herba alba* extract. The apoptotic mechanism was also activated by the crude extract of *Artemisia* included up-regulation of p53 and Bax and down-regulation of Bcl-2 expression levels with no cytotoxic effect for normal cell lines exposed to the plant extract.\(^31\)

Besides, *Artemisia* extract increased the number of cells in the G2/M phases, followed by caspase 3 upregulation, and apoptosis. Further, it is inhibited cancer cell proliferation and induced apoptosis.\(^32\) Therefore, the present study is designed to evaluate the anticancer activity of crude extract of *A. Judaica*, on human hepatocellular carcinoma (HepG2) cell line. The possible underlying antiproliferation mechanisms were investigated, by studying the effect of *A. Judaica* on cytotoxicity, cell cycle arrest and apoptosis-related genes in the human hepatocellular carcinoma (HepG2) cell line.

### MATERIALS AND METHODS

#### Plant collection and extract preparation

The aerial parts (leaves and stems) of *A. Judaica* were collected during the summer season (July 2020) from Wadi Gharandal, South Sina, Egypt. Taxonomical identification was confirmed by a botanist and voucher specimen, were deposited in the Herbarium of the National Research Center, Dokki, Cairo, Egypt. Herbs were washed and shade dried for a week and was milled to fine powder, then it was extracted with methanol at a ratio of 20 g dry powder in 200 ml of ethanol for 48 h using the maceration method. The liquid extract was filtered and concentrated under vacuum by soxhlet extraction, then stored in the dark at 4°C until use.

#### Assessment of cytotoxic and anti-proliferous activities of *A. Judaica*

Cell proliferation and viability of the cells were estimated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colourimetric assay. Human hepatocellular carcinoma (HepG2) and normal liver (THLE2) cell lines in exponential growth phase were seeded at a density of 1 x 10^4 cells per well (100 μl/well) onto a 96-well plate (Falcon, Franklin Likes, NJ, USA) in DMEM medium (GIBCO, Grand Island, New York, USA; Cat.no.A1049101). The cell density was adjusted by the trypan blue exclusion method. The whole compounds were cultured in different concentrations (ranged from 0 to 200 µg/mL) for 24 hours at 37 °C in a 5% CO\(_2\) with a 95% humidity incubator. Besides, different concentrations of cisplatin as a reference chemotherapeutic drug were added and the microplates were incubated for a further 48 hour in DMEM medium (200 µL). The medium was washed gently twice with ice-cold PBS and a volume of 200 µL MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl
tetrazolium bromide, a yellow tetrazole, (Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154)] was added to each well. The microplate was incubated at 37 °C for another 4 hours in a CO₂ incubator. About 180 μL medium/MTT was removed and 100 μL of acidified isopropanol were added per well to solubilize the formazan produced. Finally, the microplate was incubated with shaking for 15 minutes. The absorbance of each well was measured at 630 nm using a microplate reader (ELX800, Biokit, Spain). Assays were performed in triplicate on three independent experiments. Sigmoidal and dose-dependent curves were constructed to plot the results of the experiment. The concentration of the compounds inhibiting cancer cell growth by 50% of the control level (IC₅₀) was calculated using this sigmoidal curve.

**Cell cycle analysis by flow cytometry**

To evaluate the effect of *A. Judaica* crude extract on the cancereous cell division of HepG2, the cell cycle analysis was carried out using the protocol of Applied Bio-system, USA. Cells were digested with warm Trypsin-EDTA + warm 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 70% ethanol was added 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 cells were re-suspended in 500 μl warm PBS, centrifuged and the supernatant was removed. The cells were re-suspended in 100 μl PBS and stored at 4°C for up to 4 days. In the darkness, the cells were stained with 100 μl of propidium iodide (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, USA).

**Determination of the expression levels of apoptosis-regulatory genes by quantitative real-time PCR**

RNA extraction was carried out utilizing an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) for HepG2 cell lines, or treated with IC₅₀ of *A. Judaica* extract for 24 h. The procedure was performed according to the manufacturer’s instructions. The cDNA was synthesized from purified RNA with an RT2 First Strand Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s guidelines as the template for RT-qPCR. Corbett Rotor-Gene 6000 (Qiagen, Inc., Valencia, CA, USA) was used to perform quantitative real-time reverse transcriptase PCR (qRT-PCR). A final volume of 25 μl pre-mix was prepared to contain 12.5 μl of RT2 SYBR R Green ROXTM FAST master mix (Qiagen, Inc., Valencia, CA, USA), 1 μl of primers (RT2 qPCR Primer Assays, Qiagen, Inc., Valencia, CA, USA), 1 μl of cDNA, and 10.5 μl RNase-free water to make the final volume. The following primer pairs for target genes and the housekeeping β-actin gene was chosen from the Primer Bank website.

The default PCR conditions were as follows: the PCR plate was run at 95°C for 10 min to activate the enzyme, 40 cycles of 15 s at 95°C (denaturation) followed by 30 s at 60°C (annealing and synthesis). Finally, the dissociation curve was constructed immediately after the PCR run to check and verify results. The housekeeping gene β-actin was used as a reference to calculate fold change in target gene expression.

**Statistical Analysis**

Data were expressed as means ± standard error (SEM). One way ANOVA using SPSS 18.0 software was used to detect the significant difference. Values were considered statistically significant when P≤0.05. Comparison of means was carried out with Tukey’s HSD test.

**RESULTS**

**Cytotoxic activity of *A. Judaica* crude extract using MTT assay**

The cytotoxic activity of *A. Judaica* crude extract was screened on human hepatocellular carcinoma cell line (HepG2) by MTT assay (Fig. 1). The results revealed that *A. Judaica* extract (IC₅₀ = 33.76μg/ml), inhibited the proliferation and exhibited increasing in cytotoxic activity to HepG2 cell line in a dose-dependent manner, where, the percentage of cell viability declined up to 20% by *A. Judaica* dose increment, while the crude extract of *A. Judaica* didn’t induce growth inhibitory effect on normal liver cell line (THLE2) (IC₅₀ = 1041μg/ml), where, a very low cytotoxic activity was noticed by *A. Judaica* against normal liver cell line.

**Effect of *A. Judaica* crude extract on cell cycle arrest using flow cytometry**

The DNA content in *A. judaica* treated HepG2 cells was assessed using the cell cycle phase distribution analysis. The cell cycle arrest was analyzed using flow cytometry (Fig. 2). According to the inhibition rate of liver cancer (HepG2) cell viability, the results revealed that the anti-proliferative effect of *A. Judaica* was associated with cell cycle arrest using flow cytometry-based cell cycle distribution (Table 1). Compared to the control group, the treatment with *A. judaica* crude extract at 33.76 μg/ml affected the cell cycle distribution on HepG2 cells, where, the G0/ G1 phase showed decreased from 60% to 29% of cell cy-
The expression level of apoptosis-related genes modulated by A. Judaica

To evaluate the molecular mechanism of A. Judaica-induced apoptosis in HepG2 cells, the expression level of apoptosis-related genes such as p21, cyclin B1 (CycB1), cyclin-dependent kinase (CDK1), p53, Bcl-2 and Bax in HepG2 cells (Fig. 3) was estimated by quantitative real-time PCR (qRT-PCR). Results revealed that compared to the untreated HepG2 group, the expression level of p21, p53 and Bax was increased (upregulated) in A. Judaica treated HepG2 cell line, whereas, the expression level of Bcl-2, CycB1 and CDK1 genes was down-regulated (decreased) in HepG2 treated with A. Judaica compared to the untreated HepG2 cell line.

DISCUSSION

Medicinal herbs have shown their pharmacological activities as a new drug reaching the market. Different forms of cancer are constantly gaining resistance to current drugs, creating a need for the discovery of new drugs. A. Judaica extract showed remarkable cytotoxic activity against the tested liver cancer cell lines in a dose-dependent manner. This could be due to the presence of artemisinin and other sesquiterpene lactones, which have been found in A. Judaica. Artemisinin which is a sesquiterpene lactone demonstrated antitumor agent and anticancer activity when tested in vitro and in vivo.

These results agree with previous studies reporting biologically active compounds from the medicinal plant A. Judaica possess sesquiterpene lactones, essential oils and other derived phytochemicals as active constituents which have shown medicinal properties such as anticancer activities (cytotoxicity and antiproliferation) for cancer cell lines, with no cytotoxic effect for normal cell lines exposed to the plant extract.

The cell cycle arrest was analyzed using flow cytometry-based cell cycle distribution. The results indicated that A. Judaica extract inhibited the liver cancer (HepG2) cell cycle through triggering G2/M phase arrest involved transcriptional suppression, where the percentage of HepG2 cells at the G2/M phase was increased and reached about 50% (49% ± 2.08) compared to 21% for the untreated cell line. That coincided with Lang et al., who found that Artemisia extracts induced accumulation of multinucleated cancer cells within 24 h of treatment, increased the number of cells in the S and G2/M phases of the cell cycle, followed by loss of mitochondrial membrane potential, caspase 3 activation, and formation of an apoptotic cell population. These data indicated that A. Judaica exhibited an antiproliferative effect by a cell cycle blocking at the G2/M phase and apoptosis mediated cytotoxicity in carcinoma cells. However, Honda et al., found that equipotent concentrations of A. judaica extract highly arrested the cell cycle in the G0/G1 phase of cancer cell lines.

The current study inspected the ability of A. judaica crude extracts to induce apoptosis where it is known to be the most promising pathway for a cancer therapy strategy. Consequently, results showed that the extract had the highest cytotoxic activity, have ingredients or molecules involved in the activation mechanisms of one or more antiproliferative pathways. A. Judaica medicinal plant induces apoptotic process which is modulated by different tumour suppressor genes including p53. The P53 induces apoptotic cell death by direct or indirect change expression of the Bcl-2 family of proteins, Bcl-2 and Bax. The Bcl-2 gene is an anti-apoptotic gene that suppresses initiation steps of apoptosis via inhibition of the pro-apoptotic proteins. P53 may modulate susceptibility of cells to apoptosis by downregulation of BCL2 and causing up-regulation of BAX and that coincides with our findings.
CONCLUSION

Based on the findings of the study, the crude extract of A. Judaica was found to have anticancer properties specific for human tumour cells, without any toxicity on normal cells. These results indicated that A. Judaica has an antiproliferative effect on HepG2 cell lines. Moreover, A. Judaica crude extract evaluated to identify the mechanisms behind the toxicity such as cell cycle blocking at the G2/M phase using flow cytometry. The real time-PCR to measure the mRNA levels of cyclin B1 and cyclin-dependent kinase (CDK1) genes (cell cycle regulator genes) and Bcl2, where those genes showed down-regulation expression levels in HepG2 treated with A. Judaica compared to the untreated cell line. Gene expression of ribonucleic acid (RNA) study confirms A. Judaica extract to induction of apoptosis via activation of p53, p21 and Bax and inactivated of Bcl2. Taken together, these data suggest that A. Judaica could be a promising candidate species as a source of anticancer molecules. Finally, natural products such as A. Judaica are attractive sources for the development of new medicinal and therapeutic agents. Those with anticancer potential and apoptosis may be more selective and have weaker adverse effects on normal cells compared to chemotherapy used for cancer treatment. So, these research trends indicate that natural products will be among the most important sources of new drugs for cancer prevention and treatment in human beings.

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Table 1: Forward and reverse primers sequence for candidate genes

| Gene | Forward primer | Reverse primer |
|------|----------------|---------------|
| Bcl2 | AGGAAGTGAACTTCTCGGTGAC | GCTCAAGTCCTCAGGACCG |
| p21 | AGTGGACCTGGAACCTCACAG | TCTCTTGGAGAGATCAGCG |
| p53 | TACAGTCTCTGATGGGGGC | AGGACAGCAGCAACACGCAC |
| CycB1 | CTCTGTGATGTTGGAGGA | CTGATCCAGATAAACCTGA |
| CDK6 | TTGTCAGAGCTTGGGGA | CCATTGTCGAAATAGCT |
| Bax | TTTTGCTCAGGTTTTCATC | CAGGTGAATTTGCGCTGCA |
| β-actin | CACCAACTGGAGACGACAT | ACAGCCTGGATAGCAAC |

Table 2: Effects of A. judaica extract on the cell cycle distribution of HepG2 cell lines, compared with HepG2 cells alone. Values are presented as means ± SEM

| Groups | % of cells arrested in each cell cycle phase |
|--------|--------------------------------------------|
|        | Go/G1 phase (Mean± SEM) | S phase (Mean± SEM) | G2/M phase (Mean± SEM) |
| HepG2  | 60% ± 1.40 | 19% ± 1.01 | 21% ± 1.56 |
| HepG2 (A. judaica treated) | 29% ±1.62 | 22% ±1.63 | 49% ± 2.08 |
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Figure 1: Dose-response curve of different extracts of A. judaica in HepG2 (left) and liver normal THLE2 (right) cell lines. Cell viability was determined using MTT assay and data are expressed as mean±SEM, n = 3.

Figure 2: Flow cytometry analysis for the effect of 33.76μg/ml A. judaica treatment on the hepatocellular carcinoma cell cycle arrest. (a): HepG2 and (b): HepG2 treated with A. Judaica cell lines.

Figure 3: Effect of A. Judaica extract on the expression level of apoptosis-related genes, p21, CycB1, CDK1, p53, Bcl-2 and Bax in HepG2 cell lines, compared to untreated HepG2 cells.