Pharmacogn J. 2021; 13(6)Suppl: 1664-1672
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Research Article

Cytotoxic Activities of Phytochemical Components from Ethanol Extract of Ajwa Date on Human Hepatoma Cancer Cells *in Vitro*

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a primary liver cancer that occurs and develops in the liver and is among the top frequent cancer-related death worldwide. Currently, clinical treatment options can control the HCC cancer, but, in some cases, it develops resistance to standard therapies and does not respond to these treatment options. Date palm (*Phoenix dactylifera L.*) is used in traditional and alternative therapies for its various health benefits. **Objective:** The present study aims to evaluate the anticancer and cytotoxic effects of Ajwa date ethanol extract (ADX) on hepatocarcinoma (HepG2) cells. **Materials and Methods:** The polyphenolic constituents of ADX were analysed using HPLC to identify the major polyphenols in the extract. The cell proliferation and viability percentages were examined through Trypan blue dye and MTT assay. Additionally, DNA fragmentation and mRNA expression level of apoptotic genes were applied to investigate the cell death mechanism. **Results:** The ADX induced significant cytotoxic effects against hepatocarcinoma cells *in vitro*. It was reduced the viability and proliferation in HepG2 cells treated with ADX at various concentrations for different exposure times comparing to untreated cells. Furthermore, the microscopic investigation showed apparent changes in HepG2 treated cells and the results of DNA fragmentation showed an increase in the percentage of fragmented DNA. Moreover, the expression of *p53* and *Bax* genes was up regulated, while *Bcl-2* gene expression was down regulated, in HepG2 cells treated with ADX. **Conclusion:** The ADX may be a promising natural anticancer agent and can be developed as a new anticancer therapy tool.

Key words: Anticancer, Date extract, DNA fragmentation, HepG2 cells, MTT assay.

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History

- Submission Date: 23-09-2021;
- Review completed: 12-10-2021;
- Accepted Date: 21-10-2021.

DOI : 10.5530/pj.2021.13.214

Article Available online

http://www.phcogj.com/v13/i6

INTRODUCTION

Hepatocellular carcinoma (HCC) is considered one of the leading causes of mortality and is among the top frequent cancer-related death worldwide. Its incidence has increased over the last several decades, and experts believe that cases of HCC will continue to grow until 2030 worldwide.[1-2] HCC is the leading liver cancer originating from hepatocytes, occurring in more than 80% of liver cancer cases.[3-4] It is well known to be caused by chronic liver diseases, such as chronic hepatitis B and C, alcohol addiction, cigarette smoking, and dietary contaminants like aflatoxins.[5-6] Several studies have shown that the hepatitis C virus (HCV) aggravates hepatocytes' transformation into malignant cells via various routes.[7] In addition, many aflatoxins found on grains, peanuts, soybeans, and maize are correlated with the development of HCC.[8] The HCC disease is currently controlled via clinical therapeutic methods, including liver transplantation, transarterial chemotherapy, radiotherapy, and systemic therapy targeting multiple receptor tyrosine kinases using low molecules weight. [9] However, treatment selection depends on the characteristics of the tumor, the existence of the underlying liver disease, age, other medical comorbidities, and available medical resources and local expertise.[5] Unfortunately, each method presents several issues, such as drug resistance and adverse side effects. Therefore, the search for alternative products for cure with more minor or free toxic effects on normal tissues and cells continues.

Plants products have been used to treat several cancers since they have an anticancer effect against various forms of this disease.[10] These natural products have multiple action mechanisms, including cell growth inhibition, cell differentiation variance, and apoptosis initiation.[11] Various natural products, such as sulfated polysaccharides, isoverbascosides, camptothecin, tachyplesin, matrine, tylophorin, and 7-OH-4-CH(3)-coumarin, are known to have differentiation-inducing potential for hepatocellular carcinoma.[12] Many plant-derived phenolic compounds such as flavonoids are potent antioxidants with reported anti-carcinogenic effects.[13] In addition, many flavonoids and phenols play an essential role in cancer therapy by controlling genetic pathways without side effects.[14-15] Thus, researchers have sought to develop plant-derived anticancer agents promising cancer prevention and treatment activities *in vitro*.[16]

The date palm tree (*Phoenix dactylifera L.*) is an essential plant in many countries, particularly in the Middle East, North Africa, South Europe, South America, Pakistan, and India.[17] Date fruit is a natural source of antioxidants and compounds that are antimutagenic. It contains significant quantities of amino acids, vitamins such as C, B1, B2, and A, and nicotinic acid.[18-20] Ajwa is a common date fruit grown only in Al-Madinah Al-Monwarah in the Kingdom of Saudi Arabia (KSA) has many benefits and is a rich source of potassium, iron, and natural fibers.[21-22] Previous studies confirmed the numerous protective and clinical properties of Ajwa date, including antioxidant, anti-inflammatory, nephroprotective, and hepatoprotective activities.[23-26] that stem from

Cite this article: Almalki FA. Cytotoxic Activities of Phytochemical Components from Ethanol Extract of Ajwa Date on Human Hepatoma Cancer Cells in Vitro. Pharmacogn J. 2021;13(6) Suppl: 1664-1672.

the numerous chemical compounds found in the fruit. Flavonoids, glycosides, phytosterols, and polyphenols are the most abundant phytochemicals in the Ajwa date.[24] It has been found that ethanolic extracts (90%) of Ajwa date (ADX) have the highest amount of total phenolic and flavonoid contents and more potent antioxidant properties than two different types (Aseel and Zaid) of local Pakistani dates.[27] Anticancer properties of Ajwa could effectively inhibit the growth of cancer cells *in vitro* such as Caco-2 & HCT-116 colon,[28-29] human MCF-7 breast cancer,[30-29] and HepG2 cell line,[29] and *in vivo* like sarcoma-180 cells in mice.[31] ADX has shown significant beneficial effects against HCC induced by diethylnitrosamine in a rat model; in this study, the treated rats developed a typical liver architecture and showed reductions in liver enzymes and increases in antioxidant enzymes. [32] Ethyl acetate extracts of Ajwa date may cause cell cycle arrest and reduce PC3 prostate cancer cells.[33] The effect of the ajwa date pulp (ADP) extract was recently associated with reactive oxygen species (ROS) generation and depletion of the mitochondrial membrane's capacity in cancer cells. Also, ADP extract induced DNA damage in HCC cells leading to cell cycle arrest at stages S and G2/M, followed by apoptosis via a TP53-independent pathway.[34] Besides, Ajwa nano preparation has protective effects against doxorubicin-induced cardiac disorders and can increase the antioxidant of heart tissues.[35] The current study investigated the anticancer effect of ethanolic ADX on HepG2 cancer cells by evaluating cell viability and proliferation, as well as the DNA damage and changes in the treated cells morphology were examined. Therefore, the present study findings make substantial contributions to cancer therapeutics based on natural products.

MATERIAL AND METHODS

Ajwa date extract preparation

Fresh Ajwa dates were brought from a local market in Taif Province, Kingdom of Saudi Arabia. First, the dates were washed twice with double distilled water, and their pith was separated manually. Next, the pulp was dried, crushed, and then extracted with ethanol at a ratio of 1:3 (w/v) at 24 °C for 48 h. Next, the extract was passed through the Whatman filter and Millipore filter. Finally, the prepared extract was condensed and stored at -80 °C before use to produce a viscous syrup.

HPLC analysis for ADX

HPLC analysis was performed in ADX to determine its polyphenolic contents; Agilent 1260 Series. The separation of the extract was performed on a column of C18 (LI.D. 4.6 mm × 250 mm, 5 µm particle size). Water (A) and 0.02 percent trifluoroacetic acid in acetonitrile (B), with a mean flow rate of 1 ml/min, were the mobile phases. The linear-gradient (A) was programmed as follow: 0 min (80%); 0–5 min (80%); 5–8 min (40%); 8–12 min (50%); 12–14 min (80%). Exactly 10 µl of the sample solution was injected, and the absorbance was monitored at 280 nm by a multiwavelength detector. The temperature of the column was set at 36 °C, and triplicate analysis was conducted. In order to get real-time chromatograms of the normal and ADX, HPLC spectra were monitored at 254 nm.

Cell line and maintenance of cell culture

The HepG2 cells used in this study were purchased from American Type Culture Collection (ATCC) and were maintained in complete media (DMEM supplemented with 10% FBS + 1% penicillin-streptomycin) at 37 °C in a CO_2 incubator (5% CO_2 + 95% air and 100% relative humidity).

In vitro cell viability/ cytotoxicity studies

Trypan blue dye exclusion assay

Trypan blue dye exclusion assay was performed to evaluate the effects of ADX on the viability of HepG2 cancer cells. The dye is a vital stain that is not absorbed by healthy, viable cells. However, trypan blue can enter cells when damaged or dead, thereby allowing for the counting of killed cells. Viable cells have a translucent cytoplasm, with dead cells dyed blue. Briefly, 50 µl of freshly prepared trypan blue solution (0.05% distilled water) was combined for 5 minutes with 50 µl cell suspension. The mixtures were then spread onto the hemocytometer slide. Cytotoxicity was monitored by counting viable cells using a light microscope; viable cells will appear with clear, uncolored cytoplasm while the dead cells will present with blue cytoplasm.[36]

MTT assay

Cytotoxicity of ADX was determined *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium (MTT) test according to the adapted technique of Horiuchi et al.[37] In this assay, MTT salt will convert to purple formazan crystals by viable, active cells. In short, cells were seeded at $1x10^6$ cells per 100 µl in 96 well plate and incubated at 37 °C for 24 hours. Cells were treated with different ADX concentrations (5, 10, 20 or 40 mg/ml). At 37 °C, the plates were incubated with 5% CO_2 for another 24 and 48 h. A volume of 10 µl of MTT solution was added to each well and incubated at a humidified atmosphere (37 °C & 5% CO_2) for 3-4 h. DMEM without addition was used as negative power. The optical density (OD) of each well was recorded at 570 nm wavelength. Each ADX concentration was tripled, and cell viability percentage was determined using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{OD of Control} - \text{OD of ADX treatment} \times 100}{\text{OD of Control}}$$

Apoptosis detection via DNA fragmentation assay

DNA fragmentation is used as a biochemical marker to measure apoptosis.[38] In this study, the DNA content of HepG2 treated cells was degraded by nucleases activated via the caspase pathway. A DNA fragmentation assay was carried out using agarose gel electrophoresis. According to the manufacturer's instructions, following ADX treatment, DNA fragmentation induced in the treated cells was determined by a Quick Apoptotic DNA Ladder Detection Kit (Bio-Vision, USA). The DNA pellet was obtained from 1 10^6 harvested cells for the control and treated cells and was dissolved in 30 µl suspension buffer and electrophoresed in 2% agarose gel containing 0.5 µg/ml ethidium bromide for 1–2 h. The fragmented DNA bands were visualized by a UV light transilluminator and photographed. The percentages of fragmented DNA were evaluated colorimetrically at 575 nm using Diphenylamine (DPA) as defined by Burton[39] and updated by Perandones et al.[40] The DPA assay is valuable method for measuring apoptosis by determining the percentage of fragmentation of DNA into the oligosomal-sized fragment. The principle of DPA assay is based on the interaction between the DNA backbone sugar (deoxyribose) with DPA in the acidic environment producing a bluish-green color that can be detected by a wavelength ranging from 560-595 nm.

Gene expression levels

Apoptosis or programmed cell death is a tightly regulated process that is controlled via multi genes and pathways. Experimentally, apoptosis can be detected through analysis of its associated genes using cell RNA content. Briefly, total RNA was isolated using TRIzol reagent, and its purity was examined by gel electrophoresis. The RNA extracted from the control and treated cells were exposed to DNase and then used for RT-PCR to synthesize the first-strand cDNA using a reverse transcription kit (Maxime RT PreMix kit, South Korea). PCR amplification was applied in a thermal cycler (PXE 0.5 Thermo) using the primer pairs listed in Table 1 obtained from previously

published work.[41-42] The product was run on 1.5% agarose gel and visualized by a UV transilluminator. The band signal intensities were quantified using Gel-Pro software (version 3.1 for Windows 3) after scanning. The target gene-amplification product level ratio to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was calculated to normalize the initial variation in sample concentration as a control for reaction efficiency.[43-44]

Statistical Analysis

Using SPSS 11.0 software, statistical analysis was performed. A one-way ANOVA assessed significant differences between treatment groups, followed by Duncan tests.[45-46] The outcomes are expressed in the mean ± SE. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The polyphenolic constituents of ADX were investigated, and HPLC analysis revealed the occurrence of six primary polyphenols, including gallic acid (GA), naringenin (NG), catechin, chlorogenic acid, caffeic acid, and syringic acid at concentrations of 28.41 ± 1.62, 16.96 ± 1.3, 4.91 ± 0.75, 2.40 ± 0.38, 1.17 ± 0.18, and 1.17 ± 0.12 mg/g, respectively (Figures 1-2). The concentration of GA was 1.75-fold more significant than that of NG, and this compound made up approximately half (28.41 mg/g) of the total polyphenolic components (56.31 mg/g) of ADX. A previous investigation[29] demonstrated the presence of gallic, syringic, and coumaric acids as the primary phenolic contents in the aqueous extract of Ajwa date, which confirms our results. Nematallah et al.[47] examined various extraction solvents and methods to get the highest amount of polyphenols and flavonoids from Ajwa date fruit, and they reported that aqueous acetone (50%) extract is the best one.

GA and its analogs have several biological activities, including antioxidant, antimutagenic, and anticarcinogenic. It prevents proliferation and causes apoptosis in the lymphoblastic leukemia C121cell line.[48] It has also been reported that GA with other cancer drugs showed a promising anticancerous effect. In the Paclitaxel/GA combination, the highest cytotoxic effect, induction of apoptosis, and a substantial elevation in *P53* and Caspase 3 levels were observed in Hela cervical cancerous cells.[49] Epidemiological studies have revealed that GA and NG compounds exert anticancer, antiproliferative, and pro-apoptotic activities against different cancer cells.[50-51] Phenolic compounds are generally accepted to possess a broad spectrum of health-beneficial effects.[52-54] It is also reported that, *in vivo*, using mouse model, Ajwa bioactive ingredients were safe from body weight loss caused by chemotherapeutic agent doxorubicin (DOX) which is used clinically for breast cancer treatment.[55]

The six primary polyphenols were detected, including gallic acid, naringenin, catechin, chlorogenic acid, caffeic acid, and syringic acid.

The cytotoxic effect and the most significant concentration of ADX against HepG2 cells (IC_{50}) were investigated experimentally using different extract concentrations and treatment times. The screening results using Trypan blue staining are presented in Table 2 and Figure 3A. The data showed that 20 mg/ml ADX, referred to as the IC_{50} value, could strongly inhibit HepG2 cell proliferation by 48% and 60% after treatment for 48 and 72 h, respectively. The most extensive inhibitory effect on cells proliferation was obtained at 40 mg/ml; this concentration inhibited cell proliferation by 60% and 75% after treatment for 48 and 72 h, respectively (Figure 3A). Our findings are in agreement with Khan et al.[30] data. They calculated the IC_{50} value of MCF-7 breast cancer cells exposed to different concentrations of MEAD for 48 h, as 18.2 mg/

Table 1: Summarized the targeted primers sequences used in the study.

Genes	Forward Primer	Reverse Primer
Bax	5'-CCTGTGCACCAAGGTGCCGGAACT-3'	5'-CCACCCTGGTCTTGGATCCAGCCC-3'
P53	5'-TGCGTGTGGAGTATTTGGATG-3'	5'-TGGTACAGTCAGAGCCAACCTC-3'
BCl2	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'	5'-GGTGCCGGTTCAGGTACTCAGTCA-3'
GAPDH	5'-ATGGCACCGTCAAGGCTGAG-3'	5'-GCAGTGATGGCATGGACTGT-3'

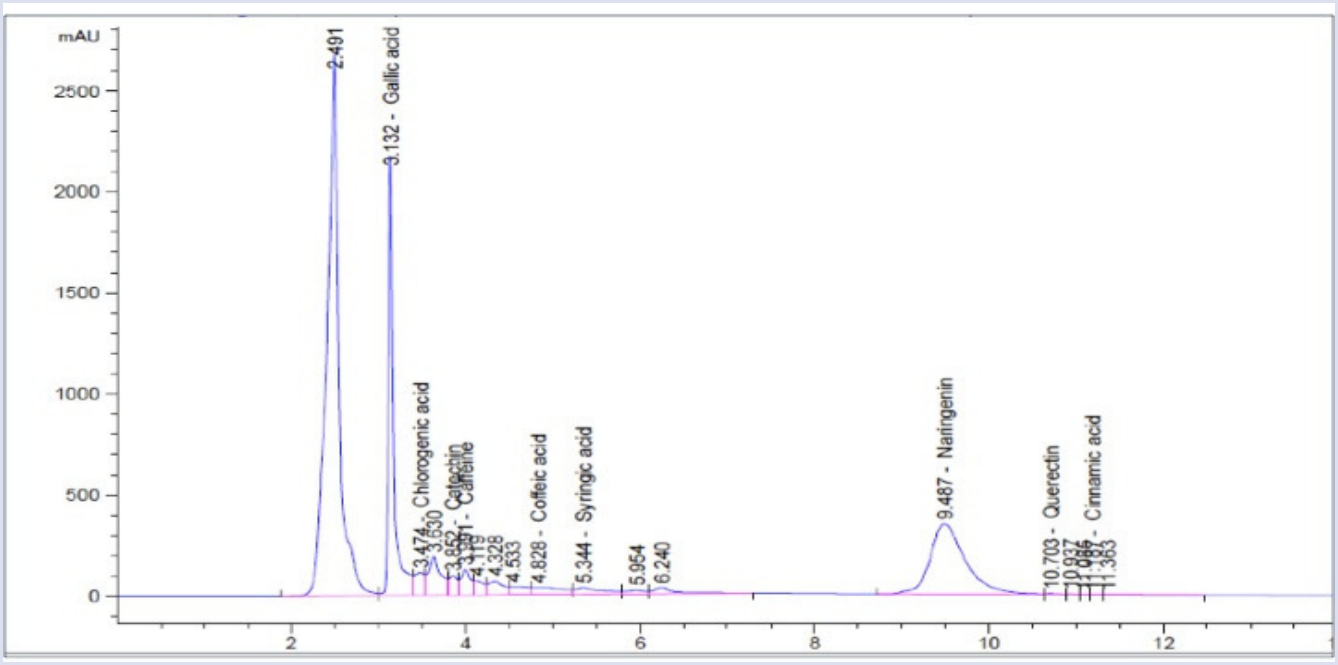

Figure 1: HPLC chromatogram of Ajwa date extract (ADX). It represents the different polyphenolic contents in ADX.

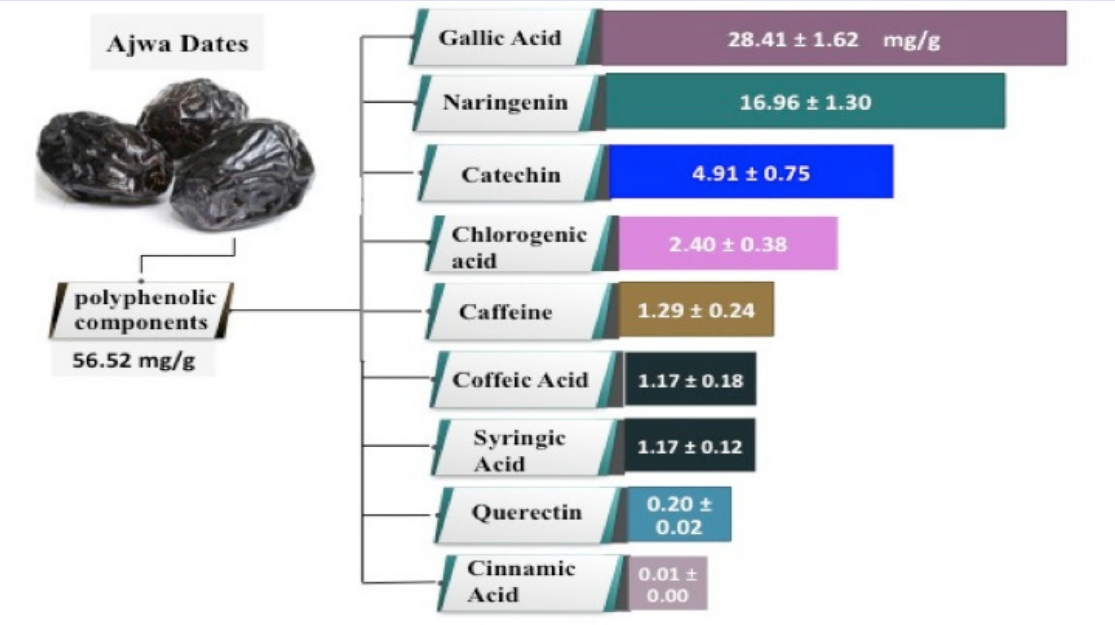

Figure 2: Diagram shows the different constituents of polyphenolic components in ADX, present as mg/g.

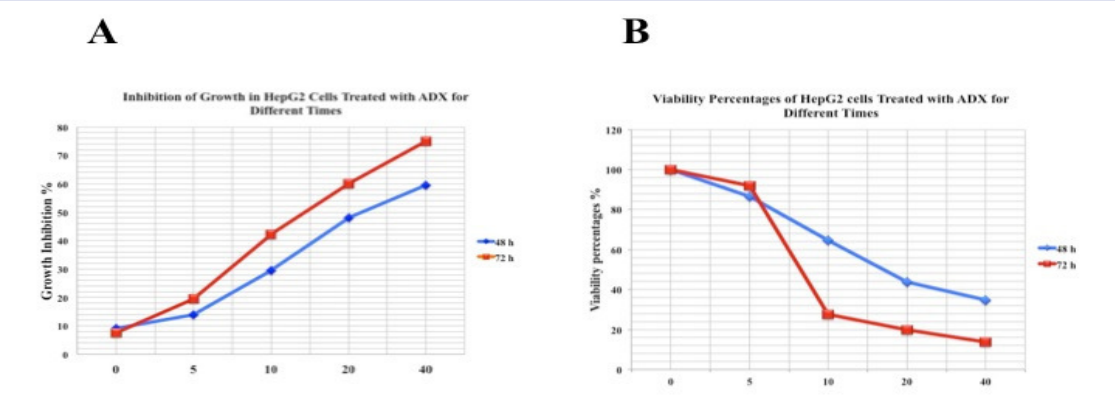

Figure 3: Charts represent the effect of ADX on HepG2 proliferation and viability.
(A) shows that the growth inhibition percentage of HepG2 cells treated with ADX is increased with the increase of the ADX concentration and treatment duration. (B) illustrates a significant drop in viability percentage of HepG2 cells exposed to different concentrations of ADX at various times. The highest inhibitory effect was noticed at 40 mg/ml of ADX for 72 hours, and the IC50 value was 20 mg/ml.

Table 2: Illustrates IC_{50} of ADX and its effect on HepG2 cancer cells' proliferative percentage at different exposure times.

Doses of ADX (mg/ml)	Proliferative Percentage of HepG2 cells at different exposure times	
	48 h	72 h
0	90.9 ± 1.50[a]	92.5 ± 1.70[a]
5	86.0 ± 1.0[a]	80.5 ± 1.85[b]
10	70.5 ± 2.44[b]	57.8 ± 2.65[c]
20	52.0 ± 2.25[c]	40.0 ± 1.90[d]
40	40.5 ± 2.75[d]	25.0 ± 0.87 [e]

ml. Moreover, Siddiqui et al.[34] reported the IC_{50} values for ethanolic Ajwa date pulp extract (ADP) as 20.03 and 16.78 mg/ml after treating HepG2 cells for 24 and 48 h, respectively.

In addition, the viability of HepG2 cancer cells after being exposed to different concentrations of ADX was analyzed using the MTT assay, and the collected data showed a dramatic decrease in the cell viability comparing to the untreated control cells (Figure 3B).

Means with different superscripts (a, b, c, d, and e) in the same column vary significantly at $P < 0.05$. Cell numbers are counted, data expressed as a percentage of untreated power.

Previous studies have proposed that some constituents of ADX, such as NG and GA, could reduce cancer cell proliferation by arresting the cell cycle[28] and inducing apoptosis in a time- and dose-dependent manner.[56] NG has cytotoxic, genotoxic, and apoptotic activities by

developing reactive oxygen species (ROS) on cancer cells. Thus, the derived NG compound could also be used as an antiproliferative agent to treat cancer.[57] Our results follow previous studies, which showed that GA is involved in the regulation of *p53*, which controls the cell cycle and has been implicated in tumor suppression.[58] Interestingly, these two compounds (GA & NG) have been determined to inhibit different cancer cells include MCF7 (breast) and CACO (intestinal) cell lines.[59-60] In addition, Al-Radadi[29] reported that aqueous Ajwa extract could be used in the biosynthesis of platinum nanoparticles and effectively reduced up to 73% of the growth of the HepG2 cells *in vitro*. Likewise, Siddiqi et al.[34] findings demonstrated a significant decrease in viability of human liver HepG2 cancer cells treated with ADP, and this action is time- and dose-dependent.

The differences between treated and untreated cells were checked morphologically using Phase-contrast microscopy to investigate the impact of ADX on the HepG2 cancer cell morphology. The images of untreated HepG2 cells showed a monolayer's characteristic epithelial nature and prolific growth. Ovoid cells with clear cytoplasm and a large central nucleus have one or fragmented nuclei (Figure 4A). This type of cell with fragmented nuclei occurs physiologically in hepatocytes and is usually present in cancer cells known as binucleated cells. By comparison, ADX-treated cells with IC_{50} dose (20 mg/ml) for 48 h, displayed mild to moderate decreases in the number of cells as present in Figure 4B. In addition, cells appeared shrinkage with condensed cytoplasm, membrane destruction, round shape, and cellular detachment. (Figure 4C). These morphological changes represent the initial features of apoptotic cell death.[61] Moreover, significant inhibition in the cell numbers and cellular crowding was noticed after 72 h, (Figure 4D). Similar to the present findings, Khan et al.,[30] Ishurd et al.,[31] and Siddiqui et al.[34] indicated that date extracts could induce cellular changes, including blebbing of the membrane, DNA fragmentation, and cell adherence loss, leading to apoptosis and cell death. These results support our morphological findings and further validate HepG2 cell apoptosis from ADX therapy.

DNA fragmentation is a degradation of a nuclear genome in specific sites between nucleosomes. It considers a hallmark indicator for apoptosis events in cells and tissues and can be evaluated colorimetrically or via agarose gel electrophoresis. Herein, DNA fragmentation analysis further investigated the major apoptotic events in HepG2 cells treated with ADX. The percentages of fragmented DNA were calculated, and the results were listed in Table 3 and Figure 5. DNA fragments were observed colorimetrically by DPA (Table 3), and DNA profiles were compared via agarose gel electrophoresis (Figure 5). In ADX-treated cells, fragmented DNA increased significantly compared to untreated cells in a dose-dependent manner (Table 3 and Figure 5). Khan et al.[30] showed that methanolic extract of Ajwa date (MEAD) induced apoptosis in a human MCF-7 cell line *in vitro,* and a reduction in living cells number was noticed while the percentage of early- or late-stage of apoptotic cells were increased. Another study[34] investigated the impact of ethanolic extract of Ajwa date pulp (ADP) against HepG2 cancer cells and found that the extract induced apoptosis and DNA fragmentation in treated cells in a dose-dependent manner. The current study findings are consistent with the results of the previous investigations.

Means with different superscripts (a, b, c, and d) between groups in the same column are significantly different at $P < 0.05$.

Table 3: Effect of ADX treatment on the DNA fragmentation percentage induced in HepG2 cells after 48 h.

Treatment	DNA Fragmentation %	
	Mean ± SE	Change
Untreated Cells	5.80 ± 1.08^{d}	0
10 mg/ml ADX	18.25 ± 1.31^{c}	+ 12.45
20 mg/ml ADX	38.00 ± 2.5^{b}	+32.2
40 mg/ml ADX	58.0 ± 1.93^{a}	+ 52.20

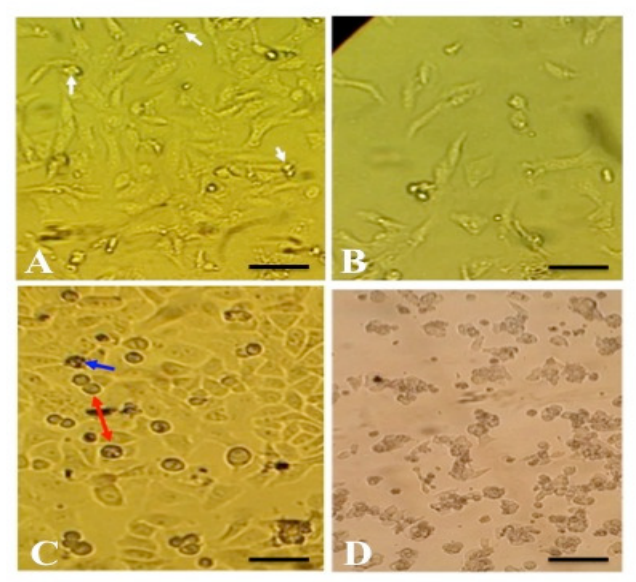

Figure 4: Inverted microscopic photographs of HepG2 Cells.
The images show untreated cells (A) that appeared in monolayer and epithelial nature with fragmented nuclei indicated by white arrows. Treated with ADX at 20 mg/ml (B) and 40 mg/ml (C) after 48 h. Arrows suggest morphologically apoptotic changes, including condensed and fragmented nuclei (red) and apoptotic bodies (blue). There were also gross decreases in cell numbers (D) with increased exposure time, 72 h. (Magnification 200X).

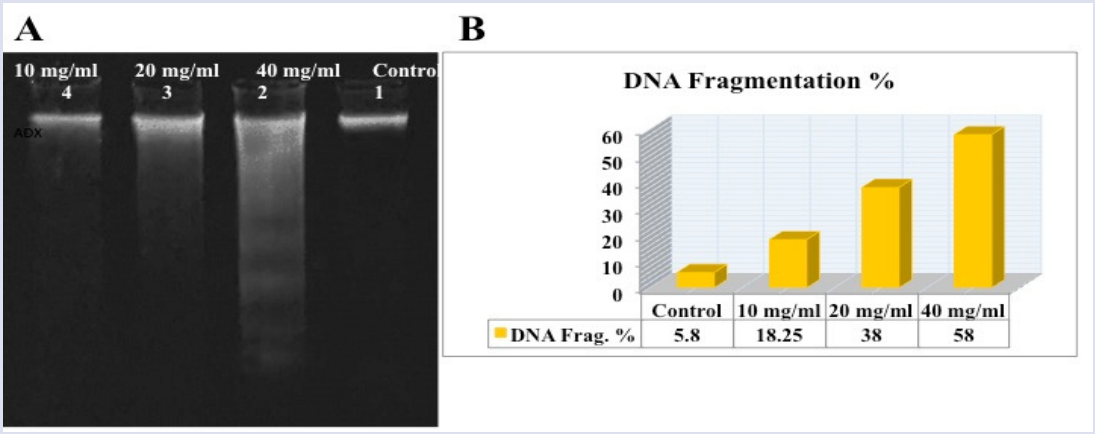

Figure 5: DNA fragmentation induced in HepG2 cells treated with ADX at the indicated doses (10, 20 and 40 mg/ml) for 48 h. (**A**) showing DNA profile on 2% Agarose gel electrophoresis, 1 × 10^6 cells were used, and 20 µl of DNA sample was loaded in each well. **Lane 1:** Control untreated cells with nonfragmented DNA. The DNA appears at the top of the gel as a high-molecular-weight band. **Lane 2:** Effect of 40 mg/ml ADX, **Lane 3:** Effect of 20 mg/ml ADX, and **Lane 4:** Effect of 10 mg/ml ADX. The damaged DNA presents as smmer in treated cancer cells, and the fragmented DNA density increased by the increasing of ADX doses as shown in Lane 2, 3 and 4. (**B**) A histogram illustrates the cytotoxic effect of ADX on the DNA fragmentation percentage of HepG2 cells in comparison to the control. Data were obtained from three independent experiments and expressed as Mean ± SEM.

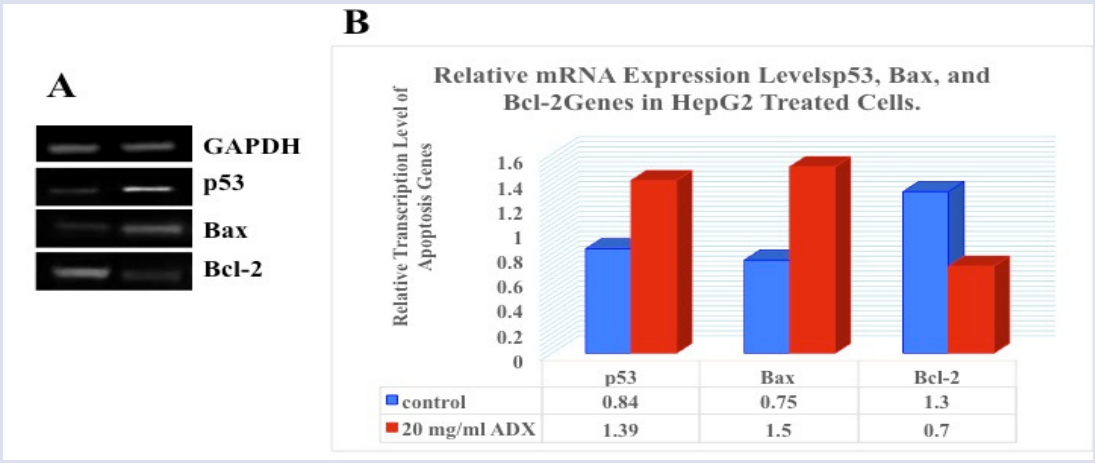

Figure 6: Shows the effects of 20 mg/ml ADX treatment in pro-apoptotic and anti-apoptotic gene expression levels. (**A**) 1.5% agarose gel electrophoresis photographs illustrate the relative transcription levels of *P53*, *Bax,* and *BCL-2* genes comparing to the GAPDH gene in HepG2 treated cells for 48 h. (**B**) the histogram shows an increase in *Bax* and *p53*gene expression levels by over 2- and 1.5-fold, respectively, compared with the control when cells were treated with 20 mg/ml ADX. A significant drop in *Bcl-2* expression by 2-fold can be observed in comparison to the control. Results were obtained from three independent experiments and expressed as Mean ± SEM.

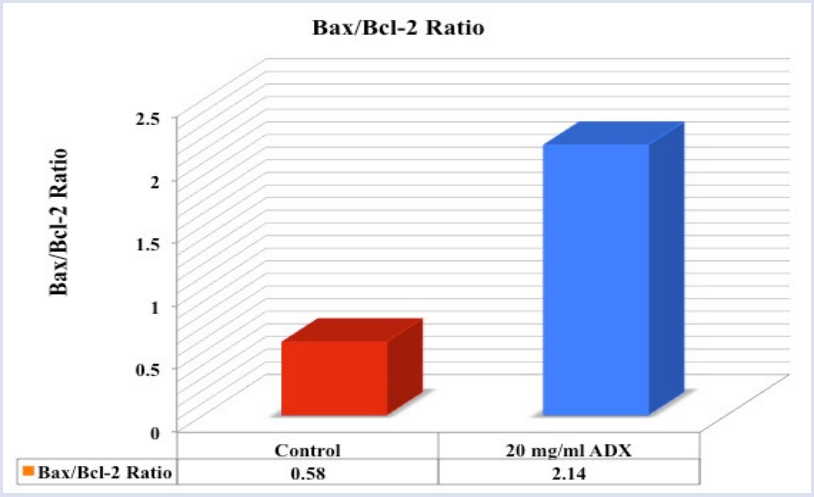

Figure 7: Histogram showing the effects of 20 mg/ml ADX treatment on *Bax/Bcl-2* transcription ratio in HepG2 treated cells for 48 h.

Numerous genes are involved in apoptosis process direction and/or regulation, some of them still under investigation or even undiscovered. B-cell lymphoma 2 gene (*Bcl2*) anti-apoptotic, BCL2-associated X protein (*Bax*) pro-apoptotic, and tumor suppressor gene *p53* are critical players in cancer generation and growth. The expression levels of apoptotic genes (*Bcl-2, Bax,* and *p53*) of HepG2 cancer cells exposed to ADX at IC_{50} value (20 mg/ml) for 48 h were analyzed using sq-PCR. Statistically, a significant increase in *Bax* and *p53* expression levels was observed over 2 and 1.5-fold, respectively, compared with the control (Figure 6). Additionally, a dramatic inhibition in the *Bcl-2* mRNA expression level was recorded by a 2-fold decrease compared with untreated cells (Figure 6). Moreover, *Bax/Bcl-2* ratio was calculated, and the results showed a significant increase in treated HepG2 cells by 4-fold compared to the untreated group (Figure 6). From this point of findings, ADX can act as an up-regulator for *Bax* and *p53* genes and down regulator for the *Bcl-2* gene, and as a result, the ADX can induce apoptosis in treated HepG2 cancer cells.

Khan et al.[30] stated that apoptotic cell death is indicated in MCF-7 breast cancer cells treated with MEAD by substantial increases in *p53*, *Bax*, *Fas,* and *FasL* genes expression and a significant decrease in *Bcl-2* gene expression level as compared to the control group. The fold increase of *p53* and *Bax* was ten-fold when cells exposed to 20 mg/ml of MEAD for 48 h, which is confirmed the present findings. Siddiqui et al.[34] reported a downregulation in the expression level of *p53* gene in HepG2 cells treated with 15 and 25 mg/ml ADP for 48 h, compared to untreated cells, which is slightly different from Khan et al.[30] finding and the current study result. The transcriptional regulation of various processes, such as the cell cycle, apoptosis, and DNA repair, is caused by the stabilization and accumulation of *p53*.[62] Pro-apoptotic genes, including *Bax*, are upregulated during apoptosis, whereas *Bcl-2* family genes are downregulated.[34]

Additionally, *P53* upregulation can modulate transcriptional activation of other associated pro-apoptotic genes. Further supporting the theory that a *p53*-dependent mechanism can trigger HepG2 cell apoptosis is the increased expression of *Bax* and *p53* genes observed in our research. A drop in *Bcl-2* expression and a four-fold increase in *Bax/Bcl-2* transcription ratio relative to control group expression (Figure 7) indicates that *p53*-mediated signaling triggers HepG2 cell death following treatment ADX, leading to apoptosis.

The *Bax/Bcl-2* ratio increased dramaticlly by 4-fold compared to the control cells, reflecting the increase in apoptosis gene (*Bax*) transcription level and the decrease in anti-apoptosis gene (*Bcl-2*) mRNA expression level.

Previous studies[29,47] confirmed the hepatoprotective and antioxidant properties of aqueous and 50% aqueous acetone ADX *in vitro* and *in vivo*. Other investigations[30,33,34] demonstrated the action of methanolic, ethyl acetate, and ethanolic fractions of ADX on different types of human cancer cells through various experimental methods. The impact of ADX was recorded to be associated with ROS induction, mitochondrial membrane potential (MMP) disruption and, DNA damage which causing cell cycle arrest at any phase (G1, S, and G2/M phases) and finally terminated with apoptosis via a *p53*-dependent[30] or independent[34] pathways.

CONCLUSION

In conclusion, ADX extract investigated in the present study showed anticancer effects decreased the proliferation and viability of HepG2 cancer cells and increased their DNA fragmentation. ADX also upregulated the expression of pro-apoptosis genes, such as *p53* and *Bax*, and downregulated the expression of anti-apoptosis genes, such as *Bcl-2*. The results of this work are of significant interest because they shed light on the phytochemical components of ADX that render it a natural anticancer agent.

ACKNOWLEDGEMENTS

The author is gratfull to Taif University, Taif, Saudi Arabia.

ABBREVIATIONS

ADX: Ajwa date extract; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; ADP: Ajwa date pulp; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GA: Gallic acid; NG: Naringenin; DOX: Doxorubicin.

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Cite this article: Almalki FA. Cytotoxic Activities of Phytochemical Components from Ethanol Extract of Ajwa Date on Human Hepatoma Cancer Cells *in Vitro*. Pharmacogn J. 2021;13(6)Suppl: 1664-1672.