

IMPACT OF AJWA DATE EXTRACT ON THE PRO-APOPTOTIC BAX GENE EXPRESSION IN HEPATOCELLULAR CARCINOMA CELL LINE

¹HANAN SALEM ALHAMED, ²DR. ANGHAM ABDULKAREEM, ³PROF. KHALID ABULNAJA

¹Research Scholar at King Abdulaziz University

halhamed0011@stu.kau.edu.sa

² Assistant professor in Biochemistry, King Abdulaziz University

aabdulkareem@kau.edu.sa

³Professor in Biochemistry, King Abdulaziz University

kabualnaja@kau.edu.sa

Affiliated to King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

For Correspondence: ³ kabualnaja@kau.edu.sa

ABSTRACT: Hepatocellular carcinoma (HCC) is the fourth cause of cancer-related death worldwide. Conventional cancer treatments are effective in eradicating the primary tumor; however, there is a great chance of disease recurrence besides the tense effects of the radio and chemo therapies. Therefore, researchers are trying to find safe alternative treatments. Natural products such as dates have shown beneficial effects in many diseases including cancer due to their important constituents. In this project, we investigated the anticancer effect of Ajwa Date Extract (ADE) on hepatocellular carcinoma (HepG2) cell line. Cells were treated with ADE in different concentrations (20, 30, 40, 50 and 60 mg/ml) for 24 hours. Results showed an inhibitory effect of ADE on cancer cells in a dose-dependent manner with apoptotic morphological changes of the cells. ADE also caused genomic DNA fragmentation, as well as a significant increase in gene expression of BAX that play a major role in apoptosis. Thus, ADE may offer a potential safe and efficient HCC treatment in the future.

KEYWORDS: Ajwa Date, Phoenix dactylifera L., apoptosis, Hepatocellular carcinoma, HCC, BAX, gene expression, date extract.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths worldwide, accounting for over 800,000 deaths annually and more prevalence in males than in females. Hepatitis B and C, excessive alcohol consumption, diabetes, obesity and some hereditary disorders are considered to be strong risk factors of HCC incidence [1], [2].

Incidence of cancer begins when apoptosis fails to occur due to mutations in the tumor suppressor genes (TSGs), which slow down cell division, repair DNA and control cell

death; as a result, cells continue to grow and divide in an uncontrol way, leading eventually to cancer [3]. BAX, a member of Bcl-2 gene family, is a pro-apoptotic gene that tightly control apoptosis. When BAX protein is activated, it binds to mitochondrial outer membrane (MOM) and changes its permeability, resulting in the leakage of the inner mitochondria chemicals to the cytosol. These chemicals lead to caspases activation and eventually cell death [4], [5].

Recently, natural products are being central topic for scientists as adjuvant treatment of cancer instead of the more aggressive traditional ways, chemotherapy and radiotherapy [6]. Flavonoids, for instance, are polyphenolic compounds extracted from the plants showed an anti-proliferating, anti-inflammatory and anti-cancerous activity [7].

Ajwa date is rich in flavonoids; it has been reported that date is considered the highest source of flavonoids among the dry fruits, also, was used for therapeutic purposes over decades due to its pharmaceutical properties [8], [9].

A study conducted in 2017 to investigate the effect of Ajwa date extract (ADE) on diethylnitrosamine-induced HCC in Wistar rats, the results showed a significant desirable effect against HCC; it restored the normal levels of antioxidant enzymes, hepatic enzymes, and cytokines [10]. Another study used ethanolic ADE on HCC cell line also reported a positive impact; the extract induced DNA damage, which was followed by apoptosis, and inhibited cancer cells growth without affecting the healthy cells [2]. Similarly, ADE induced cell cycle arrest and apoptosis in human breast adenocarcinoma (MCF7) cell line, reduced cell proliferation from 35% to 95% and up-regulated p53 and Bax proapoptotic genes [11].

In this study, we aimed to investigate the gene expression regulatory effect of the ethanolic Ajwa date extract on the pro-apoptotic BAX gene in the hepatocellular carcinoma cell line, HepG2

I. MATERIALS AND METHODS

1. AJWA DATE EXTRACT PREPARATION

ADE, the drug in our study, was prepared as follow: Ajwa dates were purchased from Al-Madina Al-Munawwarah, KSA. In a flask, 10 gram of date pulp was added with petroleum ether ethanol 80% at a ratio of 1:1 (w/v) and shaken for 24 hours by a rotary shaker. The resultant mixture was centrifuged for 10 minutes at 10000 revolutions per minute (rpm) then filtered with muslin cloth (eight layers). The resultants filtrate was vaporized at 60°C under low pressure; a thick solution was obtained and kept in the refrigerator until further use in experiments.

Stock 1 of the drug was prepared by adding 200 mg of ADE in 1 ml UltraPure DNase/RNase-Free Distilled Water (200 mg/ml); then, different concentrations were prepared (20, 30, 40, 50 and 60 mg/ml).

2. WST-1 ASSAY

According to the manufacturer’s instructions (Abcam), cell viability and proliferation were assessed using WST-1 assay kit. Briefly, 1×10^4 cells/well were seeded in 100 μ l media in 69-well plate and incubated overnight to allow attachment. Then, cells were treated with ADE in different conc. (20, 30, 40, 50 and 60 mg/ml) and the control group was treated with UltraPure DNase/RNase-Free Distilled Water and media; the cells were incubated at 37°C with 5% (v/v) CO₂ in atmospheric air incubator for 24 hours. Following the treatment period, 10 μ l of WST-1 reagent was added to the wells and incubated in 5% CO₂ incubator for 3 h at 37°C. Then, the absorbance was measured at 450 nm to evaluate viability/proliferation.

3. DNA FRAGMENTATION ASSAY

Genomic DNA was isolated from both treated and untreated cells using QIAamp DNA Mini kit. Electrophoresis of extracted DNA was performed on 2% agarose gel at 150 V for 40 min using 1x TBE buffer containing 10 μ l of ethidium bromide. An aliquot of the extracted DNA sample (5 μ l) was mixed with 2 μ l of 6X Loading dye (Bromophenol blue) and loaded into the submerged wells. The samples were electrophoresed until the dye front reached the end of the gel. DNA bands were observed under UV gel documentation system.

4. GENE EXPRESSION ANALYSIS

The qRT-PCR reaction was carried out according to the manufacturer’s protocol (QIAGEN). Data was normalized for housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The GAPDH and BAX primers’ sequences were obtained from an earlier published studies [12], [13] and are given in Table 1.

qRT-PCR was performed using 1 μ l of diluted cDNA template (1:10 dilution) combined with 9 μ l of PCR solution in a 96-well microplate. Each sample was run in triplicate in two different experiments.

Table 1: Primer sequences used for gene expression analysis

| Gene | Primer Sequence |
|-------|---|
| GAPDH | Forward: 5'- CTTTTCGTCGCCAGCCGAG-3' Reverse: 5'GCCCAATACGACCAAATCCGTTGACT- |

| | |
|-----|--|
| | 3' |
| BAX | Forward: 5'-GCTGTTGGGCTGGATCCAAG-3' Reverse: 5'-TCAGCCCATCTTCTTCCAGA-3' |

II. RESULTS

1. Effect of ADE on morphology and viability of HepG2 cells

As shown in fig. 1, treated cells exhibited morphological changes, which are characteristic apoptotic features; they became round in shape, fragmented, translucent and detached with shrinkage. In contrast, the untreated control cells were intact with regular shape, adherent to the surface and homogenous. ADE drastically increased the morphological changes in HepG2 cells in a dose-dependent manner.

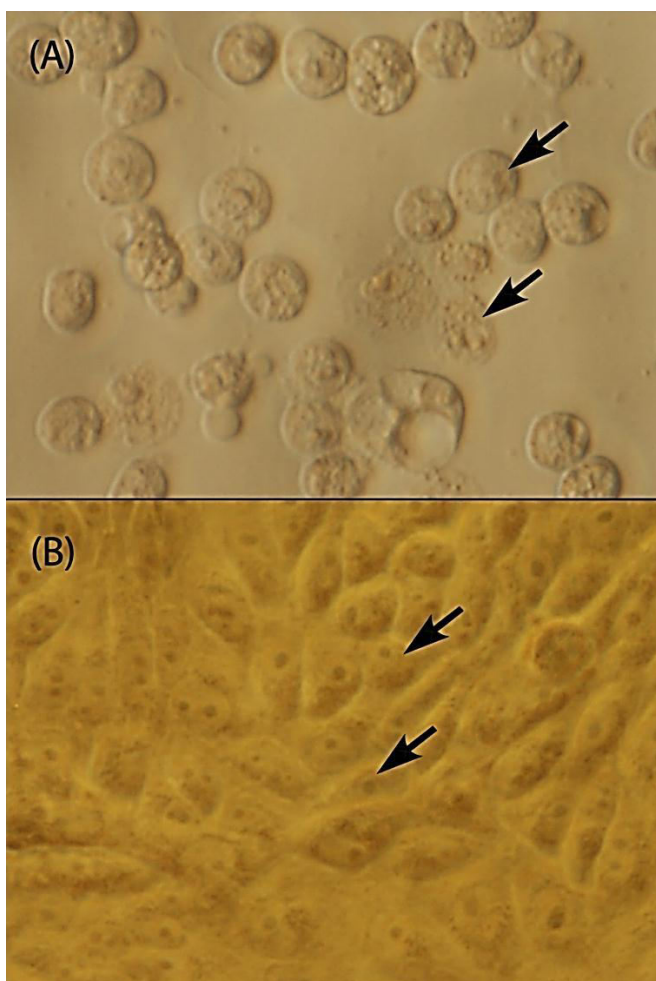


Figure 1: Morphological Changes of HepG2 Cells. (A) treated cells with ADE (60 mg/ml in picture) in comparison with the (B) untreated control (treated with H₂O instead of ADE) showed distinct apoptotic features. Image taken by inverted microscope (60x magnification).

WST-1 assay demonstrated growth inhibition of HepG2 cells following treatment with ADE for 24 h in different concentrations (20, 30, 40, 50 and 60 mg/ml) as shown in (Fig.

2). Cell viability was reduced with an IC_{50} value of 38.86 mg/ml. The decrease in proliferation was very highly significant compared to the control by using one-way ANOVA of $p < 0.0001$ for all (Fig. 3). Thus, the results suggested that ADE treatment inhibited HepG2 cell growth and proliferation in a dose-dependent manner, indicating its potential anti-proliferating effect against cancer cells.

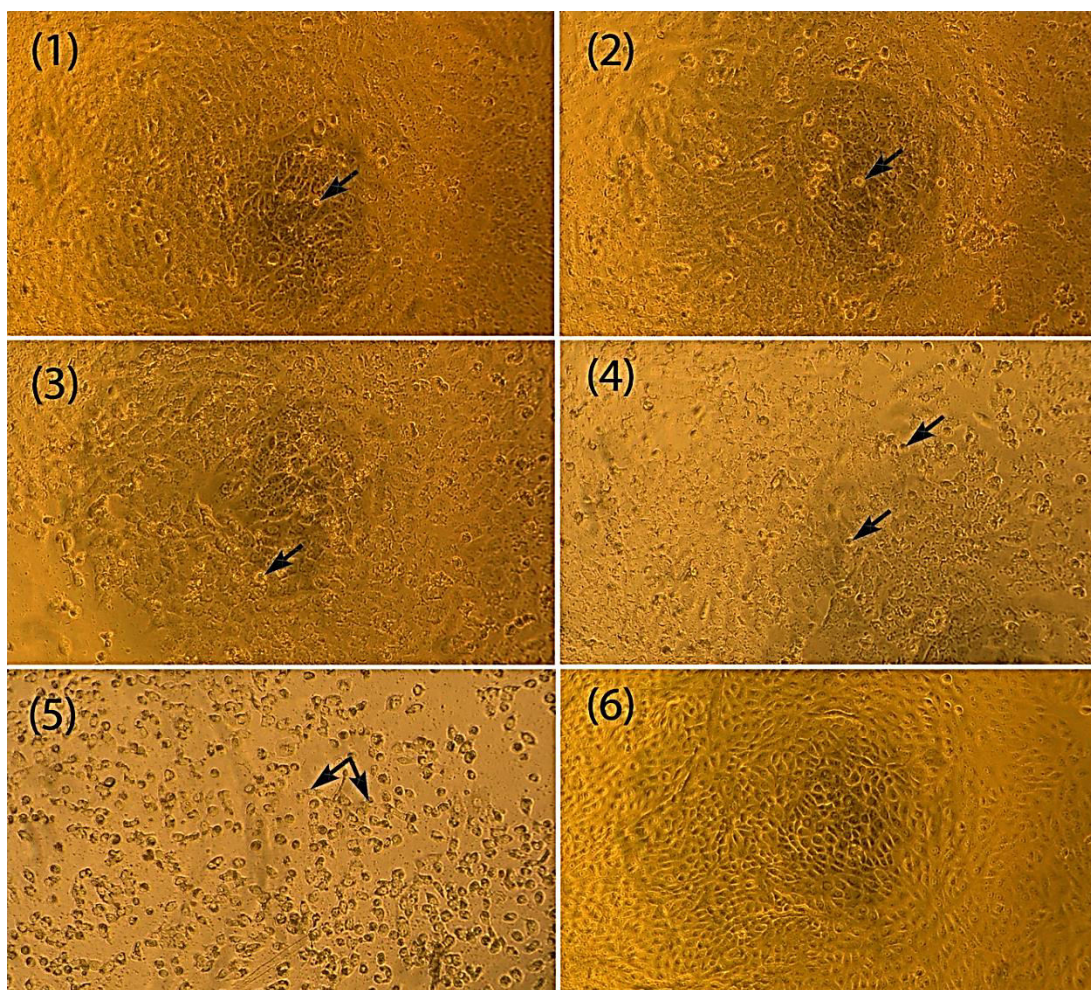


Figure 2: Cytotoxic effect of ADE on cells; microscopic observation of ADE cytotoxicity on HepG2 cells in different concentrations, 20, 30 40 50 and 60 mg/ml, (1-5) respectively, after 24 h incubation, compared to (6) the untreated aqueous control (WST-1 assay). Image taken by inverted microscope (10x magnification).

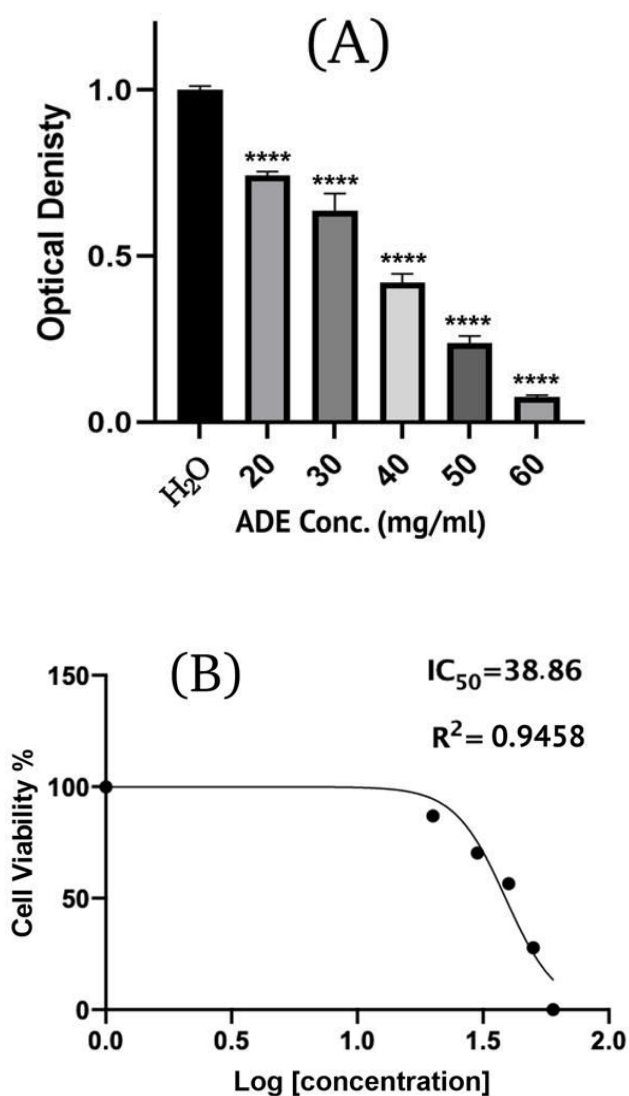


Fig 3: (A) Cell viability of HepG2 cells: HepG2 cells after exposure duration to ADE treatment, compared to the untreated aqueous control; **(B) Inhibitory effect of ADE on HepG2 cell line:** the curve is representing IC₅₀ value of ADE; the cells were treated with ADE and incubated for 24 h. The effect of ADE was assessed using WST-1 assay.

2. Role of ADE in the induction of DNA fragmentation in HepG2 cells

DNA fragmentation was visualized by UV light after treatment of HepG2 cells with ADE; laddering pattern was observed in lane 2 and 3 (30 and 40 mg/ml, respectively)

compared to the intact unfragmented control band (lane 1) (Fig. 4), confirming the degradation of nuclear DNA into nucleosomal units after treatment which is a hallmark of apoptosis which is a further support of ADE efficiency against HCC cells.

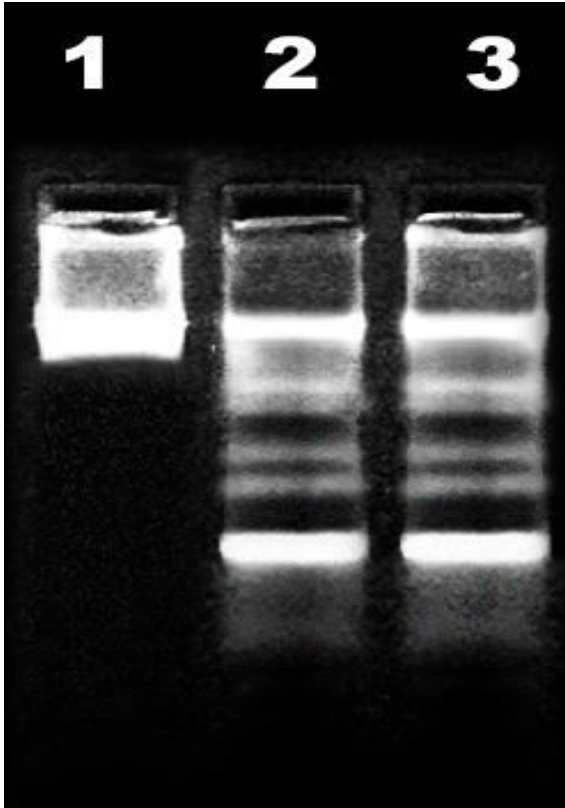


Figure 4: DNA fragmentation assay in HepG2 cells as an indication of apoptosis; Lane 1: showing untreated control; Lane 2 and 3 represent the DNA of treated cells with 30 and 40 mg/ml of ADE, respectively.

3. Effect of ADE on the expression of the pro-apoptotic Bax gene

The expression of BAX in the HepG2 cells was investigated after ADE treatment. The analysis of the RT-PCR data using one-way ANOVA showed that the expression of BAX in the cells treated with 30 mg/ml ADE was not significantly different in comparison with the control with mean equal to 1.049 ± 0.114 at $p = 0.87$. Conversely, mRNA expression in the cells treated with 40 mg/ml ADE was upregulated by 0.532-fold (53%) comparing to the untreated control; mean equal to 1.532 ± 0.131 , demonstrating a highly significant difference at $p= 0.0044$ (Figure 4.5), which confirms the incidence of apoptotic cell death at the higher concentration.

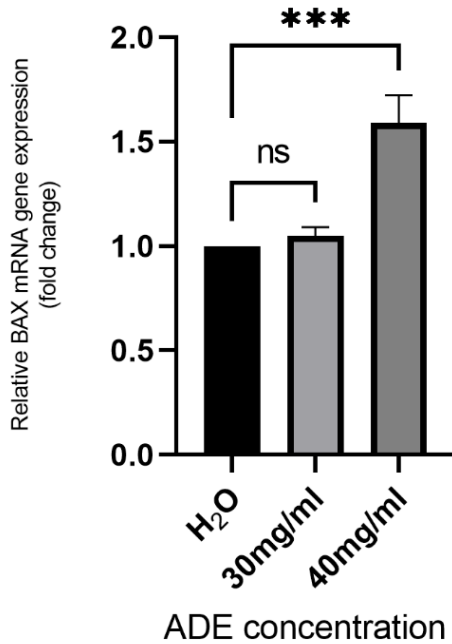


Figure 5: Molecular profiling by measurement of mRNA levels of pro-apoptotic gene BAX: HCC cells which were treated with 30 mg/ml of ADE showed a non-significant difference in the expression of BAX gene in comparison with the control; while treated cells with 40 mg/ml demonstrated a highly significant difference.

III. DISCUSSION

Cancer is a major burden worldwide, causing millions of deaths each year [14]. Current applicable treatments are considered aggressive on body cells in many perspectives; thus, scientists keep developing safer and efficient alternative treatments. Natural products are getting scientists' attention nowadays [6]. In this study, ADE was used as a drug against hepatocellular carcinoma in HepG2 cell line; The results supported the hypothesis that ADE may induce apoptosis in cancer cells; it exerted an inhibitory effect on cell growth and proliferation, triggered genomic DNA fragmentation and upregulated gene expression of the key pro-apoptotic gene BAX.

Ajwa date is well known for its health benefits in traditional and alternative medicine; it contains many active ingredients including polyphenolic compounds such as flavonoids that exert an anti-proliferative action against malignant cells [7]. Other reports reveal that they depict the highest polyphenols levels compared to any other dried fruit [16]. In an earlier study, total flavonoid content of Ajwa date extract was assessed using aluminum chloride method and was expressed as rutin equivalent (RE) per gram of dry extract; the total flavonoid content was calculated as 227.4 RE/g [17]. A recent study conducted in 2019 by Siddiqui et al. reported that ethanolic Ajwa date pulp extract reduced cell viability of HCC cells with an IC_{50} of 20 mg/ml and caused morphological alternations, these results support what we have found that ADE exerted antiproliferative effect against HepG2 cells with an IC_{50} of 38.86 mg/ml and suppress their growth and division. Other studies have also reported the antiproliferation effect of ADE against other types of cancer, such as in MCF7 and PC3 cell lines [11], [15]. It is thought that due to high sugar content in Ajwa date, ≈ 74.3 g/100 g dry weight [18], high concentrations are needed to obtain the desired effect [2].

Interestingly, in the present study, microscopic imaging of treated HepG2 cells demonstrated changes in cell morphology by displaying major apoptotic features such as roundness, transparency and detachment [19]; these observations are in accordance with existing evidence indicated that Ajwa date extract has triggered most treated cells to late apoptosis stage in HepG2 and MCF7 cell lines when analyzed by flow cytometry [2], [11]. Moreover, the genomic DNA fragmentation that formed a ladder pattern on gel indicated that programmed cell death (apoptosis) had taken place; however, the underlying mechanism of apoptosis induction by ADE is still unclear.

In line with our other results, gene expression of BAX, which impairs mitochondrial outer membrane permeability and eventually leads to programmed cell death, was significantly upregulated by 53% comparing to the control when treated with 40 mg/ml, this finding relates to an earlier published result, where methanolic Ajwa date extract increased Bax gene expression by 7 and 10-fold in MCF7 cells upon treatment with 15 and 20 mg/ml, respectively [11].

ADE efficient anti-proliferating effect may be due to the synergetic action of total flavonoids, many isolated flavonoids such as kaempferol, quercetin, fisetin, galangin and

casticin have proven the anticancer property of these compounds against different types of cancer by upregulating the gene expression of the pro-apoptotic gene BAX [20].

IV. CONCLUSION

The present study revealed the potent growth-inhibitory effect of the 80% ethanolic Ajwa date extract against human hepatocellular carcinoma HepG2 cells. ADE significantly reduced cell viability caused DNA damage in HCC cells and upregulated pro-apoptotic gene BAX that led to apoptosis which is absent in cancer.

ADE is a promising anticancer drug against HCC; however, further clinical and pharmaceutical studies must be conducted to validate the safety and efficacy of this drug. For instance, studying the active compounds' absorption, distribution, metabolism, toxicity on organs, clearance, bioavailability and elimination in the human body will assist in determining the effective dose and route of administration to achieve the drug's optimum therapeutic effect.

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