The Anti-Proliferative Activity of Ephedra Aphylla Methanol Extract Against Liver Cancer Cell Line

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Abstract

This study aimed to investigate the anti-proliferation activities of methanol extract *ephedra aphylla* against (HC) liver cancer cell line in *vitro and to identify the possible mechanism of action*. Anti-proliferative activity of the plant extracts against the cancerous cell lines was tested by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on liver cancer cell line (HC). Gene expression studies using qPCR for the proapoptotic gene P53 were also carried out. The methanol extract of *ephedra aphylla* exhibited promising anti-proliferative activity. Dose response relationship was shown after 72hr incubation. Significant cytotoxic activity of methanol extracts has identified on HC, IC50 was (6.79 µg/ml). Furthermore, methanol extract significantly up-regulated the expression of P53. Thus, these findings suggest potential utilization of *ephedra aphylla* as new source in researching for new anticancer agents.

Keywords: *ephedra aphylla, HC, antiproliferative, p53*

Introduction

Liver cancer is the second leading cause of cancer mortality worldwide and fifth most common cancer [(1)], Beside that a 5-year survival rate is only 8.9% even with intensive conventional therapy, the burden of this aggressive malignancy is growing globally, and it could be 1 million cases by 2030 [(2,3)]. lethality of liver cancer mainly due to its resistance to the current anticancer agents, a lack of biomarkers that can detect early stage of disease, and underlying liver disease that restrict the use of chemotherapeutic drugs [(4)] . Recently, in attempt to enhance patient's life style, and to increase efficacy of convention chemotherapy, the Plants became effective source of complex chemical compound that could be used as a good candidate for the development of such novel anticancer agents [(5)]. Many plants and natural agents have been investigated through in vitro and in vivo as potential an anti-HCC agent for several years [(6)].

Ephedra aphylla is amember of Ephedraceae family that known for its medicinal value in the Mediterranean area because of the ephedrine alkaloid and other chemicals in the stems of most members of this genus [(7)]. Even though there are very few content of alkaloids in *ephedra aphylla* but there are many on flavonoids and condensed tannins, triterpenes, and cardiac glycosides; compounds that were reported to have medicinal efficacy against numerous diseases [(8)]. Some studies have reported that many of phytochemical compounds that had isolated from Arial parts of *ephedra aphylla* had chemo preventive potential as it inhibits in vitro chemical carcinogenesis and exerts pro-apoptotic and anti-proliferative effects in different tissues [(9,10)].

The study of gene expression profile of cancer cells has become an important tool to understand the biological modifications involved in disease development, to generate personalized pharmacological therapies for patients, and to explore the molecular effects of drug exposure with the purpose of improving the effectiveness of treatment [(11)]. A p53 protein an important candidate target against which new anticancer treatments could be developed [(12)] as the p53 transcription factor stands out as a key tumor suppressor and a master regulator of various signaling pathways involved in this process. The many roles of p53 as a tumor suppressor include the ability to induce cell cycle arrest, DNA repair, and apoptosis [(13)] .in liver cancer , Inactivation of this multiple tumor suppressor gene plays an significant role in the progression of chronic liver damage to hepatocellular carcinoma by directly or indirectly

inducing chromosome instability, cell proliferation and neovascularization [14,15,].

Materials and Methods

Preparation of Extract

ephedra aphylla arial parts (300 g), the stems were dried in the open air and away from light and moisture then powdered, sieved (60mesh) size and stored in a well closed container.

Preparation of Methanol Crude Extract

300 g powder *ephedra aphylla* stems was divided into three parts and extracted with methanol for for 8 hours,in shaking water bath at 40c with stirring, The extraction ratio of each solvent was 4:1 W/V (100gm of powder/400ml of solvent). and then the mixture filtered, the filtrate kept in umber bottle and the residue dried. Extraction process repeated three times .the extract was concentrated using a rotary-evaporator under vacuum. The extract was then kept in desiccators at room temperature.

Serial Dilution of Methanol Extract

A 10 mg of the crude extracts were dissolved in 1 ml dimethyl sulfoxide (DMSO) to get 10mg/ml as stock solutions. Form stock solutions of each extract 5 different concentration were prepared (100, 50, 25, 12.5, 6.25 µg/ml).

Routine cell culture

HC cell lines were obtained from the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) Cell Bank Unit, Cancer cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C in a humidified 5% CO2 atmosphere, The cells used for the experiments were between passage 4 and 7[16].

Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as a measurement of cell proliferation in accordance with **Mosmann method** 1983[17]. The principle of this assay is based on the ability of living cells to change the yellow water-soluble substrate MTT into dark blue formazan product (insoluble in water) by mitochondrial succinate dehydrogenase enzymes. Only viable cell are able to reduce the yellow MTT while cells being dead following a toxic damage cannot. The precipitates can be dissolved after cell lysis, this product is proportion to the viable cell number and inversely proportional to the degree of cytotoxicity [18].

Cell lines were seeded at 1 × 104 cells/well After 24 hrs and when a confluent monolayer was achieved, cells were treated with serial concentrations of *ephedra aphylla* covering the range of 6.25–100 µg/µl while the untreated cells received only DMSO as a control. After 72 hrs, the medium had been eliminated and, and for each well added 28 µL of MTT solution which prepared by added 2 mg/mL of phosphate buffer saline (PBS). The plates were incubated for 2.5 h at 37 °C, in 5% CO2. The plates were taken out from the incubator and the supernatant layer was removed. After removing the MTT solution, the crystals residual in the wells were solubilized by the addition of 130 µl of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking. The optical density reading (OD) was recorded using a micro plate reader at 492 nm for absorbance and 650 nm as reference, the assay was performed in triplicate. The percentage of cell growth inhibition was determined as a mean ± SD, using the following equation was calculated as the following equation: **Inhibition %= 1- cell viability%** [17].

Cancer cells collecting

Hc cell lines in T75 flasks, were treated with methanol extracts of *ephedra aphylla* at concentrations equivalent to the calculated IC_{50} earlier obtained. The cells were incubated for 48 hrs. After which the media was decanted and cells washed in phosphate buffer solution (PBS) to remove any debris. Trypsinization of the cells was done.

RNA extraction

The Total RNA was extracted from the samples using Bioneer Kit following the manufacturer's instruction (AccuZol™ Total RNA Extraction Solution, Bioneer®, Australia). After followed the steps of RNA extraction according to manufacturer s instructions,

the RNA pellet was dissolved in RNase-free water by passing the solution a few times through a pipette tip. The extracted mRNA was stored at -80℃.

Real-time PCR

Real-time PCR test was used to define the effect of the *ephedra aphylla* extract on the degree of gene expressionof p53 , Real time Polymerase chain reaction was carried out in AriaMx Real-Time PCR (qPCR) Instrument (agilent technologies, United States) using one-step method .This method uses a One-Step EvaGreen qRT-PCR® Kit (abm good)that contain reverse-transcriptase (RT) enzyme which convert mRNA to complementary single stranded DNA (cDNA) and also contain a DNA polymerase enzyme that convert cDNA to double stranded DNA. All these reactions take place in the same reaction tube at the same time in a reaction mixture volume of 20 µL. GAPDH was used as a housekeeping gene. The sequence of used primers is indicated in Table 1.

TABLE (1) PRIMERS SEQUENCE USED IN THE PCR REACTION

Gene	Forward primer	Reverse primer
P53	5'GATCTGTTGCTGCCCCAGGAT3'	5'AGATGACAGGGGCCATGGAGT
GAPDH	5'TGGCCGTATTGGGCGCCT3'	5'TCTCCATGGTGGTGAAGA3

Statistics

The statistic design for this study was presented as mean ±SD (standard deviation). The value of groups were compared by the one-way ANOVA then by means of Tukey Post-hoc test (t-test) and measured significance at $p<0.05$,0.01 and 0.001 by using the GraphPad Prism software, version 8.2.1 for Windows 10**.** the concentration that inhibits 50% from the cell growth (IC50) was analyzed and calculated for *ephedra aphylla* extracts by linear regression equation: y=mx+b, where y is the percentage of inhibition and is set to be 50%, m is the slope of the standard curve, x is the concentration of the compound tested in µg/mL, and b is the y-intercept of the line of standard curve.

Results

In order to determine the effect of *ephedra aphylla* extract on the growth of HC liver cancer cell lines proliferation, MTT assay was used at 72 h after incubation with different doses of *ephedra aphylla* methanol extract. The survival curve shows that the cytotoxic effects of *ephedra aphylla* methanol extract on treated cells are dose dependent. There was a significant decrease in the viability of cells incubated for 72 h. The data suggests that the *ephedra aphylla* extract significantly inhibited HC cell proliferation in dose -dependent manners. Cell growth was inhibited considerably in 100 µg/ml and 72 h compared with control groups as shown in Figure 1, cell proliferation was decreased to 64.35 % ($P<0.05$) The IC50 value was yield from the graph for extract of *ephedra aphylla* by using the linear regression equations where (x) is the concentration Y= the percentage of inhibition The IC50 value ME was 6.79 µg/ml.

The effects of *ephedra aphylla* extract on p53 expression

The mRNA levels of p53 gene were analyzed. The genes CT values were normalized against the mRNA level of GAPDH as a housekeeping gene and the relative expression for each group was measured. Figure 2 shows that, there is a significant fold incensement in the level of P53 expression by 3.53 when compared with the control group.

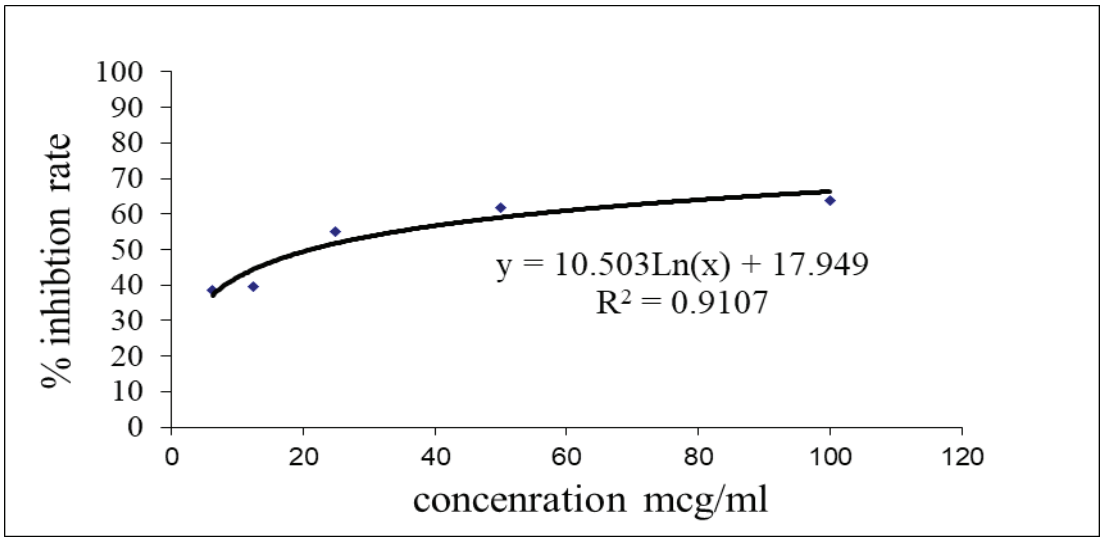

Figure (1) Dose response curve of methanol extract of *ephedra aphylla* against HC cancer cell line.

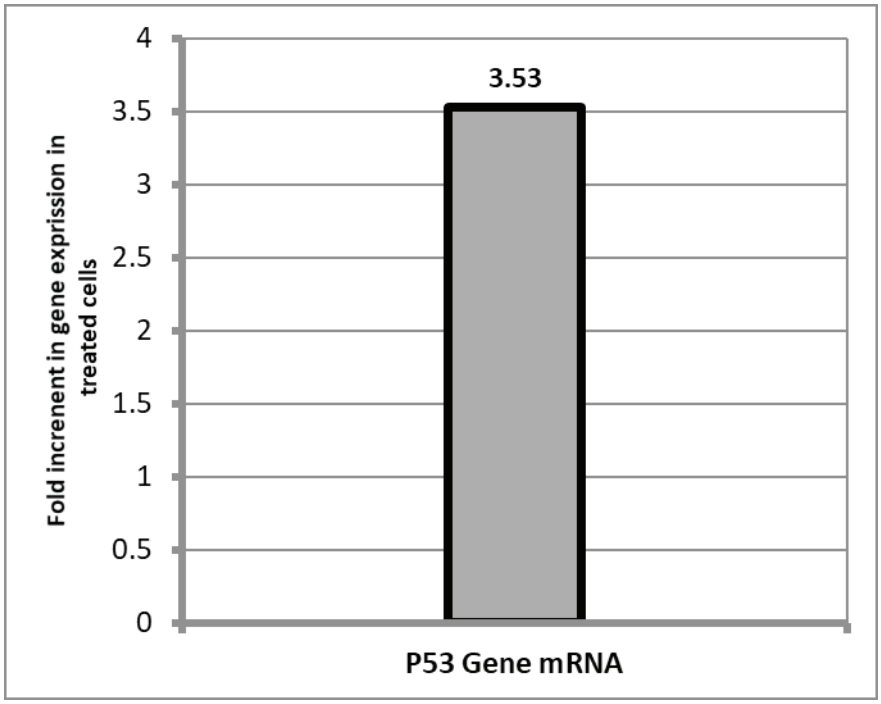

Figure (2): Fold increment in P53 gene as a result of treating the mice hepato-carcinoma cells (HC) with the plant extract.

Discussion

Methanol extract of *ephedra aphylla* has been tested against (HC) cell line to analyzed it's anti proliferative activity and measured it by using MTT assay which is one of the simple and rapid colorimetric assess and yields quantitative data [19] . The extracts showed significant dose dependent inhibition of liver cancer cell line while compared to the control (DMSO 1%) ($P<0.05$) after 72 hr. with IC50 (6.79 µg/ml) which suggesting that this sample has significant cytotoxic effect as it was lower than 20µg/ml, This is a well-recognized criterion for a compound to be judged as cytotoxic, as defined by the National Cancer Institute (NCI) [20] .

the methanol extract of *epedra aphylla* contain a wide spectrum of polyphenol and flavonoid compounds that may responsible for the anti-proliferative potential of that plant against HC cell line either by direct effect or synergistic boosting of the anticancer activities between it [21] and that suggestion support by the results of Jaradat

et al. found Methanol to be the best solvent in relation to the amount of polyphenol and flavonoid that detected in it [22].

In this study, the significant up-regulation of the P53 gene by the methanol extract of *ephedra aphylla* is an indication that it one of its way to induces apoptosis in the cancer cells (HC) via P53 dependent pathways. The activation of p53 trigger the expression of downstream genes leading to either cell cycle arrest (to allow repair and survival of the cell) at the G2/M phases [23] or it initiates apoptosis to dispose of the damaged cell through multiple mechanisms, including transactivation of specific target genes, down regulation of a distinct set of genes, and transcription-independent mechanisms [24]. Mounting evidence has confirmed that p53 is a potent activator of the caspase cascade by stimulating pro-apoptotic proteins (Bid and Bax) and stimulating the release of apoptogenic actors (cytochrome c), which lead to caspase-9 activation and in turn cleave effector caspases such as caspase-3 [25], p53 protein also activates extrinsic signaling cascades of apoptosis, that belong to tumor necrosis factor (TNF) superfamily with main signaling protein caspase 8 [26].

Conclusion

The methanol extract from *ephedra aphylla* have been demonstrated to possess significant anti-proliferative activity. The mechanism of action of the anti-proliferative activity of *ephedra aphylla* can be linked to its up-regulation of the pro-apoptotic gene p53. These findings suggest potential utilization of *ephedra aphylla* as good candidate in developing new anticancer agents.

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