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Original Article

ANTI-PROLIFERATIVE ACTIVITY OF *ZIZYPHUS SPINA CHRISTI* LEAVES METHANOL EXTRACT AGAINST RHABDOMYOSARCOMA (RD) CELL LINE

AHMED R. ABU-RAGHIF[1], GHAITH A. JASIM[2], MUNEER M. HANOON[*3]

[1]Al-Nahrain University/College of Medicine/Pharmacology Department, Baghdad-Iraq, [2]Al-Mustansiriyah University/College of Pharmacy/Department of Pharmacology and Toxicology, Baghdad-Iraq, [3]Thi Qar Health Office/Thi Qar Poison Control Center
Email: mmhanoon82@yahoo.com

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ABSTRACT

Objective: The aim of this study was to investigate the capability of *Zizyphus spina christi* methanol extract to inhibit cancer cell line proliferation.

Methods: The leaves of *Zizyphus spina christi* were extracted by cold maceration method. The anti-proliferative activity of the methanol extract against rhabdomyosarcoma (RD) cell line was tested by 3-(4, 5 Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The phytochemical constituents were identified by gas chromatography-mass spectrometry (GC-MS) analysis. The antioxidant activity was assessed by measuring free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

Results: The percentage extraction yield for leaves with methanol was 20.64%. The methanol extract showed dose dependent inhibition of RD cell line, the IC_{50} was 154.44 µg/ml. GC-MS showed the presence of flavonoid fraction and other compounds with antioxidant activity. The methanol extract demonstrated DPPH scavenging activity with IC_{50} of 33.91 mg/ml.

Conclusion: Methanol extract showed potential anti-proliferative activity against RD cell line, which could be due to its antioxidant activity.

Keywords: Anti-proliferative activity, *Zizyphus spina christi*, Methanol extract, RD cell line, MTT assay, DPPH assay

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INTRODUCTION

Cancer is one of the most leading causes of mortality in the world, accounting for almost 13% of deaths worldwide [1]. Cancer is characterized by uncontrolled growth of abnormal cells, which have the ability to invade other tissues and metastasize to other parts of the body via blood and lymph [2].

Rhabdomyosarcoma (RMS) is a malignancy that arises from skeletal muscle precursors [3]. It is the most common type of soft tissue sarcoma in children and adolescents less than twenty years old [4]. Chemotherapy is a major mode of treatment for various cancers. However, it is usually associated with adverse side effects, ranging from nausea to bone marrow failure [5], and development of multidrug resistance (MDR). Therefore, finding natural compounds from plants may provide an alternative cancer treatment [6].

Zizyphus spina christi, commonly known as Christ's thorn in English and Sidr or Nabqa in Arabic is a tree belonging to the genus *Zizyphus* in *Rhamnaceae* family [7]. It has been used in folk medicine as demulcent, emollient, and as a mouth wash [8]. The plant has been reported to possess antioxidant, antibacterial, antifungal, antidiabetic, and analgesic effects [9]. Antioxidant activity and the presence of flavonoids make the plant possible to have anticancer activity. Furthermore, a study done on 2015 approved that methanol extract of *Zizyphus spina christi* had a potent anti-angiogenic activity and, as angiogenesis is considered one of cancer treatment approaches, methanol extract was appointed to be tested against RD cell line to find out whether this extract has anticancer activity or not. So, the aim of this study was to investigate the capability of *Zizyphus spina christi* methanol extract to inhibit cancer cell line proliferation.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents used in the study are listed in table (1)

Plant material collection and extraction

Fresh and healthy leaves of *Zizyphus spina christi* were collected from various regions of An Nasiriyah city, Iraq. Leaf specimen was labeled and annotated with date of collection and locality. A voucher specimen number (2) was deposited at the College of pharmacy, Baghdad University. The leaves were washed thoroughly under running tap water and oven dried at 40 °C. The dried leaves were then grounded into fine powder using an electric grinder.

The extraction process was performed in the Department of Pharmacology, Al-Nahrain College of Medicine. The dried powdered leaves were extracted by cold maceration method. The powder (500 gm) was soaked into methanol, left in a shaking water bath at 40 °C for 8 h, then filtered through whatman No. 1 filter paper. The filtrate was pooled in an airtight dark bottle to be concentrated to dryness under reduced pressure and low temperature using rotary evaporator. The dried extract was stored in refrigerator until use [10].

Table 1: Chemicals and reagents

Chemical or reagent	Source
Ascorbic acid	Sigma Aldrich, Germany
DMSO	Sigma Aldrich, Germany
Heat inactivated fetal calf serum (HIFBS)	Sigma-Aldrich, Germany
Methanol	Sigma-Aldrich, Germany
RD cell line	American Type Culture Collection (ATCC, Rockville, MD, USA),
Trypsin EDTA	Sigma Aldrich, Germany
1,1-diphenyl-2-picrylhydrazyl (DPPH)	Sigma Aldrich, Germany
1% penicillin/streptomycin	Sigma Aldrich, Germany
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich, Germany

Assessment of anti-proliferative activity of the methanol extract against RD cell line by MTT assay

RD cell line culture and treatment

This assay was used to investigate the anti-proliferative activity of methanol extract of *Zizyphus spina christi* leaves against human rhabdomyosarcoma (RD) cell line. The cells were grown on RPMI-1640 medium supplemented with: 10% Heat inactivated fetal calf serum (HIFBS) and 1% penicillin/streptomycin. The monolayer cells were detached and a single cell suspension was made using trypsin ethylenediamine tetraacetic acid (trypsin EDTA). A hemocytometer was used to count the viable cells number. The cell suspension was diluted with RPMI-1640 medium in order to obtain final density of 1×10^4 cell/ml. The cells were seeded in 96 wells plate, with 200 µl/well and were maintained in a humidified incubator at 37 °C, in a 5% CO_2/95% air for 24 h. A stock solution of 1% of the methanol extract of *Zizyphus spina christi* in DMSO was prepared. Serial dilutions were prepared by dissolving an appropriate volume of this stock solution in the serum free RPMI-1640 medium and diluting it serially to obtain final concentrations of 100, 50, 25, 12.5, and 6.25 µg/ml. After 24 h, the cells seeding media was replaced with new media that contain the serial concentrations of the extract and the cells, supplemented with the new media were incubated for further 48 h. (200 µl) of each concentration were added to the plate well. Each concentration was tested in 8 replicates (8 wells), and the experiment was repeated twice. Serum free media without sample received 1% of DMSO and served as negative control (8 wells)[11].

MTT assay

After 48 h of incubation, 150 µl of the exposure media was removed from the wells and 50 µl of MTT in phosphate buffered saline (0.4 mg/ml) was added to each well and incubated for 4 h. After that, the medium with MTT was flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO at 37 °C for 2 h. The absorbance was measured at 570 nm using ELISA plate reader. The percentage of cell growth inhibition was determined using the following formula:

% of inhibition = $[(A_C-A_S)/A_C] \times 100\%$ Where:

A_S: Absorbance of the sample A_C: Absorbance of the control [11].

Gas chromatography-mass spectrometry (GC-MS) analysis of bioactive compounds in the methanol extract

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanol extract of *Zizyphus spina christi* was performed using a GC-MS (Model; QP 2010S, Japan). (1 µl) of the methanol extract was injected with a Hamilton syringe to the GC-MS manually for total ion chromatographic analysis in split injection technique. The bioactive compounds of the extract were identified by comparing their retention index and patterns of mass spectra with reference to Wiley Registry of Mass Spectral Data"s, New York (Wiley 8) and Fatty Acid Methyl Esters Library version 1.0 (FAME library) sources [12].

DPPH scavenging activity assay

The antioxidant activity of methanol extract of *Zizyphus spina christi* was assessed by measuring free radical scavenging activity using DPPH method. Serial dilutions of the methanol extract in methanol were prepared with the following concentrations: 500, 250, 125, 62.5, 31.25, 15.62 and 7.8 µg/ml. (1 ml) of 0.1 mmol solution of DPPH in methanol was added to 2 ml of each dilution of the extract. Ascorbic acid, which is a well-known antioxidant; was used as positive control. DPPH solution in the absence of the extract was used as control and methanol was used as blank. All the tests were performed in triplicate. After half hour of incubation in dark, absorbance was measured at 490 nm using ELISA plate reader.

The percentage of DPPH scavenging activity (P) was calculated using the following formula:

$$P = [(A_0 - As)/A_0] \times 100\%$$

Where:

A_0: Absorbance of the control.

As: Absorbance of the sample.

The IC_{50}, which is the concentration required to scavenge 50% of DPPH free radical, was calculated using dose inhibition curve by plotting the sample concentration versus the corresponding DPPH scavenging activity[13].

RESULTS

Amount of *Zizyphus spina christi* leaves crude extract

The amount obtained from the extraction of 500 g of leaves powder was 103.2 g, the percent yield was 20.64%.

Anti-proliferative activity of the methanol extract against RD cell line

The percentages of cell growth inhibition were presented as mean±SD (table 2). The results showed that all the tested concentrations of the methanol extract significantly inhibited RD cell line growth after 48 h (*P<0.05*). The inhibition was dose dependent. IC_{50} was calculated from the following logarithmic regression equation:

y=4.2329ln(x)+28.667, and was equal to 154.44 µg/ml.

Where: x= Concentration, y= The percentage of inhibition.

Table 2: Percentages of cell growth inhibition of methanol extract of *Zizyphus spina christi* leaves on human rhabdomyosarcoma (RD) cell line by MTT assay

Concentration (µg/ml)	Mean inhibition (%)±SD
6.25	37.8±0.047*
12.5	38.34±0.045*
25	40.46±0.037*
50	46.44±0.042*
100	48.42±0.024*

*= Statistically significant difference compared with the negative control (*P<0.05*).

Bioactive compounds in the methanol extract identified by GC-MS analysis

Twenty-eight compounds were identified in methanol extract of *Zizyphus spina christi* leaves (fig. 1). The major compounds identified in the extract which could explain the antioxidant and anti-proliferative activity were [4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-] (flavonoid fraction) (Line: 8), n-Hexadecanoic acid (saturated fatty acid) (Line: 21) and [2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one] (Line: 6).

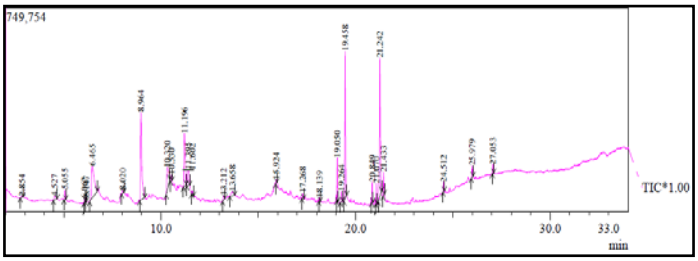

Fig. 1: Gas chromatography-mass spectrometry (GC-MS) chromatogram of methanol extract of *Zizyphus spina christi* leaves

DPPH scavenging activity

The results showed that DPPH scavenging activities of ascorbic acid and methanol extract of *Zizyphus spina christi* leaves were dose dependent. The IC_{50} values of DPPH scavenging activity were calculated using the following logarithmic regression equations:

-For ascorbic acid: y = 11.221 ln(x)+14.508, IC_{50} value was 23.64µg/ml (fig. 2).

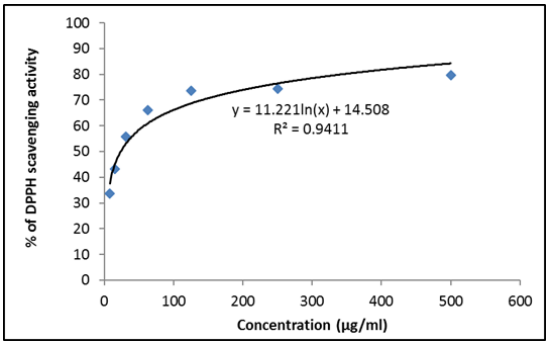

Fig. 2: 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of serial dilutions of ascorbic acid

-For methanol extract: y = 12.913 ln(x)+4.4977, IC_{50} value was 33.91µg/ml (fig. 3).

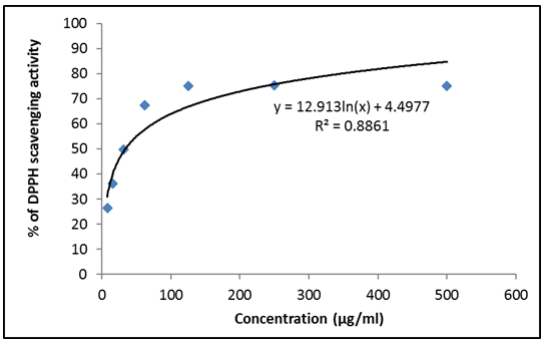

Fig. 3: 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of serial dilutions of *Zizyphus spina christi* leaves methanol extract

Where:

y = Percentage of DPPH scavenging activity and is set at 50%,

x = concentration.

DISCUSSION

The extraction process used in this study was cold maceration method. This method is suitable for extraction of thermo-liable compounds since high temperature may cause destruction of these compounds [14]. The leaves of *Zizyphus spina christi* were washed, dried and grinded before maceration. Grinding helps in getting a homogenous sample and increasing the contact area of the sample with the solvent system [15].

The anti-proliferative activity of potential antitumor agents was assessed by many experimental models, including the *in vivo* assays such as transplanted tumors [16, 17] and chemically induced malignancies [18]; or *in vitro* screening models. Among various *in vitro* models, the MTT assay, first described by Mosmann as a fastcolorimetric assay was selected as valid, easy, and semi-automated method for assessing growth inhibition in established cell lines [19].

A previous study showed that methanol extract of *Zizyphus spina christi* has a potent anti-angiogenic activity [20]. Since anti-angiogenic agents may have antitumor activity, it is wise to investigate the cytotoxic activity of methanol extract of *Zizyphus spina christi* on a tumor cell line. In the present study, the *in vitro* cytotoxic activity of methanol extract of *Zizyphus spina christi* was evaluated using human RD cell line. The results showed that all the tested concentrations of methanol extract of *Zizyphus spina christi* leaves exerted significant anti-proliferative activity against RD cell line after 48 h of treatment compared to negative control. The inhibition was concentration dependent and IC_{50} value was 154.44 µg/ml. According to United States National Cancer Institute plant screening program, a plant extract is generally considered cytotoxic if IC_{50} value, following incubation period between 48 to 72 h; is 20 µg/ml or less [21]. So, the extract has anti-proliferative but no cytotoxic activity against RD cell line.

The GC-MS spectroscopy of the methanol extract showed the presence of (4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-), which is a flavonoid fraction. Flavonoids significantly influence the cascade of immunological events associated with the cancer development and progression [22]. Many *in vivo* and *in vitro* studies showed that flavonoids may inhibit various stages in the carcinogenesis process, including tumor initiation, promotion and progression by many mechanisms of action, which may include inactivation of carcinogens, anti-proliferative action, cell cycle arrest, induction of apoptosis, anti-angiogenic action, reversal of multidrug resistance, antioxidant action or a combination of these mechanisms [23]. The other major compounds identified in the extract analyzed by GC-MS spectroscopy were: n-hexadecanoic acid and [2,4-Dihydroxy-2,5dimethyl-3(2H)-furan-3-one]. N-hexadecanoic acid has been reported to possess antioxidant, anti-inflammatory and antitumor activity [24-26]. It was demonstrated that n-hexadecanoic acid exert anti-inflammatory activity through competitive inhibition of phospholipase A_2 [27]. Phospholipase A_2 has a critical role in angiogenesis, tumorigenesis and tumor metastasis [28,29]. [2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3one] has been reported to possess antioxidant and anti-inflammatory activity [30].

The DPPH assay is based on the measurement of the scavenging capacity of antioxidants towards the stable free radical, DPPH. When DPPH solution is mixed with antioxidant, the odd electron of nitrogen atom in DPPH is reduced to the corresponding hydrazine by receiving a hydrogen atom from the antioxidant compound with the loss of violet color [31]. Free radicals are chemical species that contain unpaired electrons [32]. They are produced in biological systems and exogenously, and are known to cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and aging [33].

In this study, the methanol extract showed good antioxidant activity (in terms of DPPH scavenging activity). This agree with previous studies in which methanol was the most efficient solvent for extraction of antioxidant compounds, particularly phenolic compounds [34-36]. Phenolic compounds, such as flavonoids; possess many biological effects, mostly attributed to their antioxidant activities [37]. The mechanisms of antioxidant action of flavonoids can include: (1) Inhibition of ROS formation either by chelating trace elements or by inhibition of enzymes involved in free radical generation; (2) Scavenging ROS; and (3) Up-regulation of antioxidant defenses [38, 39].

The inhibitory effect of methanol extract of *Zizyphus spina christi* on RD cell line could be due to its antioxidant activity. Antioxidants are thought to prevent the initiation step of carcinogenesis, mainly due to their capacity to protect cells from the damage induced by free radicals. Damage to DNA by reactive oxygen species (ROS) has been widely accepted as a major cause of cancer [40, 41].

CONCLUSION

Methanol extract of *Zizyphus spina christi* showed no cytotoxic activity against RD cell line. However, the data showed a dose related inhibitory activity against RD cell line; which could be attributed to the antioxidant activity of the methanol extract as shown in DPPH assay. Furthermore, the GC-MS analysis showed

the presence of compounds with antiproliferative activity (eg, flavonoids).

CONFLICTS OF INTERESTS

Declared none

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