RESEARCH ARTICLE

Redounding of Cuscuta chinensis Lam. on BxPC-3, HepG2, and U2OS Human Cancer Cell Lines

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ABSTRACT

Objective: The present study was designed to investigate *in vitro* cytotoxic effect of aqueous extract from whole *Cuscuta chinensis* on human hepatocellular carcinoma (HepG2), biopsy xenograft of pancreatic carcinoma line-3 (BxPC-3), and children human bone osteosarcoma cell line (U2OS)**.**

Materials and Methods: The anticancer effectiveness of the methanol-watery extract of *C. chinensis* Lam. was determined by using methyl tetrazolium bromide test (MTT) assay against cancer cells by using suspensions of BxPC-3, HepG2, and U2OS cell lines. The inhibitory concentration (IC_{50}) was tested for each cancer cell line. BxPC-3, HepG2, and U2OS cell line death percent after incubation with extract for 24, 48, and 72-hours interval was compared with cisplatin death percent.

Results: The results showed that the IC_{50} of *Cuscuta* extract for BxPC-3, HepG2, and U2OS cell lines was 13, 6.5, and 0.73 µg/mL, respectively. The HepG2 cell line death%, when treated with 50 µg/mL *Cuscuta* extract at 24, 48, and 72-hour time interval, was 90.41, 91.45, and 92.93%, while cells were treated with 15 µg/mL cisplatin, the death percent was 88.8, 93.7, and 96.61%, respectively. The BxPC-3 cell line death%, when treated with 50 µg/mL *Cuscuta* extract, was 51.46, 83.37, and 91.28%, respectively, and when treated with 15 µg/mL cisplatin was 81.64, 88.02, and 96.67%, respectively. The U2OS cell line death%, when treated with 50 µg/mL *Cuscuta* extract, was 69.43, 69.75, and 88.89%, and was 74.1, 84.61, and 93.39%, respectively, when treated with 15 µg/mL cisplatin.

Conclusion: The methanol-watery extract of *C. chinensis* Lam. may have a potential role as an adjunct therapy for cancers in the future.

Keywords: Cancer cell line, *Cuscuta chinensis* Lam., IC_{50}.

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INTRODUCTION

The anticancer effect of medicinal plants was widely investigated by researchers.[1,2] Some of the currently-used anticancer chemotherapeutic agents were initially derived from plants.[3-5] *C. chinensis* Lam. (first described in China in 1786) was an annual voluble parasitic plant of the family Convolvulaceae.[6,7] It was a leafless plant and sucking nutrients by haustorians from the host plant.[8] *C. chinensis* Lam. has been used as a herbal medicine in several Asian countries for centuries.[9] Although studies concluded the cytotoxic effects, antimutagenic activity immunostimulating and antioxidant functions of this plant against tumors,[10-13] others showed that *C. chinensis* stimulated the proliferation of epidermoid carcinoma cell line, human breast cancer (MCF7) cell line,[14] lymphocytes, and rat bone cells.[13] There is, however, no direct experimental evidence for the cytotoxic effect of aqueous extract of whole *C. chinensis* on cancer cells. Therefore, the present study was designed to investigate *in vitro* cytotoxic effect of aqueous extract from whole *C. chinensis* on human hepatocellular carcinoma (HepG2), biopsy xenograft of pancreatic carcinoma line-3 (BxPC-3), and children human bone osteosarcoma cell line (U2OS) using MTT. In conclusion, results suggest that methanol-watery extract of *C. chinensis* Lam. may have a potential role as an adjunct therapy for cancers in the future.

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MATERIALS AND METHODS

Plant Collection, Identification, and Drying

The hallow plant of *Cuscuta chinensis* Lam. was collected at the duration mid of November to mid of December 2016 from gardens of Babylon University, then the plant was identified by Dr. Nedaa Adnan (Plant herbarium, Department of Biology, College of Science, University of Babylon). The collected plant was dried in shade at room temperature (RT) for 10 days. The dried plant was milled by using an electric mill.

Plant Extract Preparation

The dried and powdered plant materials were extracted with solvent methanol-water (1:1 v/v), according to Ekpenyong *et al.* With some modification, 1-gram of plant powder:10 mL of solvent was blended for 30 minutes at RT. The suspension was filtered by guise and the filtrated liquid was concentrated to dryness in an oven at 45ºC. The dried concentrated material was milled by using an electric mill and the final powder was sterilized by UV equipment for 20 minutes.[15]

Cell Culture

Biopsy xenograft of pancreatic carcinoma line-3 (BxPC-3) and children liver hepatocellular carcinoma cell line (HepG2) were purchased from American Type Culture Collection ATCC (Middlesex, UK), children human bone osteosarcoma cell line (U2OS) was originally obtained from Sigma Aldrich and stored in the Cell Bank of the Tissue Culture Centre at the University of Al-Mustansiriyah, College of Pharmacy. BxPC-3, HepG2, and U2OS cell lines were used as model cancer cells for this study.

Cell Maintenance

BxPC-3 and HepG2 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Merelbeke, Belgium) supplemented with 5% fetal bovine serum FBS (Fisher Scientific, USA) and 1% L-glutamine (Lonza, UK), as well as, to 1% penicillin-streptomycin-amphotericin B 100X (Lonza, UK) as antiseptic. U2OS cells were maintained in DMEM medium (Lonza, UK) supplemented with 5% fetal bovine serum FBS and 1% L-glutamine (Lonza, UK), as well as, to 1% penicillin-streptomycin-amphotericin B 100X (Lonza, UK) as antiseptic. Cells were cultured in 75 cm^2 flasks and incubated in 5% CO_2/ 95% humidified air at 37°C. Once the cells reached 90% confluency, flasks containing BxPC-3 or HepG2, U2OS cells were passaged under sterile conditions. The cells were washed with 5 mL of phosphate-buffered saline solution (PBS) and then incubated for 2 minutes in trypsin solution at 37°C to allow cells to detach from the bottom of the flask. An equal volume of complete growth media was added and the cell suspension was transferred into a 50 mL conical tube. Cells were then centrifuged at 1,200 rpm for 3 minutes. The supernatant was discarded and the cell pellet resuspended in fresh supplemented growth media. Cells were then counted under the microscope on a hemocytometer and used as required.

Storage and Resuscitation of Cell Lines

Following trypsinization of a confluent 75 cm^2 flask, the cell suspension was centrifuged at 1,200 rpm for 3 minutes. The cell pellet was then resuspended in 4 mL freezing medium (Life Technologies) and 1 mL aliquots were added to cryovials (Thermo Fisher Scientific, Loughborough, UK). The cells were stored at -80°C for 24 hours, and were stored under liquid nitrogen for long-term storage. Cells stored under liquid nitrogen were quickly thawed at 37°C and added to 10 mL fresh growth media. The cells were harvested by centrifugation and resuspended in 25 mL of fresh medium and transferred to a 75 cm^2 flask and grown.

Cell Viability and Inhibitory Concentration (IC_{50}) by MTT Assay

The MTT assay was used to assess the effects of methanol-watery extract of whole *C. chinensis* on cancer cell viability. A 100 µL from all cells suspensions (BxPC-3, HepG2, and U2OS) were dispensed into 96-well flat-bottom tissue culture plates (Falcon, USA) at concentrations of 5×10^3 cells per well and incubated 24 hours under standard conditions; 4×10^3 cells/well for 48 hours incubation, and 3×10^3 cells/well for 72 hours incubation. After 24 hours, the cells were treated with 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL of the plant extract. After a recovery period, 24, 48, and 72 hours, the cell culture medium was removed and cultures were incubated with a medium containing 30 µL of MTT solution (3 mg/mL MTT in PBS) (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) for 4 hours at 37°C. After 4 hours, this medium was removed by gentle inversion and tapping onto paper. Control wells received only 100 µL growth media. 100 µL of dimethyl sulfoxide (DMSO) was added to each well, the plates were then kept at room temperature in the dark for about 15 to 20 minutes. The absorbance of each well was measured by a multiscan reader at a wavelength of 540 nm and correcting for background absorbance using a wavelength of 650 nm. The cells' viability was determined according to the optical density (OD) of the wells which contained no extract. The inhibitory concentration 50% (IC_{50}) was defined as the minimum concentration of the extract that reduced viability of the incubated cells after 72 hours by 50%.

Statistical Analysis

Data were analyzed using the Microsoft Office Excel (2007) SPSS software package. Multiple comparisons of treatments were performed using one-way analysis of variance (ANOVA). A difference was considered to have significance at $p < 0.05$. Data are presented as mean ± standard deviation of three replicates. Experimental results are shown as mean ± standard error of the mean (SEM). MTT tests were replicated three times. The IC_{50} values were calculated from the linear regression analysis.

RESULTS

Percentage of Cell Death of Human Biopsy Xenograft of Pancreatic Carcinoma Line-3 Cell Line (BxPC-3) by Methanol-Watery Extract of whole *C. chinensis*

To estimate the effect of the extract on BxPC-3 cells viability, BxPC-3 cells were treated with 0.75, 1.5, 3.12, 6.25, 12.5,

25, and 50 µg/mL extract at 24, 48, and 72 hours (Figure 1) $p < 0.005$. Methanol-watery extract of whole *C. chinensis* significantly increases cell death of BxPC-3 at 50 µg/mL (51, 83, and 91%) at 24, 48, and 72 hours, respectively, $p < 0.001$, and 25 µg/mL (37, 42, and 60%) at 24, 48, and 72 hours, respectively, $p < 0.05$ *vs.* other concentrations (Figure 1). BxPC-3 cell line treated with extract in concentration 50 µg/mL compared to the cisplatin (control) showed high significant difference $p < 0.001$.

Percentage of Cell Death of Human Children Liver Hepatocellular Carcinoma Cell Line (HepG2) by Methanol-Watery Extract of whole *C. chinensis*

HepG2 cell line showed highly cytotoxicity effect at concentration 50 and 25 µg/mL (90, 91, 92.9 and 82, 83, 85%), respectively, at 24, 48, and 72 hours, as compared to other concentrations which showed low death percentage as illustrated in Figure 2, $p < 0.0001$. HepG2 cell line treated with methanol-watery extract of whole *C. chinensis* in concentration 50 and 25 µg/mL compared to the cisplatin (control) showed high significant difference $p < 0.0005$ and $p < 0.0001$, respectively.

Percentage of Cell Death of Children Human Bone Osteosarcoma Cell Line U2OS by Methanol-Watery Extract of whole *C. chinensis*

To determine the effect on U2OS cell viability, MTT assay was conducted. The results of the MTT assay showed that 50 µg/mL methanol-watery extract of whole *C. chinensis* was clearly capable of reducing cell viability after 72 hours, $p < 0.0001$ (Figure 3). U2OS cell line treated with methanol-watery extract of whole *C. chinensis* in concentration 50, 25, and 12.5 µg/mL compared to the cisplatin (control) showed a high significant difference $p < 0.0001$.

Half Maximal Inhibitory Concentration (IC_{50}) Value of Methanol-Watery Extract of whole *C. chinensis*

The dose-response curve generated by Origin 9.1 using nonlinear regression analysis for methanol-watery extract of whole *C. chinensis* in BxPC-3, HepG2, and U2OS cells are shown in Figures 4a to c. The IC_{50} values were obtained to a range of concentrations of methanol-watery extract of whole *C. chinensis* from 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL by MTT assay. The results of IC_{50} for methanol-watery extract of whole *C. chinensis* were 13, 6.5, and 0.73 µg/mL in BxPC-3, HepG2, and U2OS cells, respectively (Figure 4).

DISCUSSION

The anticancer effect of medicinal plants and components isolated from them is the focus of many pieces of research.[17] They regarded as promising anticancer side to side with other conventional anticancer strategies. Phytochemicals "plant-derived compounds" have been identified to have an antineoplastic effect by induction of apoptosis and/or inhibition of cell proliferation.[16] The low or non-toxic effect of some phytochemicals and their availability in an ingestive form makes them suitable to be used and encourage many researchers to study the precise mechanism of actions. In this study, we aimed to determine the cytotoxicity of methanol extract of whole *C. chinensis* on BxPC-3, HepG2, and U2OS

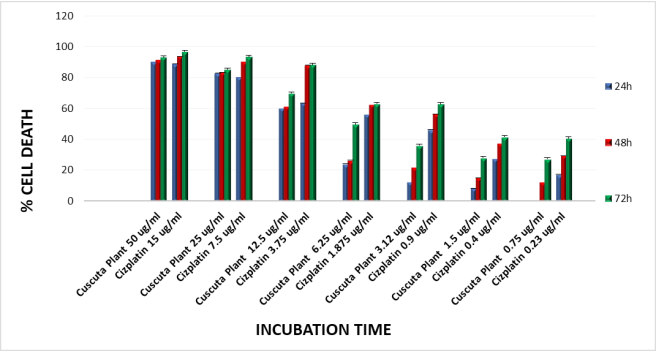

Figure 2: *In vitro* cell death percentage of the human Children liver hepatocellular carcinoma cell line (HepG2), was estimated by MTT assay in 96-well plates following 24, 48, and 72 hours exposure to 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL methanol-watery extract of whole *C. chinensis*; data is shown as % mean ± SEM of cell death for three separate experiments; treated were significantly different from the untreated controls $p < 0.0001$

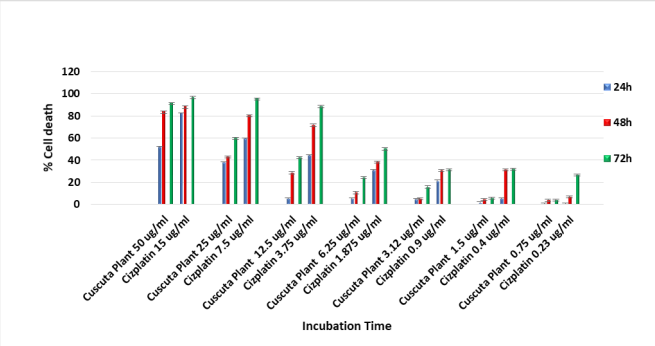

Figure 1: *In vitro* cell death percentage of the human biopsy xenograft of pancreatic carcinoma line-3 cell line (BxPC-3) was estimated by MTT assay in 96-well plates following 24, 48, and 72 hours exposure to 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL methanol-watery extract of whole *C. chinensis*; data is shown as % mean ± SEM of cell death for three separate experiments; treated were significantly different from the untreated controls $p < 0.001$

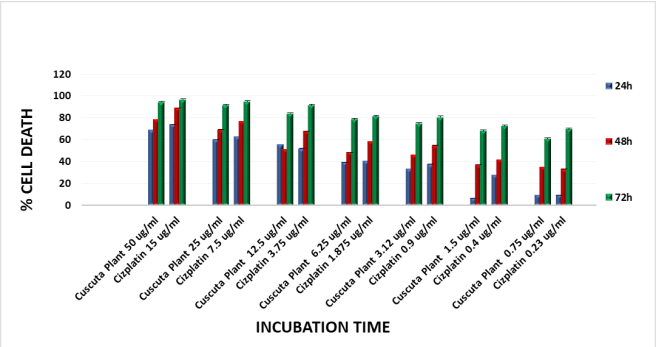

Figure 3: *In vitro* cell death percentage of the children human bone osteosarcoma cell line (U2OS) was estimated by MTT assay in 96-well plates following 24, 48, and 72 hours exposure to 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL methanol-watery extract of whole *C. chinensis*; data is shown as % mean ± SEM of cell death for three separate experiments; treated were significantly different from the untreated controls $p < 0.0001$

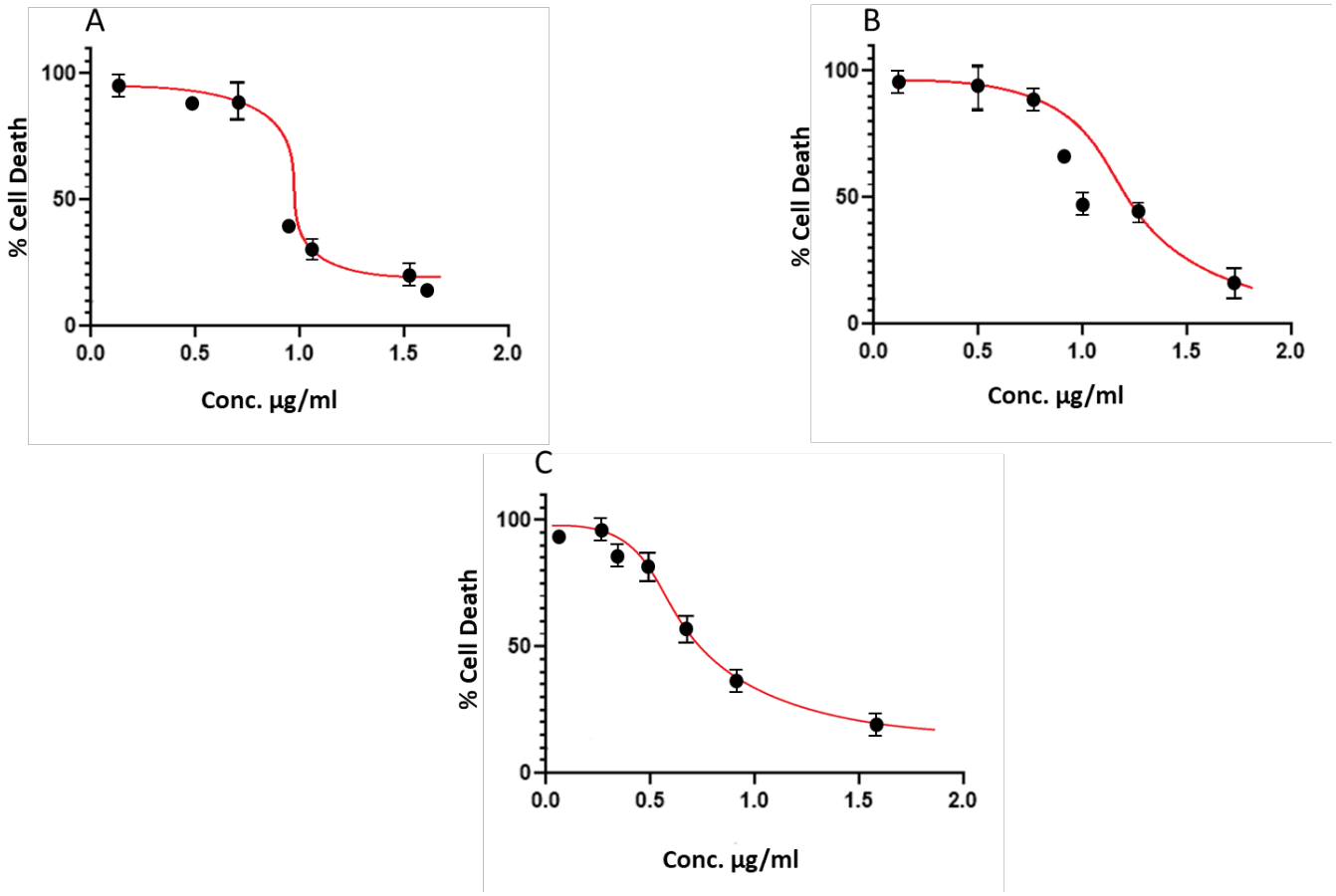

Figure 4: Dose-response curves of IC_{50} for methanol-watery extract of whole *C. chinensis*: **A.** BxPC-3, **B.** HepG2, and **C.** U2OS cells were treated for 72 hours with 0.75, 1.5, 3.12, 6.25, 12.5, 25, and 50 µg/mL dose ranges of the extract; the normalized dose-response for extract was plotted over log-transformed methanol-watery extract of whole *C. chinensis* concentrations; IC_{50} values were determined using nonlinear regression analysis (Prism 8); error bars represent the standard error of the mean (SEM) for triplicate data

cell lines using MTT assay with the measurement of IC_{50}. Comparison of cytotoxic activity of *C. chinensis* extracts to cisplatin chemotherapy for these cell lines exhibited by IC_{50}, which was 13, 6.5, and 0.73 µg/mL, respectively. The extract showed an anti-proliferative effect in all cell lines that had been used in this study with maximum results obtained in concentration 50 µg/mL and after 72 hours. The growth inhibition of HepG2, BxPC-3, and U2OS cell line when treated with 50 µg/mL *C. chinensis* extract at 72 hours was 93, 91, and 89%, respectively. The anti-neoplastic activity of *C. chinensis* also had been noticed against some cancer cell lines, such as, human Caucasian acute lymphoblastic leukemia (CCRF-CEM) and a human lymphocyte.[19] Donnapee *et al.* confirmed that *C. chinensis* possess a high level of total flavonoids and total phenolic acids, caffeic acid, kaempferol, quercetin, and beta-carotene,[20] all these chemicals have a strong antioxidant and anti-proliferative activity against various cancer cell lines and tumors.[21,22] Al-sultany in 2018 demonstrated that *C. chinensis* was rich in berberine and by using HPLC approve its presence in a concentration of 246 ppm.[23] Berberine could induce cell death in various cancer cells, such as, breast cancer, liver cancer, and lung cancer.[24] Induction of an effective apoptosis is the cause of cell death in many cell lines and xenografts.[25] It had been shown that berberine induces PARP cleavage and nuclear condensation on FaDu cells largely by activating intrinsic apoptotic pathway (mitochondrial pathway) with subsequent release of scytochrome c and AIF into the cytosol.[26,27] Mitochondria is a key target in many apoptotic events, for this reason the effect of berberine on this organelle was studied. Berberine inhibits mitochondrial complex I and interacts with the adenine nucleotide translocation.[28-31] It up regulates the expression of pro-apoptotique Bax and Bak proteins, and decreases the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL.[32-34] Finally, a number of caspases, such as, caspase-3, 4, 7, 8, and 9 may be directly activated by berberine.[30,31] With subsequent studies, researches highlight the berberine as an apoptotic enhancer by activating extrinsic apoptotic pathway.[35] In human cervical carcinoma cells (HeLa), berberine increases the expression of Fas, FasL, TNF-α, and TRAF-1, and then activates caspase-8 and 3.[36] On the other hand, berberine may regulate caspase-independent cell death by inducing the breakage of DNA strands by topoisomerase inhibition.[37] Many other molecules related to apoptosis have also been reported to be involved in cell apoptosis induced by berberine. Molecular studies showed that berberine upregulates the expression of p53 and p27 that were playing a pro-apoptotic role and inhibit

H-ras, c-fos oncogene in T24 bladder cancer cell line,[29-36] and down-regulate expression of mir-21 and increase apoptosis in the U266 multiple myeloma cancer cell line.[40] Berberine has been identified to suppress the activity of RET proto-oncogene by binding and stabilizing the RET G-quadruplex, which further activated the apoptotic mechanism validated with the increasing activity of caspase-3 in the human medullary thyroid carcinoma cell line (TT) and HEK-293 cell line.[41] In addition to berberine and its role as anti-cancer phytochemical, *C. chinensis* was rich in other important anticancer compounds, like kaempferol, quercetin, and β–carotene.[23,42]

In summary, the study documents the antineoplastic effect of *C. chinensis* Lam. extract on cancer cells. The results presented here suggest that methanol-watery extract of whole *C. chinensis* might be valuable in the treatment of these types of cancers. More studies are needed, for example, the cellular mechanism of action of the methanol-watery extract of whole *C. chinensis* mediated apoptosis of cells, besides, the methanol-watery extract of whole *C. chinensis* induced apoptosis needs to be investigated in suitable *vivo* models.

CONCLUSION

The methanol-watery extract of whole *C. chinensis* may have a potential role as an adjunct therapy for pancreatic, liver, and bone osteosarcoma cancers in the future.

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CONTRIBUTORSHIP STATEMENT

All authors participated in study design, data analysis, and writing of this manuscript. The plant extract was prepared by Dr. Fadia in the postgraduate laboratories of the Biology Department, College of Science, Babylon University. MTT protocol was conducted by Dr. Basma in postgraduate laboratories of Pharmacology and Toxicology Department, College of Pharmacy, Mustansiriyah University.

ETHICAL APPROVAL

All authors declare that the research was registered and approved by the local ethical committee of the College of Pharmacy, Mustansiriyah University, Iraq.

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