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ANTIPROLIFERATIVE ACTIVITY OF *ALLIUM AMPELOPRASUM VAR. PORRUM* AND METFORMIN AGAINST LIVER CANCER CELL LINE

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Abstract

Cancer is an uncontrolled increase in abnormal cells in the body. Cancer treatment by natural compounds and their semi-synthetic analogs both *In vitro* and *in vivo* show promising results against different malignancies, natural compounds can be substituted or used in combination with existing drugs. Allium genus is rich in sulfur compounds, steroidal saponins, flavonoids, and so on, with anticancer, antioxidant, antiplatelet aggregation, antiatherosclerosis, antimicrobial and lower blood lipids and blood glucose biological activity. Metformin has recently received increased attention to its potential antitumorigenic effects, which are thought to be independent of its hypoglycemic effects. The study aimed to investigate the antiproliferative activity of *Allium ampeloprasum var. porrum*, metformin and *Allium ampeloprasum var. porrum* combined with metformin against liver cancer cell line, and to investigate the possible effect of *Allium ampeloprasum var. porrum* on gene p53. Dry powdered leaves of the *Allium ampeloprasum var. porrum* were extracted with methanol. The Crude extract and metformin was tested for antiproliferative activity against hepatocellular carcinoma cell line by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide as *In vitro* assay, metformin was added to methanol extract and the combination was tested against hepatocellular carcinoma cell line. Quantitative analysis of p53-targeted gene expression by real-time polymerase chain reaction was performed for methanol extract. The results showed a dose dependent inhibition for liver cancer cell growth after 72 hours for methanol extract, metformin and combination of *Allium ampeloprasum var. porrum* leaves methanol extract with metformin. The concentration which exhibited 50% cytotoxicity values for methanol extract was 38.475µg/ml, for metformin was 38.99µg/ml and for combination of (metformin and methanol extract) was 1.3635µg/ml. *Allium ampeloprasum var. porrum* leaves methanol extract showed a fold increment in P53 gene expression of 0.53. It can be conclude that Methanol extract of *Allium ampeloprasum var. porrum* leaves and metformin exhibited antiproliferative activity against hepatocellular carcinoma cell line. Methanol extract of *Allium ampeloprasum var. porrum* leaves combined with metformin had synergistic effect. *Allium ampeloprasum var. porrum* leaves methanol extract showed upregulation of P53.

Key words: Antiproliferative, Allium ampeloprasum *var.* porrum, Metformin, liver Cancer

Introduction

Cancer is an uncontrolled increase in abnormal cells in the body (Safarzadeh *et al*., 2014). It is a group of diseases that can occur in any tissue in the body and can be classified as benign or malignant, if malignant cancer cells are allowed to grow uncontrollably, they may eventually lead to the death of the patient, while benign cancer cells cannot spread through tissue invasion or metastasization (Alldredge *et al*., 2013). Liver, gallbladder and bile ducts are some of the most common primary and metastatic cancer sites (Kerr *et al.,* 2016). Primary liver cancer includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) and other rare types (Petrick *et al*., 2016). More than half a million new cases reported annually worldwide and close to the same incidence and mortality rates of HCC remain one of the most intractable gastrointestinal cancers (Asnacios *et al*., 2008). Unlike other tumor types, treatment is primarily dictated by liver functional reserve and patient performance status, and only secondary to tumor size (O'Sullivan *et al*., 2015). Chemotherapeutic drugs are cytotoxic and can kill both cancer cells and rapidly divide normal cells, cancer treatment by natural compounds and their semi-synthetic analogs both *In vitro* and *in vivo* show promising results against different malignancies, natural compounds can be substituted or used in combination with existing drugs (Aung *et al*., 2017). *Allium ampeloprasum var. porrum* is a worldwide grown

vegetable, although its importance is most significant in the temperate zone of Europe (Bernaert *et al*., 2011). Allium genus is rich in sulfur compounds, steroidal saponins, flavonoids and so on, with anticancer, antioxidant, antiplatelet aggregation, antiatherosclerosis, antimicrobial and lower blood lipids and blood glucose biological activity, S-alk(en)yl-l-cysteine sulfoxides are secondary cysteine metabolites highly accumulated in the Allium genus (Zeng *et al*., 2017).

Metformin has recently received increased attention to its potential antitumorigenic effects, which are thought to be independent of its hypoglycemic effects, evaluated in multiple *In vitro* and in vivo studies, and is currently being tested in clinical trials as an adjunct to classical chemotherapy regimens (Kourelis and Siegel, 2012).

Materials and Methods

Collection and identification of plant material

Allium ampeloprasum var. porrum leaves was obtained from local market in Baghdad. The plant was identified and authenticated by the Department of Pharmacognosy and medicinal plants at AL-Mustansiriyah University / College of Pharmacy.

Allium ampeloprasum var. porrum leaves were well washed with tap water and washed out of dust and dirt, then left to air dry for 5 days, then powdered, sieved and stored in a well closed container.

Preparation of Methanol Crude Extract

300g of *Allium ampeloprasum var. porrum* leaves powder was extracted with methanol, soaked with the solvent and remained in the shaking water bath at 40°C for 24 hours, then filtered by Whatman no.1 filter paper to obtain a clear extract. The extract was concentrated by rotary evaporator with vaccum (Al-Zubaidy *et al*., 2016).

Stock solution preparation

Crude methanol extract of *Allium ampeloprasum var. porrum* leaves were stored in a freezer at -20°C until used. 10 mg of the crude extract were dissolved in 1 ml dimethyl sulfoxide (DMSO, Santacruz Biotechnology) the yield was 10mg/ml as stock solution (Badgujar *et al*., 2017).

Metformin stock solution

The stock solution of metformin is (10mg/1ml DMSO). 5µl was taken from metformin and serum free media (995µl) to 1 ml as a stock solution stored at 4°C in between uses (Ali *et al*., 2016).

Serial Dilution of Methanol Extract

From stock solution of methanol extract and metformin five different concentration were prepared (100, 50, 25, 12.5, 6.25 µg/ml).

Preparation of (DMSO) solution as control

Control was prepared by placing 20µl DMSO in 980µl CGM in eppendrof tube to make 1ml.

Preparation of hepatocellular carcinoma cell line (HC) for cytotoxicity assays

Cancer cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/ mL penicillin and 100µg/ml streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week and incubated at 37°C in a humidified 5% CO_2 atmosphere (Al-Shammari *et al.*, 2016).

MTT assay

The MTT cell viability assay was done using 96-well plates. Cell lines were seeded at 1×10^4 cells/well. After 24 hours when a confluent monolayer was achieved, cells were treated with serial concentrations of *Allium ampeloprasum var. porrum* extract and metformin. After 72 hours the medium was removed and 28 µl of MTT solution was added to each well. Followed by incubating the plates for 2.5 hours at 37°C in 5% CO_2, the plates were taken out from the incubator and the supernatant layer was removed. After removal of the MTT solution; the remaining crystals in the wells were solubilized by addition of 130 µl of DMSO (Dimethyl Sulphoxide) followed by 37°C incubation for 15 min with shaking. The absorbance analysis was occupied at 492 nm also the reference at 650nm by micro-plate reader; the assay was performed in triplicate (Jabir *et al*., 2019).

The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:

Cell viability % = Mean sample absorbance/ mean control absorbance × 100%.

Inhibition %= 1- cell viability %.

IC_{50} values were calculated by the linear and logarithmic correlation equation.

A plot of % cytotoxicity against sample concentrations was assist to calculate the concentration which exhibited 50% cytotoxicity (IC_{50}).

Determination of P53 gene expression in HC cell line

Cancer cells collecting

After cells were exposed to the determined inhibition concentration 50% of the *Allium ampeloprasum var. porrum* leaves methanol extract for the determined time of incubation in the tissue culture vessels, methanol extract

had been nominated for further experiment, cells were harvested. The attached cells were collected from the vessel with trypsin versene solution, then transferred to a 10 ml centrifuge tube and centrifugation carried out for 10 min at 1500 rpm in 4°C. The cells pellet was re-suspended in 1 ml of PBS and centrifuged again at the same conditions, then the supernatant was discarded and the cells pellet was re-suspended in 200 µL of PBS and kept in deep freeze (-85°C).

Extraction of cellular RNA

After cells were thawed the cellular RNA was extracted using AccuZol™ solution, it's a ready-to-use reagent for the isolation of total RNA from various sample materials. The procedure used according to the manufacturer instruction.

Real-time polymerase chain reaction PCR

Real-time polymerase chain reaction was carried out in AriaMx real-time PCR (qPCR) instrument (agilent technologies, United States) using one-step method. The thermal profile used, where the first cycle time was 15 minutes at 42°C, the second cycle was 10 minutes at 95°C. The DNA amplification was carried out at 95°C for 10 seconds, 56°C for 15 seconds and 72°C for 20. DNA Disassociation thermal temperature consist of 95°C, 55°C and 95°C for 30 seconds for each time.

After the thermal profile was set and all the above reaction mixture was vortexed for short time and spent at 6000 rpm for 1 minute then was placed at the real-time PCR device and the run was started amplification reaction according to the manufacturer's instructions.

Determination of level of expression

The Delta Delta Ct method was used to determine the level of P53 gene expression and the fold level of mRNA increased in both *Allium ampeloprasum var. porrum* leaves methanol extract treated and untreated cancer cell line, another gene needed to be determined in the same run used to be called a house keeping gene, this study used 18rS gene (or muGAPDH for HC cells). The fold of mRNA increase or decrease was calculated using the formula 2^-(ΔΔCt) (Pfaffl, 2004) (Rao *et al*., 2013).

Statistical analysis

The statistical design for this study was presented as mean ±SD (standerd deviation). Group values were compared by one-way ANOVA then by the Tukey Post-hoc test (t-test) and measured significants 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 *Allium ampeloprasum var. porrum* extract 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 8ml and b is the y-intercept of the line of standard curve.

Results

In vitro screening of *Allium ampeloprasum var. porrum* methanol extract and metformin on hepatocellular carcinoma cell line (HC), the results showed a dose dependent inhibition for cancer cell growth after 72 hours for methanol extract and metformin, the extract concentrations used were 100, 50, 25, 12.5 and 6.25 µg/ml.

The IC50 value was deduced from the graph for each extract of *Allium ampeloprasum var. porrum* leaves by using the following linear regression equations: for methanol [y = 12.865ln (x) + 3.0427], for metformin [y = 9.7272ln (x) + 14.366] and for combination of (metformin and methanol extract) [y = 6.8371ln (x) + 47.88]. Where Y = the percentage of inhibition and X = concentration. The IC50 values for methanol extract was 38.475µg/ml, for metformin was 38.99µg/ml and for combination of (metformin and methanol extract) was 1.3635µg/ml.

Fig. 1, 2 and 3 show the dose response curve of ME, metformin and combination of (metformin and methanol extract) respectively.

Determination of P53 gene expression in HC cell line

Fig. 4 represent the amplification curves of the real-time RT-PCR. The Ct values of both the HKG and the GOI obtained from the real-time RT-PCR are represented in table 1. The increment in P53 gene expression represented in Fig. 5.

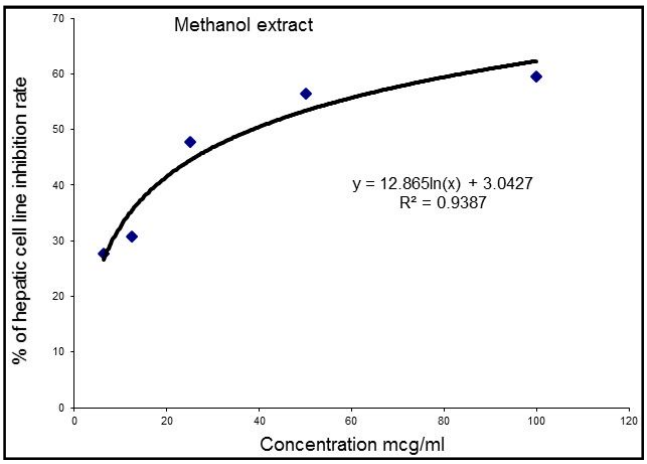

Fig. 1: Dose response curve of methanol extract of *Allium ampeloprasum var. porrum* for HC cancer cell line.

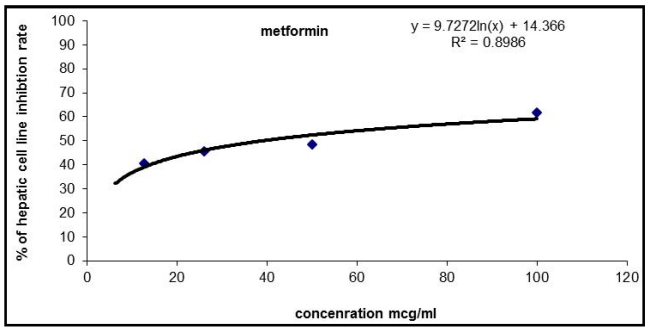

Fig. 2: Dose response curve of metformin for HC cancer cell line.

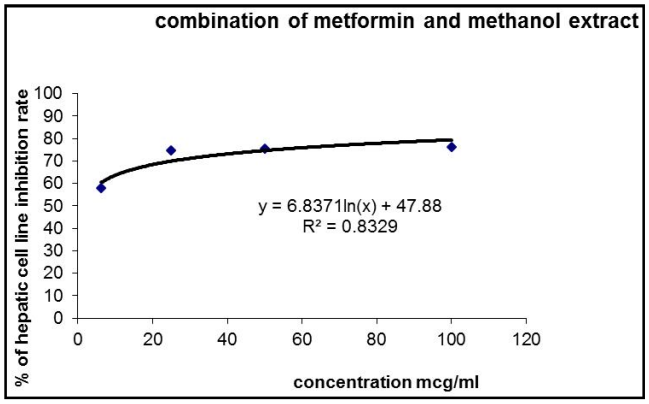

Fig. 3: Dose response curve of combination(metformin and methanol extract of *Allium ampeloprasum var. porrum*) for HC cancer cell line.

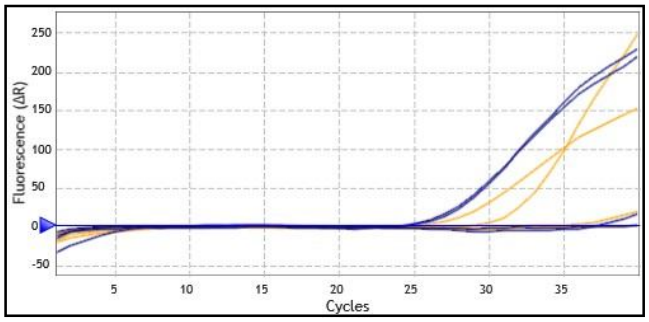

Fig. 4: Amplification curves of RT-PCR for GAPDH and P53 gene in hepatocellular carcinoma cell line (HC) treated with *Allium ampeloprasum var. porrum* extract.

Table 1: Cycles threshold (Ct) of the house keeping gene (GAPDH) and gene of interest (P53).

Gene Name	Ct Control untreated cells	Ct Cells treated with *Allium ampeloprasum var. porrum* extract	Δ Ct	ΔΔ Ct	Fold increment In P53 gene expression
GAPDH	36.39	38.39	9.95 (control)	1.47	0.53
P53	26.44	29.91	8.48 (treatment)		

Discussion

Allium species have been recognized as rich sources

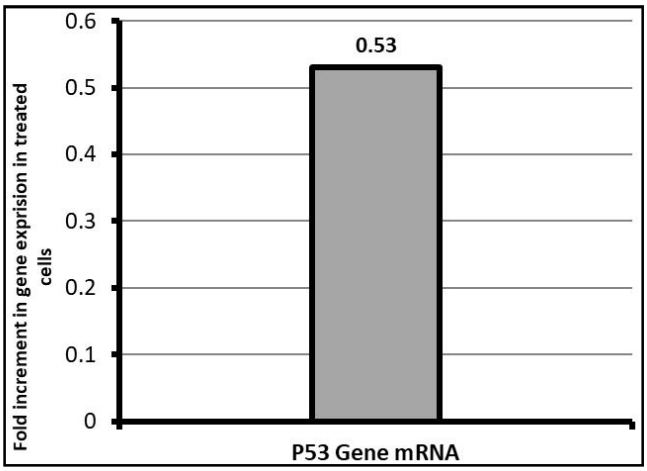

Fig. 5: Fold increment in P53 gene as a result of treating the hepatocellular carcinoma cell line (HC) with *Allium ampeloprasum var. porrum* extract.

of secondary metabolites, such as polyphenolic compounds, including phenolic acids, flavonoids and flavonoid polymers with associated health benefits (Bernaert *et al*., 2013). Extraction is the essential first step in the analysis of medicinal plants, as it is necessary to extract the desired chemical components from the plant materials for further separation and characterization (Sasidharan *et al.*, 2011). Dry fine powder of *Allium ampeloprasum var. porrum* leaves have been used as raw materials for extraction, the advantage of using dried plant material instead of fresh plant material for herbal products is that dried plant materials is easier to store and also has a longer shelf life (Phrompittayarat *et al.*, 2007). Extraction method used in this study was cold method or maceration method, since this method is the most appropriate method of extracting dried leaves, promoting high yield of crude extract, the highest content of total phenolics, total flavonoids, major active compounds, and the most potent antioxidant activity (Vongsak *et al*., 2013).

The antiproliferative activity was tested by the MTT assay. *In vitro* screening of *Allium ampeloprasum var. porrum* extract and metformin against hepatocellular carcinoma cell line showed a dose dependent inhibition for cancer cell growth after 72 hours for methanol extract and metformin. The 50% inhibition of cell growth (IC_{50}) value was deduced from the graph for *Allium ampeloprasum var. porrum* leaves extract and metformin, it was 38.475µg/ml for methanol extract and 38.99µg/ml for metformin. The criteria used to categorize the activity of *Allium ampeloprasum var. porrum* leaves extract against hepatocellular carcinoma cell line based on IC_{50} values, were modified from those of NCI and Geran *et al.*, as the IC_{50} of *Allium ampeloprasum var. porrum* leaves extract between 21 - 200 µg/ml, so it can

be considered as moderately active (Srisawat *et al*., 2013). In the present study, the leaves extract of *Allium ampeloprasum var. porrum* and metformin significantly reduced the viability of the hepatocellular carcinoma cell line compared to control. It has recently been noted that the Allium genus has shown extensive *In vitro* and *in vivo* antiproliferative, anti-motility and cytotoxic potentials against cancer cells (Asemani *et al*., 2019). A number of studies showed potent anticancer action of Allium derivatives in model tumor systems (Pinto and Rivlin, 2001).

In vitro results of a study by Fattorusso *et al*., (2000) implied that the *Allium porrum L.* had cytotoxicity against various cancer cell lines; the results of present study might be supported by what Fattorusso and coworker found.

Saito *et al.,* (2013) investigated the impact of metformin on tumor-initiating HCC cells; it suppressed cell growth and induced apoptosis in a dose dependent manner death in agreement with the findings of present study.

In the current study the inhibitory effect of *Allium ampeloprasum var. porrum* leaves methanol extract combined with metformin were investigated, the IC_{50} for this combination was 1.3635 µg/ml, which determined by MTT assay. Depending on the IC_{50} value, the combination exhibited lower IC_{50} value than the IC_{50} value of extract alone. Therefore the methanol extract combination may have synergistic effect (Ali *et al*., 2016). It is important to mention that the anticancer effect of metformin is potentiated in the presence of other substances, flavone, epothilone and quercetin as antioxidants enhance the activity of metformin as anticancer (Aldalaien *et al*., 2018).

TP53 (tumor suppressor gene p53) is one of the most well-studied tumor suppressor genes because of its role in the protection against malignancies, and its signaling is triggered by cellular events ranging from DNA damage to hypoxia, stress and a large number of other causes (Hientz *et al*., 2017).

In our study, we used methanol extract of the *Allium ampeloprasum var. porrum* leaves for the real-time RT-PCR, the results showed a fold increment in P53 gene expression of 0.53, here we have shown the molecular mechanism responsible for the antiproliferative activity of *Allium ampeloprasum var. porrum* leaves methanol extract against hepatocellular carcinoma cell line (HC) through upregulation of P53.

Polyphenols used p53 signaling pathway to produce anticancer activity through apoptosis in variety of cancers (Khan *et al*., 2020). Lee *et al*., (2014) concluded that polyphenols isolated from *Allium cepa Linn*, a member of the family *Liliaceae*, are up-regulating p53 protein in agreement with the findings of present study.

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