Research Article

The Anti-proliferative Activity of *Vitis vinifera* Leaves of Methanol Extract Alone and in Combination with Doxorubicin against Liver Cancer Cell Line

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ABSTRACT

The aim of this study was to investigate the capability of *Vitis vinifera* leaves of methanol, water extracts alone and in combination with doxorubicin to inhibit HepG2 liver cancer cell line proliferation, and to identify the possible mechanism of action. The anti-proliferative activity of the methanol and water extracts tested by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on liver cancer cell line ($HepG_2$). Dose response relationship was shown. Significant cytotoxic activity of methanol and water extracts has identified on $HepG_2$, the IC_{50} were 7.80µg/ml and 20.8µg/ml respectively. When in combination with doxorubicin Dose response relationship was shown. Significant cytotoxic activity of the methanol extract combination but no significant cytotoxic activity of the water extracts combination, the IC_{50} were 4.2µg/ml and 31.5µg/ml respectively. These finding showed that methanol and water extracts alone had a significant dose-dependent efficiency against the growth of the cells $HepG_2$; At the same time in combination with doxorubicin methanol extract significant dose-dependent efficiency against the growth of the cells $HepG_2$ (synergistic). However, the water extracts combination did not show any cytotoxic action at the applied dose, so no toxic effect against the $HepG_2$ cell line can be expected *in vitro* (contrast action).

Keywords: *Vitis vinifera*, methanol, doxorubicin, HepG2.

INTRODUCTION

Cancer is considered as a major cause of mortality worldwide[8]. The main problems that exist with chemotherapeutic agents are severe adverse effects and multi-drug resistance formation. Some of the methods by which cancer cells become resistant to therapies are drug efflux systems, amplification of drug targets, or changes in drug kinetics[7,9]. There has been a growing interest in the use of complementary and alternative medicines (CAM), due to the disadvantages associated with conventional cancer chemotherapies and the supposed advantages of more natural treatment options[10]. Traditional use of natural compounds in cancer treatment is relatively cheap due to the availability of plants and the simple methods used in product preparation. However, commercialization of natural compounds for cancer treatment may result in dwindling of natural resources and problems with producing a consistent quality of adulteration[3].

Vitis vinifera L. (Vitaceae)[4]. The leaves of plant are rich in tannins, phenol, flavonoids and procyanidins. Additionally, the leaves also contain organic acids, lipids, enzymes and vitamins[2,5]. Grape extracts have been studied widely due to their beneficial effects on human health. Some studies have reported that grape extracts showed cytotoxicity towards cultured cells[16] as well as inhibited tumor growth in animal models[19]. Different molecular mechanisms have been proposed for these protective effects of *Vitis vinifera* extracts, such as inhibition of enzymes playing an essential role in cell proliferation (e.g. human topoisomerase I)[18] and inhibition of angiogenesis[1].Plant polyphenols account not only for antioxidant but also for anticancer activity[17]. Leaves of *Vitis vinifera* have also shown hepatoprotective effect on acetaminophen induced hepatic DNA damage[11].

MATERIALS AND METHODS

Preparation of Extract

Vitis vinifera leaves (5kg), the leaves 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

Five Kg powder *vitis vinifera* leaves was extracted with methanol for 24 hours at room temperature and with stirring; filter and the solvent is evaporated to dryness under reduced pressure at 50° C using a rotary evaporator.

Serial Dilution of Methanol Extract

There extracts dissolved in dimethyl sulfoxide (DMSO) which was referred as stock solution (10mg/ml). Form stock solutions of each extract 6 different concentration were prepared (200, 100, 50, 25, 12.5, 6.25 µg/ml).

Cell Lines and Culture Maintenance

Human Cancer cell lines used as targets were $HepG_2$ (hepatocellular carcinoma). Cell was obtained from Iran. Cells Were routinely grown as monolayer cell cultures in $50cm^2$. Flasks In an atmosphere containing 5% CO_2 In air, and 100% Relative humidity at 37 °C and sub-cultured twice a week, restricting the total number of cell passages

below 20. The Culture medium used was Dulbecco's modified Eagle's medium, DMEM (Gibco, Glasgow, UK), Supplemented with 10% Foetal bovine serum (Gibco, Glasgow, UK), 2 mM glutamine (Sigma), 100 g/ml streptomycin and 100 IU/ml penicillin. Cell passages were carried out by detaching adherent cells at a logarithmic growth phase by addition of 2–3 ml of PBS, 0.05% Trypsin– 0.02% EDTA (Gibco) 500µl and incubation for 2–5 min at 37°C. MTT added on the cell and incubated for 4 hours prior to the absorbance measurements at 570nm[13]. The loss of membrane integrity, as a morphological characteristic for cell death. The Number of cells that were alive was estimated through a haematocytometer and phase-contrast microscopy[6].

Cell Proliferation Assay

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay was used to identify the cell line proliferation capability in according to Mosmann method. All of the cells were between passages 4-7.

The cells were treated with numerous concentrations of *Vitis vinifera leaves* extract for 48 hrs. All Extracts were tested triplicate dilutions in complete growth medium, starting with a peak concentration of 200µ g/µl. The Cytotoxic activity of all agents was tested in concentrations covering the range of 6.25–200 µg/µl. For the experiments, cells were plated (100 per well). MTT was prepared by adding 5mg/ml in PBS (phosphate buffer saline). 20µl of MTT was used per well and the plates were incubated at 37°C, in 5% CO2 for 5hrs.

The plates were removed from the incubator and the supernatant was removed. (200µl) of DMSO was added to all wells. The plates were shaken vigorously for one minute at room temperature to dissolve the dark blue crystals. The absorbance was taken at 570nm and the reference at 650nm by using enzyme-linked immunosorbent assay (ELISA). The absorbance of cells cultured in control media was taken to represent 100% viability. The viability of treated cells was determined as a percentage of untreated control.

Each concentration was tested in quadruplicate, and the experiment was repeated twice. The concentration of the cells in each well was $1x10^4$, the percentage of cell line inhibition was determined as the mean ± SD. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value ("blank" is the mean optical density of the background control wells). Mean values from trireplicate wells were calculated automatically[12]. Results were expressed as triplicate Determinations gave a CV (standard deviation/mean %). Extract Potency against cancer cell growth was expressed in terms of IC_{50} Values (50% Inhibitory concentration) calculated from the plotted dose effect curves (through least-square regression analysis).[14] The cytotoxicity (%) was calculated as follows, and corrected for cytotoxicity due to DMSO in the control. Inhibition %= 1- $\{(OD_{sample}-OD_{blank})/OD_{control} - OD_{blank}\}\times100$.[15]

Statistical Analysis

Data were expressed as mean ± standard deviation (SD, n = 3) and percentage of inhibition of $HepG_2$. Statistical followed by IC_{50} significant cytotoxicity. IC_{50} is the concentration that inhibits 50% from the cell growth. Cells proliferation (IC_{50}) was analyzed and calculated for the *vitis vinifera leaves* extracts by linear regression equation: y=*m*x+b Where y is the percentage of inhibition and it set to be 50%, *m* is the slope of the standard curve, x is the concentration of compound tested in µg/mL, and b is the y-intercept of the line of standard curve[20].

RESULTS

Activity of *Vitis vinifera* methanol leaves extracts alone on $HepG_2$ liver cancer cell line, (Figure 1) show the dose response curve for the *In vitro* screening of *Vitis vinifera leaves* methanol extract on liver cancer cell line $HepG_2$, which was in passage 7 the results showed a dose-dependent inhibition on the cell growth after 48hr. The extracts concentrations used were 200, 100, 50, 25, 12.5 and 6.25µg/ml, with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ± standard deviation (SD).

The IC50 value was deduced from the graph for the methanolic extract of *Vitis vinifera* leaves, was calculated by using the following linear regression equation mention in Figure below respectively where Y=the percentage of inhibition and X= concentration. The IC50 value for ME= 7.80µg/ml.

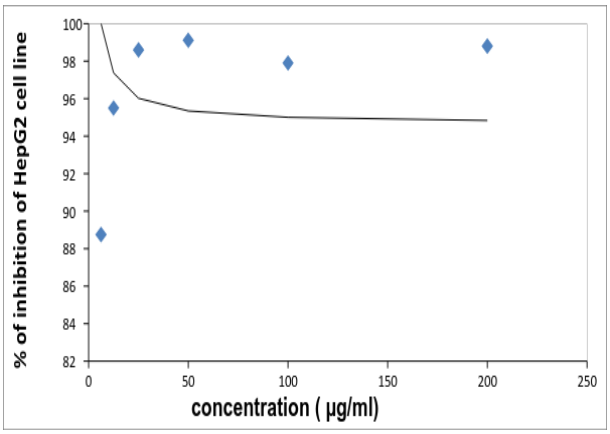


Figure 1: Dose response curve of Methanol extract of *vitis vinifera* against $HepG_2$ cancer cell line

Activity of *Vitis vinifera* leaves of methanol extracts in combination with doxorubicin on $HepG_2$ liver cancer cell line.

(Figure 2) show the dose response curve of *In vitro* screening of *Vitis vinifera* leaves methanol extracts in combination with doxorubicin on $HepG_2$ liver cancer cell line, which were in passage 6.

The results showed a dose-dependent inhibition on the cell growth after 48hr. The extract concentrations used

were 200, 100, 50, 25, 12.5 and 6.25µg/ml, with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ± SD. The IC50 value was deduced from the graph for the methanolic extract in combination with doxorubicin of *Vitis vinifera leaves*, was calculated by using the following linear regression equation below in figure where Y=the percentage of inhibition and X= concentration. The IC_{50} value for ME = 4.2µg/ml.

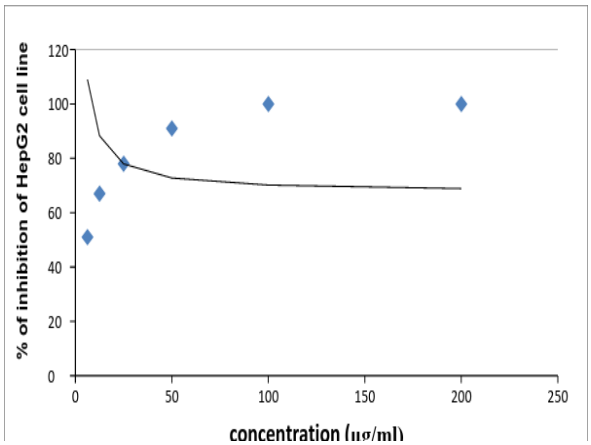
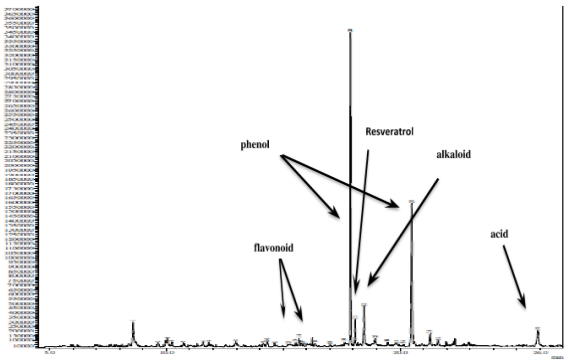


Figure 3: Dose response curve of Methanol extract of *vitis vinifera leaves in* combination with doxorubicin against $HepG_2$ cancer cell line.

Identification of Methanol Extract by Gas Chromatography

The functional groups of the chemicals in *Vitis vinifera* leaves of methanol extract tested by GS showed that flavonoids and alkaloid, Resveratrol, acid and phenols are exist in the extract. There compounds have anti proliferative activity.



DISCUSSION

methanol extract of *Vitis vinifera leaves* has been tested against the ($HepG_2$) cell lines to determine if the anti-proliferative activity observed was due to the compounds being cytotoxic or to direct inhibition.

Methanol extract of *Vitis vinifera leaves* showed the highest percentage of anti-proliferative activity alone and in combination when comparison to chloroform, petroleum ether and water extract against $HepG_2$ liver cancer cell line. Because the extract has IC_{50} values below 20µg/ml, this finding further implies that methanol is anti-proliferative. Methanol extract of *Vitis vinifera leaves* exerted dose-dependent inhibitory effects on $HepG_2$. The $HepG_2$ experiment was as the preliminary assay to assess if the activity of methanol extract was due to its cytotoxic activity.

The extract inhibited cell proliferation at 200µg/ml, also cytotoxic activity was observed below 20µg/ml. In the crude extract, the IC50 value was lower than 20µg/ml, suggesting that this sample has significant cytotoxic effect. This is a well-recognized criterion for a compound to be judged as cytotoxic, as defined by the National Cancer Institute (NCI)[21]. In the present study the growth passages of the $HepG_2$cells that were used in all experiments were between4-7 to avoid advanced passages that may has lost their parental characteristics or morphology, thereby producing unreliable data[22]. Methanol decreases the viability of $HepG_2$ liver cancer cell line[23,24].

These finding showed that methanol extract alone had a significant dose-dependent efficacy against the growth of the cells $HepG_2$. At the same time, these agents have cytotoxic activity at the applied dose. As these agents have high percentage of anti-proliferative activity than other extracts. While in combination with doxorubicin there were high significant different between extract alone and combination in combination significant dose dependent efficacy against the growth of the $HepG_2$ these combination have high cytotoxic can be expected as synergistic activity.

CONCLUSION

Methanol extract alone and in combination of *Vitis vinifera leaves* showed high significant cytotoxic activity against liver cancer cell lines ($HepG_2$) in combination synergistic activity.

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