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THE ANTI-PROLIFERATIVE ACTIVITY OF *VITIS VINIFERA* LEAVES OF WATER 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 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 water extracts tested by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on liver cancer cell line (HepG2). Dose response relationship was shown. Significant cytotoxic activity of water extracts has identified on HepG2, the IC_{50} was 20.8µg/ml. When in combination with doxorubicin Dose response relationship was shown. No significant cytotoxic activity of the water extracts combination; the IC_{50} was 31.5µg/ml. These finding showed that water extracts combination did not show any cytotoxic action at the applied dose, so no toxic effect against the HepG2 cell line can be expected *in vitro* (contrast action).

KEYWORDS: *Vitis vinifera*, water, doxorubicin, HepG2.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Medicinal plants have been widely used for the treatment of diseases in traditional way for several years. An interaction between ancient medicine and biotechnological tools is to be established towards newer drug development. The interface between cell biology, structural chemistry and *in vitro* assays will be the best way available to obtain valuable leads. The value of medicinal plants lies in the potential access to extremely complex molecular structure that would be difficult to synthesize in the laboratory. In spite of

an increasing awareness and expenditure of resources, the incidence of chronic diseases like cardiac, cancer, diabetes etc. has not declined and in fact is rising at an alarming rate. Cancer may be the most feared disease of our time and the number of deaths continues to increase steadily. Medicinal plants represent a vast potential resource for anticancer drugs and continue to be subject to extensive screening worldwide in an attempt to develop still more effective anticancer treatment.[1]

Vitis vinifera, a species of grape, is native to the Mediterranean region, central Europe and South-western Asia. It has been planted all over the world and is used for both medicinal and nutrition-al value. Previous studies on some grape varieties have shown that most of the cultivars possess medicinal properties, such as anti-inflammatory and anti-cancer effects.[2, 3] Through extracting natural compounds from fruits and leaves of *Vitis vinifera* in recent decades, a number of phenolic compounds, such as gallic acid, catechin, resveratrol and a wide variety of procyanidins have been isolated and studied for their biological activities and health-promoting benefits.[4] Phenolic substances are synthesized during the process of plant growth, whereas the presence of some stress factors, like ultraviolet radiation and disease also increase the synthesis of them.[5] The cytotoxic activities of *V. vinifera* cultivars have been investigated against different cancer cell lines (HL- 60, MCF-7, HT-29 and HeLa).[6,7]

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 water Crude Extract

Five Kg powder *Vitis vinifera* leaves was extracted with water 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 HepG2 (hepatocellular carcinoma). Cell was obtained from Iran. Cells were routinely grown as monolayer cell cultures in 50cm2. 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.[8] 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.[9]

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 37oC, in 5% CO_2 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 1x104, 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 automatically12. Results were expressed as triplicate Determinations gave a CV (standard deviation/mean %). Extract Potency against cancer cell growth was expressed in terms of IC50 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}×100.[10]

RESULTS

Activity of *Vitis vinifera* water leaves extracts alone on HepG2 liver cancer cell line, (Figure 1) show the dose response curve for the *In vitro* screening of *Vitis vinifera leaves* water extract on liver cancer cell line HepG2, 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 water 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 WE=20.8µg/ml.

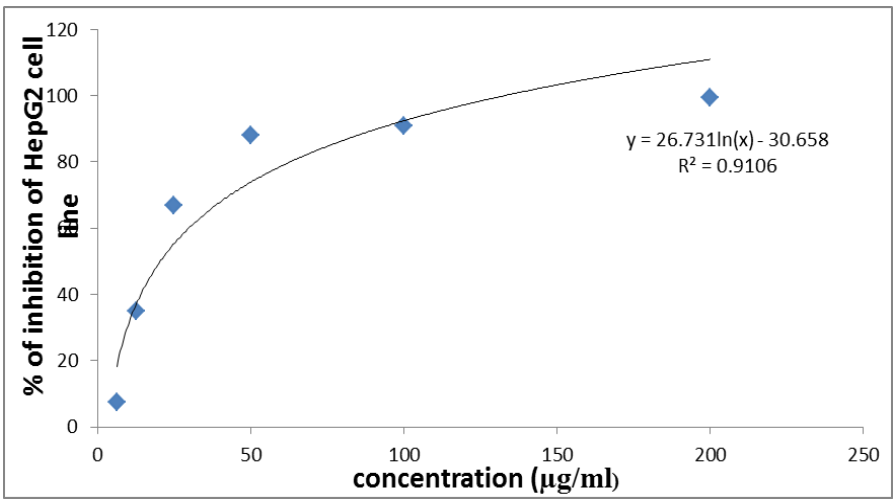

Figure 1: Dose response curve of Water extract of *Vitis vinifera* against HepG2 cancer cell line.

Activity of *Vitis vinifera* leaves of Water extracts in combination with doxorubicin on HepG2 liver cancer cell line. (Figure 2) show the dose response curve of *In vitro* screening of *Vitis vinifera* leaves water extracts in combination with doxorubicin on HepG2 liver cancer cell line, which was 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 IC_{50} value was deduced from the graph for the water 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 WE= 31.5µg/ml.

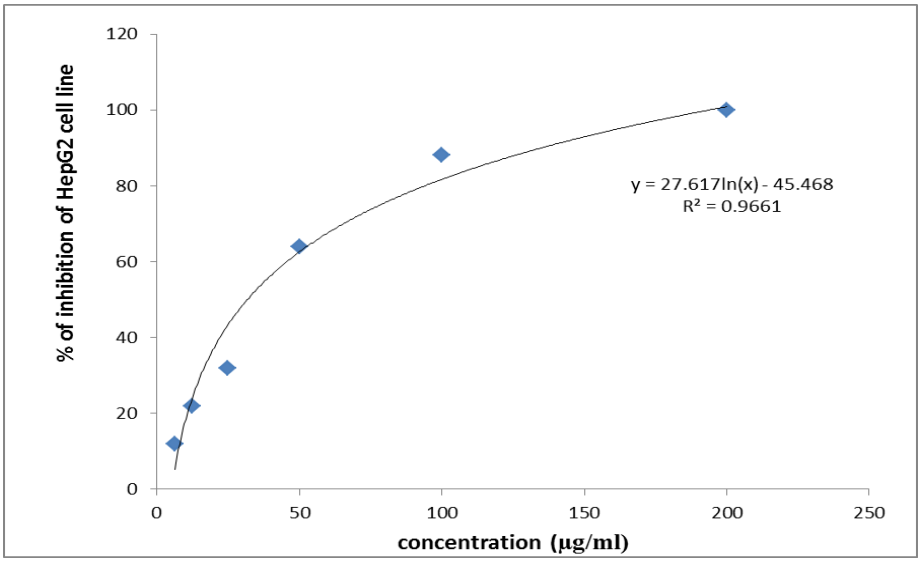

Figure 2: Dose response curve of Water extract of *Vitis vinifera leaves in* combination with doxorubicin against HepG2 cancer cell line.

Identification of Methanol Extract by Fourier Transform Infrared Spectroscopy (FT – IR)

The absorbance peaks of the chemicals in *Vitis vinifera* leaves of water extract tested by (FT – IR) showed that aromatic and nitro compound, alcohol, acid and phenols are exist in the extract. There compounds have anti proliferative activity.

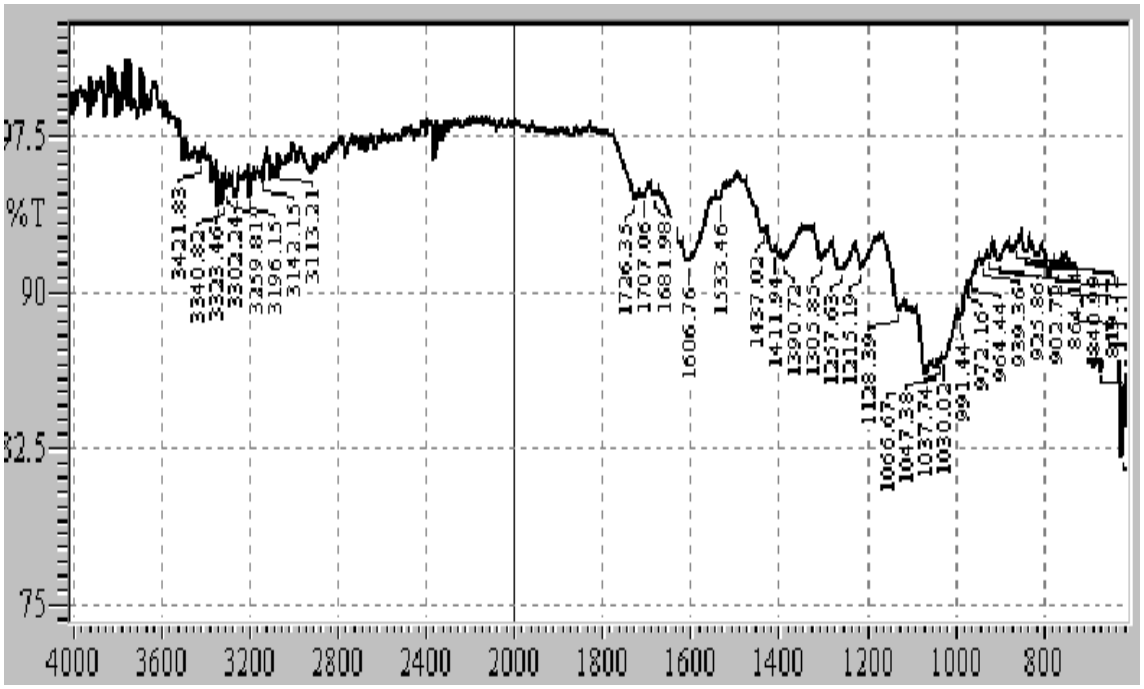

Figure 3: FT – IR peaks absorbance chart for Water extract of *Vitis vinifera leaves.*

Table 1: Peaks absorbance and their functional groups for Water extract of *Vitis vinifera leaves*

Peak Absorbance	Functional Groups
3340	O-H Stretch (alcohols, phenols)
1726	C=O Stretch (esters, saturated aliphatics, carboxylic acids, carbonyls)
1437-1600	C-C Stretch (in ring) (aromatic)
1128	C-O Stretch (alcohols, carboxylic acids, esters, ethers)
1207	C-H Wag (-CH2X) (alkyl halide)
1523	N-O Asymmetric stretch (nitro compound)

DISCUSSION

Water extract of *Vitis vinifera leaves* has been tested against the (HepG2) cell lines to determine if the antiproliferative activity observed was due to the compounds being cytotoxic or to direct inhibition. Water extract of *Vitis vinifera leaves* showed the highest percentage of anti-proliferative activity alone and minimum in combination when comparison to chloroform and petroleum ether extract against HepG2 liver cancer cell line. Because the extract has IC_{50} values below 20µg/ml, this finding further implies that water is IC_{50} was 20.8µg/ml anti-proliferative. Water 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 water 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 IC_{50} 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).[11] 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.[12] Water decreases the viability of $HepG_2$ liver cancer cell line.[13,14]

These finding showed that water extract alone had a significant dose-dependent efficacy against the growth of the cells HepG2. 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 no significant dose dependent efficacy against the growth of the $HepG_2$ these combination have no cytotoxicity can be expected as contrast action.

CONCLUSION

Water extract alone of *Vitis vinifera leaves* showed high significant cytotoxic activity but in combination with doxorubicin show no cytotoxic activity against liver cancer cell lines ($HepG_2$) antagonize effect.

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