Journal of
Cancer Science & Therapy

Hassanein et al., J Cancer Sci Ther 2011, 3.10
DOI: 10.4172/1948-5956.1000097

Research Article Open Access

Extracts of Five Medicinal Herbs Induced Cytotoxicity in Both Hepatoma and Myeloma Cell Lines

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Abstract

The aim of this study is to assess the effect of the crude methanol extracts of five herbs (*Pelargonium zonale, Terminalia bellerica*, *Philodendron selloum, Ulmus pumila* and *Ulmus parvifolia) on human hepatoma and murine myeloma cell lines.* Assessment included *in vitro* neutral red cytotoxicity assay and cytopathological diagnosis.

IC50 values obtained in both cell lines using neutral red assay showed that the plant extracts of both *Terminalia bellerica* and *Philodendron selloum*, were the most effective in inducing cytotoxicity in HepG2, IC50 (16.25±0.20 µg/ml, 17.51±0.70 µg/ml respectively). The result showed that there was no significant difference in the IC50 between the different plants extract in Myeloma cell line. However, in hepatoma cell line, the IC50 of both *Terminalia bellerica and Philodendron selloum* were significantly lower ($p< 0.01$) than the IC50 of *Ulmus pumila*, *Ulmus parvifolia* and *Pelorgonium zonale*. The results indicated that the *in vitro* NR assay of cytotoxic activities induced by plant methanol extracts were supported by the cytopathological changes on the same population of cells.

Conclusions: Neutral red cytotoxicity assay is a suitable test for screening anti-cancer potential of natural products materials. The present results showed that *Terminalia bellerica* and *Philodendron selloum* methanol extracts induced cytotoxicity on HepG2 cells. The identification of the effect of individual constituents of each plant methanol extract recommended. *In vivo* study on experimental level is needed to verify the mechanism of action.

Keywords: Human hepatoma cell line; Murine myeloma cell lines; *Terminalia bellerica*; *Philodendron selloum; cytotoxicity*

Introduction

Cancer is a class of diseases in which a group of cells display the traits of uncontrolled growth, invasion, and sometimes metastasis . The resistance of tumor cells to chemotherapeutic agents is a major problem in the clinical treatment of cancer; so a wide array of selective and potent compounds is needed to match the growing problems associated with cancer [1].

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in adults. It remains one of the most common solid tumor malignancies worldwide [2,3]. The drug resistance developed by many cancers in patients with standard anticancer agents is a serious problem encountered in cancer chemotherapy and may develop in a cell population through repeated exposure to treatment with that particular drug. The cell population may subsequently show broad cross-resistance to other anticancer agents even though it has never been exposed to those agents and this phenomenon is called multidrug resistance (MDR). More effort is needed to search for new cancer drugs by better screening of medicinal plants and other natural sources [4].

Natural products offer a valuable source of compounds with a wide variety of biological activities and chemical structures, and provide important prototypes for the development of novel drugs. It has been estimated that 20-25 % of all medicines are derived from natural products. Most of anticancer agents have been derived from natural sources obtained as pure, native compounds or as semi synthetic analogues. The plant constituents able to kill cancer cells exhibit a very large range of structural types, for example alkaloids, coumarins, diterpenes, flavonoids, tannins, iridoids, lignans, monoterpenes, steroids and triterpenes. Hundreds of plant constituents have been found to be cytotoxic against one or more tumor cell types in culture [5,6].

The extracts of some medicinal herbs showed various biological activities, e.g. *Pelargonium zonale* [7], *Terminalia bellerica* [8], *Terminalia arjuna* [8,9,10] , *Philoendron sellom* [11], *Ulmus pumila* [7,12] and *Ulmus parvifolia* [13, 14].

Cell-based assays are becoming an increasingly important part of the preclinical pharmaceutical discovery and validation process, as researchers directly study the effects of chemical compounds upon a wide variety of cell types. *In vitro* cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals. It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed [15].

The application of mammalian cell cultures cytotoxicity assays for quantitating the potencies of cytotoxin agents included either the MTT colorimetric cell viability assay [16] or neutral red cell viability assay

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Received November 03, 2011; **Accepted** November 27, 2011; **Published** December 01, 2011

Citation: Hassanein HI, El-ahwany EG, Salah FM, Hammam OA, Refai L, et al. (2011) Extracts of Five Medicinal Herbs Induced Cytotoxicity in Both Hepatoma and Myeloma Cell Lines. J Cancer Sci Ther 3: 239-243. doi:10.4172/1948-5956.1000097

(NR) [17]. The NR assay has been found to be more sensitive than the MTT assay [15]. The NR assay is based on the incorporation of the supravital dye into the lysosomes of viable cells. If toxic agents injure the lysosomal membrane, dead or damaged cell cannot retain the dye. After NR dye has been extracted from lysosome, it is quantitated spectrophotemetric [17].

This study aimed to assess the effect of the crude methanol extracts of five herbs (*Pelargonium zonale, Terminalia bellercia*, *Philodendron selloum, Ulmus pumila* and *Ulmus parvifolia)* on *human hepatoma* and *myeloma cell lines.* Assessment included *in vitro* neutral red cytotoxicity assay and cytopathological diagnosis.

Materials and Methods

Plant Materials

The following medicinal plants *Pelargonium zonale*, *Terminalia bellerica*, *Philodendron selloum*, *Ulmus pumila* and *Ulmus parvifolia*, were collected from Orman Garden, Giza, Egypt. The identification of the plants was carried out by specialist (Manager of Egyptian Agriculture Museum). The selection of these plants was based on literature survey.

Extraction of the plants

The plants were dried at room temperature and powdered by an electric mill. Each of plant powder was extracted with 85% methanol (Merck). The solvent was evaporated by rotatory evaporator under reduced pressure and at temperature 75°C. The dried extract was dissolved in dimethyl sulfoxide solution (DMSO) (Sigma) at concentration of 200 mg/ml and kept as a stock sample.

Cell culture

Non-secreting murine myeloma cell line (p3X63 Ag8) and human hepatocellular carcinoma HepG2 (kindly provided by the Department of Medicine, Case Western Reverse School of Medicine, Cleveland, OH and Biology Department of the American University in Cairo, Egypt, respectively) were used in testing the anticancer activity. The myeloma and the HepG2 cell lines were cultured at 37°C in a 5% CO_2 incubator for 72 hours (hrs).When the growth was at confluence phase, each of the two cell lines were dissociated from the culture flask by 0.025% trypsin (Sigma) and the culture flasks were gently shaked to ensure that a single-cell suspension was obtained. The cells were counted using a Neubauer heamocytomter. The cell viability was tested by the trypan blue dye exclusion method [18].

Neutral red cytotoxicity assay

Neutral red cytotoxicity assay based on the initial protocol described by [16] and modified by [17] was carried out. The cells from mother flasks were seeded in a 24-well microtitre plate (Corning) ($1X10^6$) cell/well. The plates were incubated at 37°C in 5% CO_2 for 24 hrs to achieve monolayer confluence. Culture medium containing different concentrations of each plant extract (10, 25, 50 and 100 mg/ml) were added in triplicate. Medium without plant extracts served as untreated control. The dye-medium was removed and the plates were washed with formol-calcium (10 ml 40% formaldehyde, 10 ml 10% anhydrous calcium chloride and 80 ml water). Five hundred µl of acetic acid-ethanol (one ml glacial acetic acid in 100 ml 50% ethanol) was added and the plates were kept for 15 min at room temperature to extract the dye. Plates were then shaked for a few seconds, so that complete dissolution was achieved. The absorbance of the extracted dye was measured by spectrophotometric reading (Spectra max 190-Molecular devices) using with 540 nm filter. The mean of three measurements for each concentration was determined (n=6).

Cytopathological diagnosis

On the same cell population, the cells were trypsinized and washed with phosphate buffered saline (PBS), pH= 7.4 and collected into centrifuge tube. Collected sample was centrifuged at a rate of 1200–1500 rpm for 15 minutes using Shandon Cytospin (Thermo Fisher Scientific, Waltham, Massachusetts). The sediment was smeared on slides that were pretreated with 3-APTES (3-amino-propyl-triethoxy saline, Sigma-Aldrich Ireland Ltd, Dublin, Ireland). Slides were fixed immediately in 95% ethanol for 24 hours and then stained with hematoxylin and eosin.

Calculations and statistics

Cytotoxicity assay was measured as optical density at 540 nm. Dose-response curves were plotted, and 50% inhibitory concentrations of plants extracts (IC50) were calculated through Graph Pad Prism software program. Data are presented as mean ± SD. For statistical analysis of data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the LSD test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. Data were analyzed using SPSS (version 11; Chicago, IL, USA).

Results

The effects of the following crude medicinal plants methanol extract of *Pelargonium zonale*, *Terminalia bellerica*, *Philodendron selloum*, *Ulmus pumila* and *Ulmus parvifolia* on the proliferation of HepG2 and myeloma cell lines were determined using the neutral red cytotoxic assay.

All cell lines were growth inhibited in a dose-dependent manner after exposure to the plant extracts. The results presented by the optical density (OD) and they indicated that all the different plants methanol extract which mentioned above have a cytotoxic effect in both cell lines (Figures 1,2).

IC50 values obtained in both cell lines using neutral red assay showed that the *Ulmus pumila* was the most effective in inducing

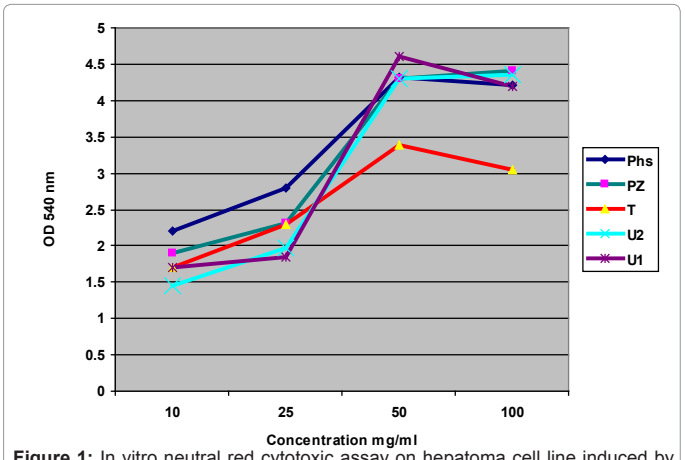

Figure 1: In vitro neutral red cytotoxic assay on hepatoma cell line induced by crude methanol extracts of five medicinal plants.U1=*Ulmus pumila*, U2=*Ulmus parvifolia*, T=*Terminalia bellerica*, PZ=*Pelargonium zonale*, PHS= *Philodendron selloum*.

cytotoxicity in Myeloma cell line, IC50 (14.27±2.13µg/ml) and the plant extracts of both *Terminalia bellerica* and *Philodendron selloum*, were the most effective in inducing cytotoxicity in HepG2, IC50 (16.25±0.20 µg/ml, 17.51±0.70 µg/ml respectively) (Table 1 and Figure 3).

There was no significant difference in the IC50 between the different plants extract in Myeloma cell line. In hepatoma cell line, the IC50 of both *Terminalia bellerica* and *Philodendron selloum* were significantly lower (p‹ 0.01) than the IC50 of Ulmus *pumila*, *Ulmus parvifolia* and *Pelorgonium zonale*.

However, there was no significant difference in the IC50 between the best two plants extract in myeloma and hepatoma cell lines (*Ulmus pumila* and *Philodendron selloum* respectively).

Cytological Examination of Smears Prepared from Hepatoma and Myeloma Cell Lines

Hepatoma cell line cells (HepG2) treated with different plant methanol extracts showed increase number of apoptotic and degenerated cells which were not seen in HepG2 cells not treated. Examination of smears prepared from HepG2 cells without treatment by light microscope showed malignant polyherdral shape hepatocytes forming acini and clusters of cells with enlarged nuclei and increased nucleocytoplasmic ratio.

Treated HepG2 cells and mainly *Philodendron selloum* and *Terminalia bellerica* plant resulted in apoptotic changes in the form

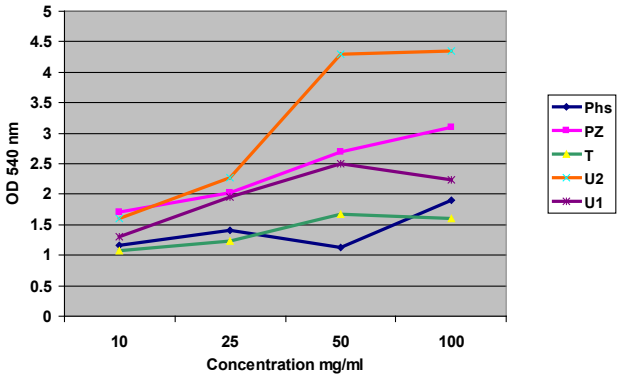

Figure 2: In vitro neutral red cytotoxic assay on myeloma cell line induced by crude methanol extracts of five medicinal plants.U1=*Ulmus pumila*, U2=*Ulmus parvifolia*, T=*Terminalia bellerica*, PZ=*Pelargonium zonale*, PHS= *Philodendron selloum*.

	IC50 values of the different plants methanol extract				
Plants methanol extracts (mg/ml)	U1 Mean ±SD	U2 Mean ±SD	T Mean ±SD	PZ Mean ±SD	PHS Mean ±SD
Hepatoma cell line	27.72±1.16[c,e]	25.11±1.93[c,e]	16.25±0.20[a,b,d]	24.80±3.4[c,e]	17.51±0.70[a,b,d]
Myeloma cell line	14.27±2.13	15.83±3.04	18.33±3.62	16.20±2.61	17.18±2.40

Data are shown as mean ±SD from three separate experiments (Exp.)
The IC50 Values Unit is µg/ml
[a] p‹ 0.01 compared to U1
[b] p‹ 0.01 compared to U2
[c] p‹ 0.01 compared to T
[d] p‹ 0.01 compared to PZ
[e] p‹ 0.01 compared to PHS.

Table 1: IC50 values of the different plants methanol extract in Myeloma and Hepatoma cell lines.

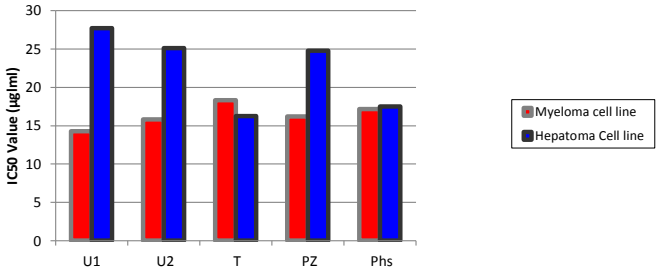

Figure 3: The IC50 Values of the different plant methanol extracts in Myeloma and Hepatoma cell lines. U1= *Ulmus pumila*, U2=*Ulmus parvifolia*, T=*Terminalia bellerica*, PZ=*Pelargonium zonale*, Phs=*Philodendron selloum*.

of reduction in cell volume, cell shrinkage, reduction in chromatin condensation and formation of numerous clear cytoplasmic vacuoles and blebs Hepatocytes also degeneration as structural alteration and decrease in the number of normal HepG2 cells and with necrotic debris (Figures 4A-B, 5A-B).

Comparing, the changes with those of cells treated with plant extracts, similar changes was observed in Myeloma cell line cells compared to untreated cells.

Examination of myeloma cells by light microscope showed malignant lymphocytes (small and large) with enlarged nuclei and increased nucleocytoplasmic ratio. After treatment with different plants methanol extracts, mainly *Ulmus pumila*, lymphocytes showed cell degeneration with necrotic debris, other cells showed apoptotoic changes and decrease in the number of normal lymphocytes (Figure 6A-B).

In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals [19, 20]. It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed [21].

In the present study, the inhibition of cell viability of the five plant methanol extracts was assessed in two malignant cell lines. The human liver cancer cell line HepG2 and mouse myeloma cell lines were used. The human hepatoma HepG2 cell line is widely used as an experimental model for *in vitro* study of HCC [22,23].

In the present work, the cytotoxic activities of the plant extracts were studied by neutral red cytotoxic assay. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is one of the most common cytotoxicity tests used with many biomedical and environmental applications [24]. The NR assay is based on the incorporation of the supra-vital dye into the lysosomes of viable cells. If toxic agents injure the lysosomal membrane, dead or damaged cell cannot retain the dye. After NR dye has been extracted from lysosome, it was quantitated spectrophotemetrically [17].

The present results showed that the five medicinal plant extracts, induced cytotoxicity in both cell lines tested in a concentration-dependent manner. These extracts may induce cell death which indicated the cytotoxic activities of these plant extracts. Probably multiple bioactive compounds in the herbal extracts having cytotoxic potentiality are involved. However the plant extracts of *Terminalia bellerica* and *Philodendron selloum*, were the most effective in inducing

cytotoxicity in HepG2, IC50 (16.25±0.20 µg/ml, 17.5±0.70 µg/ml respectively).

Philodendron selloum extracts are rich with phenolic compounds especially tannins and flavonoids. These classes of compounds showed high cytotoxic effect against tumour cell line through different mechanisms as antioxidant effect, regulation of the host immune system, induction of apoptosis, etc. [25].

Plant derived polyphenols including tannic and gallic acid were reported to be the main constituents in *Terminalia bellerica*, *Ulmus pumila* and *Philodendron selloum*.

The inhibition of malignant cells *in vitro* induced by the tannic acid was reported. Marienfeld et al. [26] reported that tannic acid (TA) could inhibit the malignant cholangiocyte *in vitro* and *in vivo* and also enhance sensitivity of Mz-ChA-1 cholangiocarcinoma cells to camptothecin cytotoxicity which might involve an effect on xenobiotic metabolism [27].

On the other hand *Ulmus pumila* induce remarkable cytotoxic effect on myeloma cell line, and have been used as traditional Chinese medicine to treat edema, mastitis, gastric cancer and inflammation [28]. Tannins and triterpenes were isolated from the plant [8]. According to literature the plant is rich with triterpenoid and sesquiterpenoid compounds which are isolated from its root bark [12]. The high toxicity

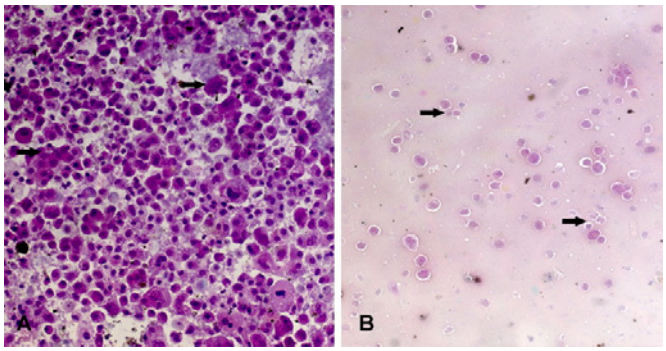

Figure 4: A) Smear prepared from HepG2 cells showing malignant hepatocytes forming acini and clusters of cells with enlarged nuclei and increased nucleocytoplasmic ratio, arrows (H&E, 200). B) HepG2 cells after treatment with *Philodendron selloum* methanol extract swing small shrinking hepatocytes with apoptotic and degenerative changes, arrows (H&E, 200).

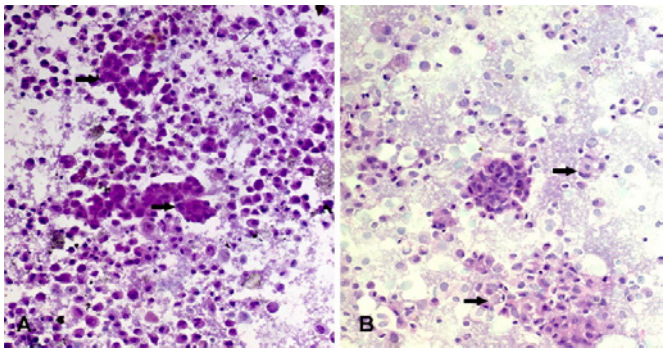

Figure 5: A) Smears prepared from HepG2 cells showing malignant hepatocytes forming acini and clusters of cells with enlarged nuclei and increased nucleocytoplasmic ratio, arrows (H&E, 200). B) HepG2 cells after treatment with *Terminalia bellerica* methanol extract showing small shrinking hepatocytes with apoptotic and degenerative changes, arrows (H&E, 200).

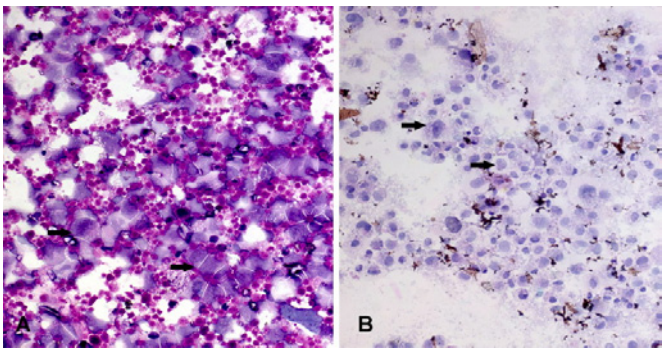

Figure 6: A) Myeloma cell line showing malignant lymphocytes (small and large) enlarged nuclei and increased nucleocytoplasmic ratio, arrows (H&E, 400). B) Myeloma cell line after treatment with plant extract *Ulmus pumila* methanol extract, showing scattered degenerated lymphocytes and lymphocytes with degenerative changes, arrows (H&E, 400).

of the plant against myeloma cell line may be due to the presence of terpenoidal compound.

On comparing IC50 of different plant methanol extracts on HepG2, the *Philodendron selloum* methanol extract showed remarkable cytotoxic effect than the other plant methanol extracts. However the *Ulmus pumila* methanol extract showed remarkable cytotoxic effect on myeloma cell line.

The different sensitivity observed in the two cell lines with the used plant methanol extracts denotes a different response in each cell line. The mechanism of action of the plant extracts should be further investigated.

The present data indicated that the *in vitro* NR assay of cytotoxic activities induced by plant methanol extracts were comparable to the cytopathological changes on the same population of cells. Studies have shown that cytotoxic effect of the phenolic compounds on different tumors is mediated through apoptosis. For instance, gallic acid selectively induces cell death in various transformed cell lines such as PLC/PRF/5 (human hepatoma), HL-60, RG (human promyelocytic leukemia) and P-388D (mouse lymphoid neoplasma) [29]. It was reported that certain products from plants can induce apoptosis in neoplastic cells but not in normal cells [30,31].

In our study light microscopic observation of *Philodendron selloum* and *Terminalia bellerica* plant methanol extracts treated HepG2 and myeloma cells showed apoptosis and degenerative changes of HepG2 cells. Hepatocytes degeneration, decrease in the number of HepG2 cells and necrotic debris were observed. Comparing the changes with those of cells treated with plant methanol extracts, similar changes were observed in myeloma cell line cells compared to untreated cells.

The results obtained from the cytotoxicity assay indicated that there were difference between two cell lines. Concerning the sensitivity to different plant extracts, HepG2 appeared to be more sensitive to *Philodendron selloum* while myeloma cell line appeared more sensitive to *Ulmus pumila*.

Conclusions

Neutral red cytotoxicity assay is a suitable test for screening anti-cancer potential of natural products materials. The present results suggests that *Terminalia bellerica* and *Philodendron selloum* extracts induced cytotoxicity on HepG2 cells, while *Ulmus pumila* showed

the highest cytotoxic effect on myeloma cell line, but not significant from the other plant methanol extracts. These plant extracts contain a bioactive compound capable of killing malignant cells by apoptosis. The identification of the effect of individual constituents of each plant extract recommended. *In vivo* study on experimental level is needed to verify the mechanism of action.

Acknowledgment

This work was financially supported by TBRI internal project No.487D. Principle Investigator: Prof. Dr. Hanaa hassanein. We acknowledge Associate Prof. Dr. Naglaa M. I. El-Lakkany and Associate Professor. Dr. Sayed H.H. Seif El.Din for their help in the calculations of the IC50 of all the plant extracts.

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