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In-vitro cytotoxicity evaluation of aqueous fruit and leaf extracts of *Grewia asiatica* using MTT Assay

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ABSTRACT

The research was conducted to investigate the cytotoxic activities of crude aqueous extracts of fruits and leaves of Grewia asiatica plant. In-vitro cytotoxic activity of Grewia asiatica was determined by using methyl thiazolyl tetrazolium (MTT) assay using cell lines HEK-293 (Epidermal Kidney Cancer cell line), NCI-H522 (Cell Lung cancer cell line), HELA (Cervical Cancer cell line), Hep – 2 (Laryngeal Cancer cell line), and MCF-7 (Breast cancer cell line). Grewia asiatica (G. asiatica), a shrub from the Malvaceae family, is commonly used among Native Americans and occasionally utilized to treat illnesses with cancer-like symptoms. The results found were quite promising. The statistically processed results support the conclusion, that the aqueous extracts of leaves and fruits showed significant anticancer activity against liver cancer and breast cancer.

Key words: Cytotoxic, HELA, Lung cancer, MTT, Cervical cancer.

INTRODUCTION

Cancer is a genetic disease1. Cancer arises from an accumulation of mutations in oncogenes, tumor suppressor genes and genes that maintain the genomic integrity of the cell. Although environmental and other nongenetic factors have roles in many stages of tumorigenesis, it is widely accepted that cancer arises due to mutations in cancer-susceptibility genes[1-3]. The alteration of one gene, however, does not suffice to give rise to full blown cancer. For progression toward malignancy and invasion, further mutational hits are necessary. Hence the risk of cancer development does not only depend on mutations initiating tumorigenesis, but also on subsequent mutations driving tumor progression[4-5]. There are two basic types of *in-vitro*

cancer screening method - (a) chemo-sensitivity and (b) chemo-resistance. Human breast carcinoma cell lines (MCF-7, MDA-MB453), Human Non-Small Cell Lung cancer cell line (NCI-H23, NCIH522) and HEK-293T (Human Embryonic Kidney 293 normal cells) are used for the *in-vitro* screening of newly synthesized compounds. Natural drugs are under investigation for their selective cytotoxicity to cancer cells. *Grewia asiatica* (*G. asiatica*), a shrub from the Malvaceae family, native to India, Nepal, Cambodia, Laos and Thailand, is commonly used among Native Americans and occasionally utilized to treat illnesses with cancer-like symptoms is known for its medicinal properties[6-8]. Grewia asiatica contains anthocyanin type cyanidin 3-glucoside vitamin C, A, minerals,carotenes and dietary fibers etc [9].

MATERIALS AND METHODS

Collection and Extraction

Grewia asiatica leaves and fruits were collected from Dehgam, Gujarat. Leaves and fruits were dried in an oven at 50±0.5ºC up to approximately 5-7% moisture content. Leaves and fruits were pulverized to coarse powder and passed through 60# sieve. This powder was extracted with water in a soxhlet apparatus for 6 hours. The extract was concentrated under reduced pressure by a rotary vacuum evaporator. Preliminary qualitative analysis of aqueous extracts showed the presence of antioxidants such as vitamin C, anthocyanin, carotenoids. This extracts was dissolved in DMSO to prepare series of concentration in a range of 0.01 -100 µg/ml. These aqueous extracts were screened for its anti-cancer activity.

Media

Leibovitz L-15 Medium with L-Glutamine, FBS (Fetal Bovine Serum), SFM HEK-293 (Serum Free Media, Hyclone), Thioglycollate medium (TGM), Tryptone soya broth (TSB) and Cell proliferation kit (MTT) 1000 tests.

Cell lines

Cell lines require for the in-vitro anticancer activity like HEK-293 (Epidermal Kidney Cancer cell line), NCI-H522 (Cell Lung cancer cell line), HELA (Cervical Cancer cell line), Hep – 2 (Laryngeal Cancer cell line), and MCF-7 (Breast cancer cell line) were purchased from NCCS, Pune.

Microbial and Fungal Culture

Microbial and fungal culture for activity like *Candida albicans, Bacillus subtilis, Candida sporogenes* were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Subculture of Adherent Cell Lines (MCF-7, HEC 293, NCI-H522, HeLa and HEp-2) [10-11]

The degree of confluency and the absence of bacterial and fungal contaminants were confirmed by observing cultures using an inverted microscope. PBS without Ca^{2+}/Mg^{2+} used to wash cell monolayer with volume equivalent to half the volume of culture medium. After washing cell monolayer, Trypsin/EDTA was added on to the washed cell monolayer using 1 ml per 25 cm^2 of surface area in a flask and flask was rotated to cover monolayer with trypsin and placed in incubator for 2-10 minutes. Using an inverted microscope the cells were examined to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing HEK-293 medium among it 100-200 µl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed HEK-293 medium and incubated as appropriate for the cell line.

Determination of Bacteria and Fungi in Normal and Carcinoma Cell Lines [10]

Cell line was cultured in the absence of antibiotics at NCCS, Pune. Using this cell line, cell suspension was prepared by scrapping attached cells with the use of a cell scraper and maintained the pH 7.5- 8.0. In 1.5 mL cell suspension, 2 mL thioglycollate medium (TGM) and 2 mL tryptone soya broth (TSB) were added and inoculated with two different strains; *Candida albicans* (0.1 mL) *Bacillus subtilis* (0.1 mL). Then in 1.5 mL cell suspension, 1 mL TGM was added and inoculated with 0.1 mL *Candida sporogenes* and 2 mL (TGM), 2 mL (TSB) were left uninoculated as negative controls. Broths were incubated at 32 ºC. Test and Control broths were examined for turbidity after 14 days.

Anti-cancer Activity

MTT Assay[10]

The cells in a concentration of 1×10^6 cells/ml were preincubated in culture medium for 3 hrs at 37 °C and 6.5 % CO_2. Then, the cells were seeded at a concentration of 5×10^4 cells/well in 100 µl culture medium and at various concentrations (0.005-100 µM/ml) of standard Methotrexate and aqueous extract of G.asiatica fruit extract (dissolved in 2 % DMSO (dimethylsulphoxide) solution) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 hrs at 37 °C and 6.5 % CO_2. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37 °C and 6.5 % CO_2. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader at wavelength in between 550 and 600 nm according to the filters available for the ELISA reader. The reference wavelength should be more than 650 nm. By plotting a graph of Log (concentration of compound) *vs* % cell inhibition, IC_{50}, the concentration of compound required to inhibit 50 % cell growth, was determined. A line drawn from 50 % value on the *Y* axis meets the curve and interpolate to the *X* axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC50 value.

Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where, At = Absorbance of Test,

Ab= Absorbance of Blank (Media),

Ac= Absorbance of control (cells)

% cell inhibition = 100 − % cell survival

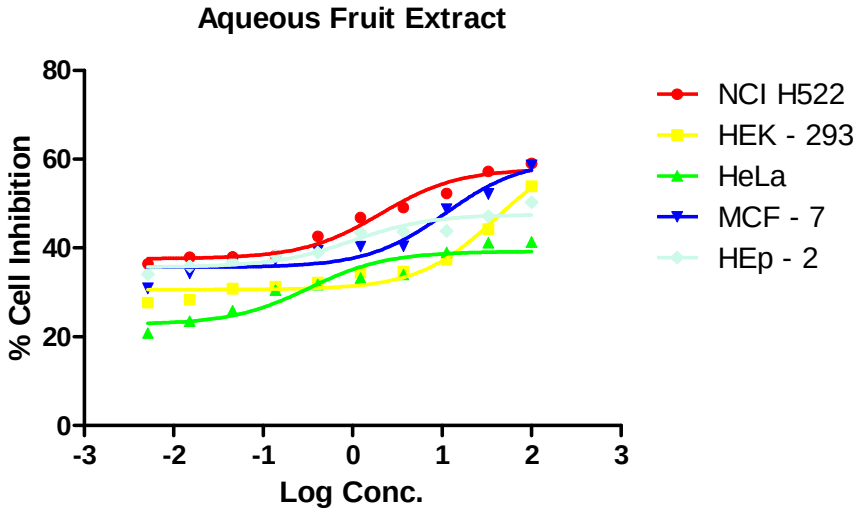
RESULTS AND DISCUSSION

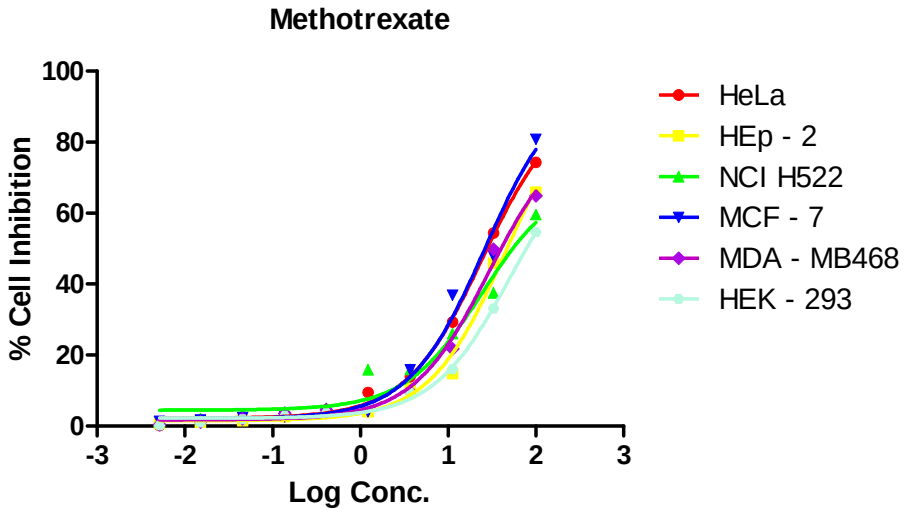
Total bacterial and fungal count

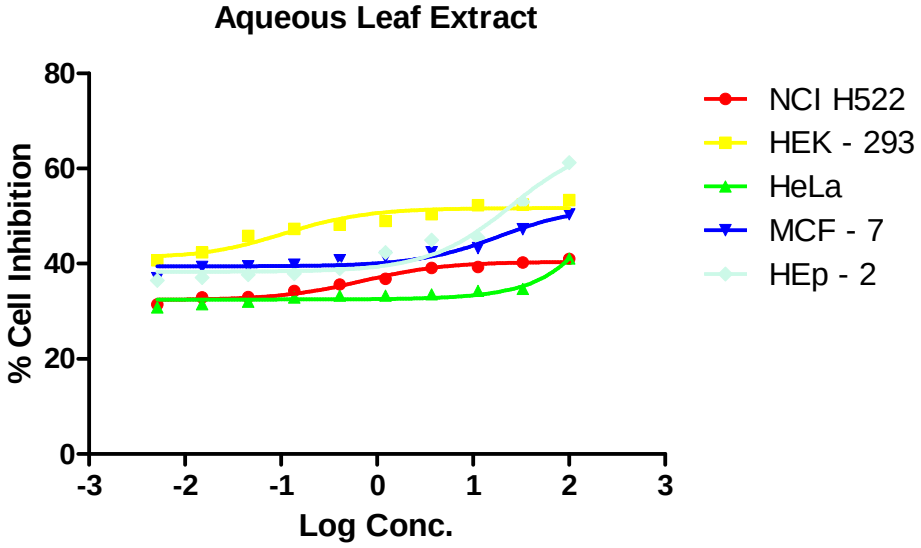
After 14 days incubation, the examinations of the test and control broths were confirmed for the absence of turbidity. Absence of turbidity in the test and control broths means that there was no evidence of bacterial, fungal and cross contamination.

Cytotoxicity Assay

The effect of aqueous extracts of *G. asiatica* (test) and Methotrexate (standard) on the growth of MCF-7, HEC 293, NCI-H522, HeLa and HEp-2 cell lines were examined by the MTT assay. Concentration in the range of 0.01 -100 µg/ml and 0.01 – 100 µM for aqueous extracts of G.







asiatica and Methotrexate (control) respectively were used for the study and dose response curves constructed by decreasing number of viable cells with increasing concentration of aqueous extract of G. asiatica as well as Methotrexate (Figure 3). *Graph Pad Prism Software (Ver. 5.01)* was used to calculate IC50 value (Figure 1 and 2). The survivability of cells to the aqueous extract of G. asiatica and Methotrexate was characterized by IC50 and R2 values (Table 1). It indicates that the cytotoxic effect steadily strengthens with increase in the concentration.

Table 1: IC50 and R^2 values of various extracts of G. asiatica and Methotrexate on various cell lines by MTT Assay.

Compound	Log Conc.	NCI H522	HEK - 293	HeLa	MCF - 7	HEp - 2
Aqueous fruit extract	IC_{50} (mg/ml)	59.03	53.88	>100	58.65	50.31
	R^2	0.9711	0.9540	0.9034	0.9281	0.9024
Aqueous leaf extract	IC_{50} (mg/ml)	>100	53.34	>100	50.37	61.23
	R^2	0.9738	0.9158	0.9058	0.9278	0.9550
methotrexate	IC_{50} (mg/ml)	24.21	49.53	24.59	26.34	44.99
	R^2	0.9619	0.9951	0.9973	0.9828	0.9874

NCIH522 – Lung cancer cell line
HEK – 293 – Human Epidermal Kidney Cell line (Normal cell line)
HeLa – Cervical cancer cell line
MCF – 7 – Breast cancer cell line
HEp – 2 – Larynx cancer cell line

The results of our study show that aqueous extracts of leaves and roots of G. asiatica has a cytotoxic effect on HEK-293 (Epidermal Kidney Cancer cell line), NCI-H522 (Cell Lung cancer cell line), HELA (Cervical Cancer cell line), Hep – 2 (Laryngeal Cancer cell line), and MCF-7 (Breast cancer cell line) concentration dependent manner but the extract showed better therapeutic value. Comparable cytotoxicity of aqueous extracts of G. asiatica was found against lung cancer cell line and breast cancer cell line (figure.1 &2). For aqueous fruit extract of G. asiatica was found to be active on Lung cancer cell line with IC50 of 59.03µg/ml and 58.65µg/ml in MCF-7 cell line (Table.1) while aqueous leaf extract of G. asiatica was found to be active on Breast cancer cell line with IC50 of 50.37µg/ml and 61.23µg/ml in HEp – 2 cell lines.

CONCLUSION

Aqueous leaf and fruit extract of G. asiatica showed in-vitro anticancer activity against Lung cancer cell line, Breast cancer cell line, Cervical cancer cell line and Larynx cancer cell line. This activity may be due to either of the presence of antioxidants such as vitamin C, anthocyanin, folate carotenoids. These results suggest aqueous leaf and fruit extract of G. asiatica may be an attractive option for the “drug hunters” as a potential agent for the management of human cancer. The constituent which is responsible for this activity is remains for further investigation.

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