ORIGINAL ARTICLE

Anti-proliferative or Cytopathic Potential of *Thapsia garganica, Citrus sinensis, Citrus limon* and *Vinca rosea* Extracts Against Human Embryonic Kidney Carcinoma Cell Line

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ABSTRACT

Objective*: Developing anti-cancer drugs from natural products is receiving increasing interest worldwide due to limitations and side effects of anti-cancer drugs. The purpose of this study was to explore the anti-proliferative or cytopathic potential of natural compounds derived from plant sources as alternatives of synthetic compounds on human embryonic kidney carcinoma (HEK) cell line.*

Methods*: In this study, aqueous and methanolic extracts were obtained from various plants,* viz*,* Thapsia garganica*,* Citrus sinesis*,* Citrus limon *and* Vinca rosea*. Extracts were serially diluted into 96-well microtitre plates and were screened for anti-proliferative potential against the HEK cell line via the neutral red dye uptake assay.*

Results*: The findings revealed that methanolic extracts of* T. garganica *leaf and* V. rosea *leaf were the most effective as anti-proliferative or cytotoxic against the HEK cell line, with* IC_{50} *at 32-fold dilution of the extract.*

Conclusion: *The extracts of* T. garganic *and* V, rosea *have been used as anti-proliferative drugs but after trial in experimental animals for being not toxic.*

Keywords: Anti-proliferative, cytopathic potential, human embryonic kidney carcinoma cell line, natural compounds, plants.

INTRODUCTION

Cancer is the leading cause of mortality worldwide, with 14 million new cases and 8.2 million cancer-related deaths in 2012 (1). It is influenced by various environmental and genetic factors. There are two fundamental methods for the treatment of the cancer. Surgery is the most widely used for the treatment of benign tumours, while gamma radiation and chemotherapy along with surgery are the cornerstones for the treatment of malignant neoplasms. The problem with radiation and chemotherapy is that the normal cells can also die along with the cancerous cells (2). There is a surge in developing an alternative treatment for cancer. The plant sources can be exploited to obtain the compounds those can be helpful in treating cancers.

Thapsia, commonly known as the deadly carrot (3), belongs to the family Apiaceae that consists of poisonous plants. Thapsigargin is the chemical compound that has been isolated from *Thapsia garganica.* It has been used to produce a prodrug synthetically. This prodrug has been used efficiently during clinical trials for cancer treatment. By addressing the calcium balance, this active compound kills the tumour cells. Hundreds of bioactive compounds are found in citrus fruits that possess anti-cancerous properties (4). This includes vitamin C, a common antioxidant and several chemicals such as flavanoids and monoterpenes, including tangeritin, limonene and nobiletin. These chemicals are naturally occurring in citrus fruits in high concentrations. They function to decrease cell proliferation (5). Traditional folklore medicine use

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the leaf extracts of plant *Catharanthus roseus* for the treatment of diabetes. Vinblastine and vincristine are complex indole alkaloids also discovered and isolated from *C. roseus*. They are used in the treatment of various cancers. These compounds are structurally similar but they differ in their toxic properties and the type of tumour they affect (6). The extracts have depicted significant anti-cancer activity against various cell types (7). Plants have always been an important source of traditional and modern medicine (8). Medicinal plants are an important source of life-saving drugs for the majority of the world's population. Some of these plants exhibit magnificent anti-proliferative or anti-cancer properties. In Pakistan, however, there is not much work regarding the anti-proliferative properties of plant extracts except a few. Therefore, the present study was planned to identify the significant anti-proliferative potentials of plant extracts. Human embryonic kidney (HEK) carcinoma cells, also often referred to as HEK 293, 293 cells or HEK cells, are a specific cell line originally derived from HEK cells grown in tissue culture. HEK 293 cells are very easy to grow and transfect very readily and have been widely used in cell biology research for many years. In the present study, extracts of commonly available medicinal plants such as *T. garganica* leaf, stem and delicate stem, orange and lemon peel and *Vinca rosea* leaf were used to find the anti-proliferative potentials or cytopathic effects against the HEK cell line. The objectives of the study were to optimize the bioassay condition of natural extracts isolated from *Citrus limon* (lemon peel), *Citrus sinensis* (orange peel), *T. garganica* and *V. rosea* and to determine the inhibitory concentration (IC 50) by using HEK carcinoma cell line.

MATERIALS AND METHODS

Collection of explants

The explants of the study were collected from various sources. *Vinca rosea* was collected from the botanical gardens of Lahore College for Women University Lahore (LCWU). Citrus plants, namely oranges and lemon, were collected in large number from the fruit shop at LCWU. *Thapsia garganica* was collected from Margalla Hills Islamabad. The leaves of *V. rosea* were removed and washed thoroughly, and were dried completely. The powder was prepared in a grinder. The citrus plants, oranges and lemons, were properly washed to remove any dust and peel was removed. All fibres attached to the peel were carefully removed, were dried under shade and ground into a fine powder. The *T. garganica* leaves and stem were used after proper washing and adopting the same method as described above.

Preparation of plant extract

All powdered plant samples (*V. rosea*, *C. limon*, *C. sinensis* and *T. garganica* leaf, stem and delicate stem) were separately blended with water for preparing a water extract. For this purpose, 1 g of powdered plant sample was blended with 3 ml of sterile distilled water. For methanol extraction, all the powdered plant was blended with the methanol for preparing methanolic extracts. For this purpose, 1 g of powdered plant sample was blended with 3 ml of pure methanol and stored in the Eppendorf tubes at –20°C. The tubes were properly labelled during the storage. The extracts were removed from the Eppendorf tubes with the help of a sterile syringe. Separate syringe was used for separate extracts. The needle was removed and the syringe was attached to the microfilter. For each extract, a separate microfilter was used. Carefully, the syringe was pressed so that the liquid came out drop by drop. It was stored in sterile Eppendorf tubes. All processes were carried out in laminar flow hood. The tubes were labelled and stored in a freezer at –20°C.

Culturing of HEK cell line

(i) Propagation of cell line

(A) Preparation of GMEM (1 l)

Approximately 12.3 g of Glasgow Modified Essential Medium (GMEM) prepared powder was dissolved in 800 ml distilled water. After dissolving, 3.75 g/L of $NaHCO_3$ (sodium bicarbonate) was added. Then 1 ml/L of penicillin, 1 cm^2/L streptomycin and gentamycin were added to the mixture. The mixture was placed on a stirrer for half an hour, and pH was adjusted to 5. Before use in cell culture, 10% (100 ml) of 29.5 g/L tryptose phosphate broth and 10% foetal calf serum were added to the medium which was incubated until required.

(B) Method for cell propagation

The frozen cell line was placed into the beaker of water. The beaker was then placed into the water bath at 37°C to maintain temperature. As soon as the vial started to melt, it was poured into a centrifuge tube. Then, 10 ml of growth medium GMEM (10% calf serum) was added dropwise into the tube. The cells were centrifuged at 1000–1200 RPM for 5–10 minutes. This process was repeated once. The supernatant was discarded and 1–1.5 ml of the medium was used to suspend the pellet after which it was added into a Roux bottle. The 10–15

ml of the medium was added to cover the bottom layer of the bottle after which the cells were incubated for 48 to 72 hours.

(ii) Cell splitting

Preparation of phosphate-buffered saline (g/L)

Solution A

NaCl: 8 g, KCl: 0.2 g, $Na_2HPO_4 \cdot 7H_2O$: 2.17, $Na_2HPO_4 \cdot 2H_2O$: 1.44 g, $Na_2HPO_4 \cdot 7H_2O$ anhydrous: 1.15 g, KH_2PO_4: 0.2 g.

Solution B

$CaCl_2$: 0.1 g, $CaCl_2 \cdot 2H_2O$: 0.134 g, $MgCl_2 \cdot 6H_2O$: 0.1 g.

Solution C

Penicillin (100 000 IU), streptomycin (0.1 ml), double distilled water (1000 ml).

Solution A was dissolved in 800 ml double distilled water, solution B was dissolved in 100 ml double distilled water and solution C was dissolved in 100 ml double distilled water. Solutions B and C were added to solution A slowly with constant stirring. Antibiotics were added. The solution was sterilized by filtration through a 0.2-µm filter after which a sterility test was carried out. The solution was stored at 8°C.

Preparation of 0.25% trypsin

The following chemicals were used to prepare the 0.25% trypsin solution: NaCl (8 g), KCl (0.2 g), $NaHPO_4 \cdot 7H_2O$ (2.183 g), KH_2PO_4 (0.2 g), trypsin (2.5 g), penicillin (100 000 IU), streptomycin (0.1 ml), deionized water (1000 ml). Each ingredient was dissolved in deionized water in the given order. The trypsin solution was placed on a magnetic stirrer, and the solution was stirred for an hour after which it was filtered into a glass bottle and was checked for sterility as described by The International Pharmacopoeia and was stored at 4°C.

Preparation of 2% versene (ethylenediaminetetraacetic acid) solution

The following chemicals were used to prepare the 0.25% trypsin solution: NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g), KH_2PO_4 (0.2 g), EDTA disodium salt (22.1 g), phenol red (1 g). All the reagents were dissolved in deionized water, and volume was made up to 1000 ml. The solution was distributed in 100 ml volume screw capped bottles and was autoclaved at 121°C for 15 minutes and stored at 4°C.

Method for cell splitting

Cells were rinsed with phosphate-buffered saline (PBS) by adding 10 ml of PBS to the Roux bottles. One to four drops of versene solution were added to the 0.1 ml trypsin solution to make the trypsin versene solution. This solution was added to the PBS solution. Roux bottles were incubated with a trypsin versene solution for 1–2 minutes. When cells were visible on the bottom surface of the bottle, 20 ml GMEM medium was added. Approximately 10 ml was added to one bottle, while the remaining 10 ml was added into another. Both flasks were incubated at 37°C for 48 hours for cell growth.

Preparation of a cell suspension

Approximately 50 ml cell suspension was prepared. First, the old media was washed out of the flasks with dense cell growth. About 10 ml of PBS was added into the flasks after which they were incubated at room temperature for 3–4 minutes. The PBS was washed out and 1% trypsin, 0.5% versene solution (made from stock 2.5:2%) was added. This allowed the detachment of cells from the surface monolayer, and from each other. The 6–8 ml of medium was added into each flask, after which the medium from all flasks was transferred into a falcon tube. The volume was made up to 50 ml by adding GMEM suspension.

Determination of anti-cancerous potential of natural extracts absorbance before analysis

In an Eppendorf tube, about 100 µL cell suspension was added to 100 µL neutral red dye solution. The Eppendorf tube was incubated at room temperature for 30 minutes, after which it was centrifuged for 3 minutes at 7000 rpm. The supernatant was discarded and about 100 µL acidified ethanol was added to the pellet. The tube was incubated for 5 minutes at room temperature after which it was centrifuged at 8000 rpm for 3 minutes. The supernatant was transferred in a fresh Eppendorf tube and absorbance was checked at 540 nm.

Cell viability and anti-proliferative analysis

Plant extracts were centrifuged at 7000 rpm for 2–3 minutes to remove any precipitates formed during freezing. In a 96-well microtitre plate, 100 µL GMEM medium was added to the first 10 wells. Then, 100 µL of the respective plant extract was dispensed into the 3rd well (C3) and was serially diluted till the 10th well (C10) each by 2-fold dilution. This was done repeatedly for all the other extracts. Then, about 100 µL of cells from the cell suspension was pipetted into each well from C1 to C10.

Cells were checked under an inverted microscope to check for contamination. The plates were then incubated for 48 hours in a CO_2 incubator with adequate conditions (37°C, 5% CO_2, 95% atmosphere). The 50 ml neutral red dye medium (40 mg neutral red) was prepared and incubated at the same temperature as the cells.

Morphological examination of treated cells

After 48 hours of incubation, plates were washed with PBS and were subjected to microscopic evaluation under an inverted microscope to ensure that they have not been contaminated and to analyse the density of live cells with an increase in the dilution of extracts.

Neutral red uptake assay

The neutral red dye medium was centrifuged at 1800 RPM for 10 minutes to remove any precipitated dye crystals. The medium was decanted out of cells, after which the wells were washed with 0.5× PBS by immersing the wells with PBS followed by gentle tapping. The 100 µL of red dye was added to each well after which the plates were incubated for 2 hours at 37°C. The red dye was then removed from the wells and plates were washed as done previously with PBS. The plates, after being washed with PBS, were analysed under an inverted microscope to study the morphological changes with the increase in dilutions of every extract, with reference to the control. Then, about 150 µL of acidified ethanol was added to each well and the plate was rapidly shaken to allow the ethanol to remove the dye and form a homogeneous solution. Absorbance was checked at 540 nm using a microtitre plate reader.

Cell viability analysis

The absorbance of samples recorded at 540 nm was used to deduce the percentage of viable cells. This was done by determining the IC_{50} using the equation.

Percentage of viable cells = Average no of viable cells (absorbance) in each well/Average no of cells (absorbance) in control × 100.

The fold dilutions and absorbance that showed 50% viable cells were indicative of the IC_{50} value for that specific extract. The IC_{50} values for every extract were plotted against their respective absorbance with reference to a consensus controls. The results at the highest fold dilution would mean the most effective extract with respect to their abilities to inhibit cancer cell growth.

The anti-proliferative effect of the extracts was analysed by culturing the HEK cell line with the extract and analysing the effects morphologically. The amount of viable cells was then determined by the neutral red uptake assay. The extracts were seeded with the cell line and were incubated in a CO_2 chamber for 48 hours. After incubation of plates for 48 hours, cells were washed with PBS, which was used to dissociate dead cells. Live cells were observed under an inverted microscope to analyse the morphological changes in every well as the dilution of the extract increased.

RESULTS

The cell growth patterns were similar in all cells treated with the extracts, as with an increase in the dilution of the extract, the cell growth started to decrease. There was a slight change in the shape of the HEK cells. This was observed in both control wells and in the experimental wells. However, no contamination was present and cells remained attached to the monolayer even after washing with PBS.

The density of cells in control wells, those treated with *T. garganica* and orange extracts were greater than the density of cells treated with other extracts. Cell viability in wells at the zero-fold dilution was less than 20% in water extracts of *T. garganica* leaves, citrus lemon peel and *V. rosea* leaf, while it was between 20% and 30% for other water extracts (Table). Similarly, cell viability was less than 20% for methanolic extracts of *T. garganica* stem, *Citrus lemon* peel and *V. rosea* leaf, while it was between 20% and 30% for other methanolic extracts. The results of water extracts revealed 50% or higher cell viability in experimental extracts at 8-fold (*T. garganica* stem), 16-fold (*T. garganica* delicate stem, *C. sinensis* peel, *V. rosea* leaf) and 32-fold (*T. garganica* leaf, Citrus lemon peel) dilution. The methanolic extract revealed 50% higher cell viability in experimental extracts at 32-fold (*T. garganica* stem, *C. sinensis* peel) and 64-fold (*T. garganica* leaf, *T. garganica* delicate stem, *C. limon* peel, *V. rosea* leaf) dilutions (Table).

DISCUSSION

Cancer is a serious disease condition that can affect any part of the body. The most common types of cancers are colon, lung, stomach, liver and breast. There is a difference in the most frequent types of cancers among men and women. About 70% of all cancer deaths in 2008 occurred in low- and middle-income countries. Deaths from cancer are projected to continue rising, with an estimated 13.1 million deaths in 2030 (9). Natural extracts in the form of infusions, herbal medicines and decoctions have been used traditionally for several years for the treatment of various infectious diseases. Plant extracts

Table: Analysis of anti-proliferative effect of different dilutions of various extracts on HEK (293t) cell line by the neutral red dye assay[a]

Solvent	Plant	Explants	Fold dilution							Control
			0	2	4	8	16	32	64	
Water	*Thapsia garganica*	Leaf	0.04 (23)	0.042 (24.8)	0.069 (40.8)	0.069 (40.8)	0.078 (46.1)	0.1 (59.1)	0.14 (82.8)	0.169
		Stem	0.024 (15)	0.044 (25)	0.06 (37.5)	0.068 (50)	0.088 (55)	0.1 (62.5)	0.13 (81.3)	0.16
		Delicate stem	0.045 (26.4)	0.052 (30.5)	0.06 (35.2)	0.068 (40)	0.088 (51.7)	0.1 (58.8)	0.13 (76.4)	0.17
	Citrus sinensis	Peel	0.041 (25)	0.044 (27.5)	0.063 (39.3)	0.068 (42.5)	0.081 (50.6)	0.12 (75)	0.14 (87.5)	0.16
	Citrus limon	Peel	0.024 (15)	0.042 (26.2)	0.066 (41.2)	0.068 (42.5)	0.078 (48.75)	0.12 (75)	0.15 (93)	0.16
	Vinca rosea	Leaf	0.02 (13.3)	0.04 (26)	0.067 (44.6)	0.069 (46)	0.088 (58.6)	0.12 (80)	0.13 (86.6)	0.15
Methanol	*Thapsia garganica*	Leaf	0.046 (29.2)	0.048 (30.5)	0.05 (31.8)	0.064 (40.7)	0.068 (43.3)	0.0712 (45.3)	0.15 (95.5)	0.157
		Stem	0.026 (16.3)	0.036 (22.5)	0.047 (29.3)	0.064 (40)	0.066 (41.25)	0.0812 (51.2)	0.12 (75)	0.16
		Delicate stem	0.046 (25.5)	0.056 (31.1)	0.067 (37.2)	0.068 (37.7)	0.069 (38.3)	0.0812 (45.1)	0.16 (88)	0.18
	Citrus sinensis	Peel	0.036 (22.5)	0.046 (28.7)	0.057 (35.6)	0.064 (40)	0.067 (40)	0.082 (51.2)	0.13 (81.25)	0.16
	Citrus limon	Peel	0.028 (17.5)	0.035 (21.8)	0.049 (30.6)	0.054 (33.7)	0.069 (43.1)	0.0712 (44.5)	0.12 (75)	0.16
	Vinca rosea	Leaf	0.026 (16.2)	0.036 (22.5)	0.047 (29.3)	0.064 (40)	0.067 (41.8)	0.0712 (44.5)	0.12 (75)	0.16

[a] The absorbance value of neutral red dye was taken at 540 nm.
[b] The values in parenthesis in this table are percentages.

possess biologically significant properties, *ie* antibacterial, anti-mutagenic, anti-carcinogenic, anti-thrombotic and anti-diabetic. The feasibility of a therapeutic agent depends on the solvent used for the preparation of the natural extract. There are various factors that affect the choice of solvent. These include the quantity of phytochemicals, the diversity of different compounds, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants. Since the end product contains traces of the solvent, it is essential that the solvent does not interfere with the bioassay and is non-toxic (10). In the present study, two solvents, *ie* water and methanol, were used. Water is a universal polar solvent that is used traditionally for the isolation of inorganic compounds. Methanol is also a polar solvent but is used to isolate the organic compounds.

The solvents work by diffusing into tissues of plants and solubilizing active compounds of the same polarity, resulting in the exploitation of the active compounds. The present study was based on the anti-cancer properties of the extract of selected plants. The drug, called G202, is chemically derived from a common weed called *T. garganica*, and has been shown to destroy cancers and their direct blood supplies, avoiding damage to healthy blood vessels and tissues. In laboratory studies, researchers found reduction of the size of human prostate tumours in a 3-day treatment of G202. The researchers also reported that G202 produced at least 50% regression in models of human breast cancer, kidney cancer and bladder cancer (11). In addition, citrus fruits such as lemons and oranges were also tested and have been used in ancient medicine. *Catharanthus roseus* is a novel antimicrobial plant since most of the bacterial pathogens are developing resistance against many of the currently available antimicrobial drugs. The anti-cancer properties of *C. roseus* have been explored (12).

During the present study, the controls showed dense growth, which indicates that the solvents did not have suppressive effects on the cell growth. However, there was a slight distortion in the shape of cells which can be due to the incorporation of the solvents into the cell membrane. Photographic evidence indicated that with the increase in dilution of the extracts, there were a higher proportion of live cells. Wells with the highest concentration of the extract, therefore, caused the maximal death

of cells. At 64-fold dilution of the extract, the number of cells in the growth wells was highest which reflected that lower concentrations of extract had less effect on the cell growth. The neutral red uptake assay is a cell viability test that relies on the ability of viable cells to bind the red dye. In dead cells, the neutral red dye is not retained as cell death/damage leads to lysosomal damage and the membrane no longer acts as a barrier to preserve the dye in the cells (13).

The study made use of various extracts that were incubated with the HEK cell line. The absorbance of cells was proportional to the number of live cells. With the increase in dilutions, the absorbance increased as more live cells took up the dye. IC_{50} inhibitory concentration of cells is used to test the efficacy of a drug. We used the IC as the study was aiming to explore the anti-proliferative agents. IC_{50} is the concentration at which 50% of the cells were alive.

The results showed that *V. rosea* leaf, *T. garganica* stem and *C. limon* had better results than other water extracts at 0-fold dilution. The highest dilution inhibiting more than 50% growth was 16-fold for *T. garganica* leaf and *C. lemon* peel. However, most other water extracts inhibited more than 50% growth at 8-fold dilution including *T. garganica* delicate stem, *C. sinensis* and *V. rosea*. The water extract of *T. garganica* stem inhibited more than 50% growth at 4-fold dilution. The results of methanolic extracts also revealed that *V. rosea* leaf, *T. garganica* stem and *C. limon* inhibited growth better than other methanolic extracts at 0-fold dilution. This suggests that both water and methanolic extracts of these plant sources have the same kind of effect. However, it was also revealed that the methanolic extract, *T. garganica* leaf and delicate stem, *C. limon* and *V. rosea* inhibited more than 50% growth at 32-fold dilution suggesting that the methanolic extract has stronger compounds those can prevent growth at higher dilution than water extracts. Furthermore, it was noted that the *T. garganica* stem and *C. sinensis* prevented more than 50% growth at 16-fold dilution again suggesting that the methanolic extracts of these plant sources were also effective at higher dilution than their water extracts. The results are encouraging and demand further investigations on lab animals to prove that they are not toxic at the same dose level.

CONCLUSION

Methanol proved to be the most effective solvent for the preparation of the extracts. Methanolic extracts of *T. garganica* leaf and *V. rosea* leaf were the most responsive with regard to their anti-proliferative properties as both achieved IC_{50} at the 32-fold dilution.

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