Studies of Cytotoxic Potential of Cucumis melo. Linn Fruit Aqueous Extract in Prostate Cancer Cell lines PC-3 Using MTT and Neutral Red Assay

SIBI P ITTIYAVIRAH, ANN GEORGE, ANJU M SANTHOSH, SUDHI T KURIAN, PRINSY PAPPACHAN and GIFTY JACOB

ABSTRACT
The objective of the study was to evaluate the cytotoxic effects of aqueous fruit extract of Cucumis melo in human prostate cancer cell line (PC-3) using MTT and neutral red assays. The crude aqueous extract of C. melo was prepared by cold maceration method, filtered, concentrated and tested on PC-3 cell line. Dose-dependent cytotoxic activities were exhibited by human prostate carcinoma PC-3 cell line. As the dose of the extract increased, the number of viable cells decreased. This confirms the anti-cancer and cytotoxic potential of the fruit of C. melo.

Keywords: Cytotoxicity, MTT assay, Neutral red assay, Human prostate carcinoma cell lines (PC-3), Cucumis melo

Carcinoma of the prostate gland is the malignancy of the male genitourinary tract and is a disorder in older men, with mean age at presentation about 70 years. Treatment of kidney stones, cancer, cardiovascular when diagnosed by the presence of symptoms. The risk disorders and stroke. Three components found in of developing prostate cancer is affected by racial and melons are Cucurbitacin-β, Lithium and Zinc which environmental factors. The magnitude of familial risk exhibit promise in cancer prevention, fighting increases with number of first degree relatives who are depression, dandruff, and ulcers and stimulates the affected, and also if the affected relatives diagnosed immune system. C. melo is also rich in antioxidants, with prostate cancer at an early age. Prostate cancer risk flavonoids such as β-carotene, lentin, xanthin and has been inversely associated with several dietary cryptoxanthin. These antioxidants have the ability to components including the essential non-metallic trace protect cells and structures in the body from oxygen free element selenium. Chromosomal alterations are radicals, hence offer protection against prostate, colon, associated with an inherited predisposition to prostate breast, lungs, endometrial and pancreatic cancer. The cancer and prostate cancer and its development. Two cucurbitacea family includes several species of prostate cancer susceptibility genes that have been cultivated plants that has great economic importance identified are the RNASEL and MSR1 genes both of like water melon (Citrullus lanatus.L.), squash which are associated with response to infections. (Cucurbita maxima.L.), cucumber (Cucumis sativus.L.)

Cucumis melo fruit is round in shape, tan to greenish and cantaloupe(Cucumis melo.L.) [1]. Earlier studies on tan with a rough texture and orange pink flesh. It is well known for its sweet taste and fragrance. It is native to Persia, Armenia, etc. Many phytochemicals having properties [2]. The active principles in the vegetable potential benefits are present in C. melo. It is rich in extracts are principally water soluble or lipophilic
Effect of *cucumis* on prostate cancer

Plant material

Carrot seeds were collected from local fruit stalls in Cherthala, Alappuzha District in the month of November 2012 and authenticated at Department of Environment Sciences, Mahatma Gandhi University, Kottayam, Kerala, India. In vitro methods were used for assessing the cytotoxic activity and they were in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC).

Reagents for phyto-chemical analysis

Bismuth nitrate, Nitric acid, Potassium iodide, Sodium carbonate, Mercuric chloride, Sulphuric acid, Hydrochloric acid, Sodium hydroxide, Ferric chloride, Alpha naphthol, Copper sulphate, Zinc chloride 3-(4,5)-diphenyl tetrazolium bromide (MTT), Isopropanol, Phosphate buffer saline (PBS), Dimethyl sulfoxide (DMSO), Calorimeter,

MTP assay and neutral red assay

PC-3 cell line-PC-3 prostate cell lines purchased from National centre for Cell Sciences (NCCS), Pune, Maharashtra, India. 1M Potassium dihydrogen phosphate, CO2 Incubator, PBS, Elution medium (ethanol/acetic acid), Spectrophotometer.

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Preparation of extract: cold maceration

The fruit was washed and the outer skin was peeled off. The remaining fleshy part was cut in to small pieces. Then it was soaked in water for seven days and was kept in a dark place. During this period shaking was done occasionally. After seven days, the liquid was added. Formation of yellow color indicated the strained and marc was pressed. The expressed liquid presence of glycosides.

Phytochemical analysis

The following tests were carried out to analyze the possible phytochemicals present in the aqueous extract of *C. melo*.

Alkaloids-Dragnetoff's test

Eight gram of bismuth nitrate was dissolved in 20 ml nitric acid and 2.72 g of potassium iodide in 50 ml water. These were mixed and allowed to stand. When potassium nitrate crystals out, the supernatant was discarded off and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with sodium carbonate followed by extraction of the liberated base with ether. To 0.5 ml of alcoholic solution of extract was added 2.0 ml of hydrochloric acid. To this acid medium, 1.0 ml of reagent was added. An orange red precipitate was produced immediately indicated the presence of alkaloids.

Mayer's test

The amount of 1.36 g mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. To 1 ml of acidic aqueous solution of extracts, a few drops of reagent was added. Formation of white or pale brown precipitate showed the presence of flavonoids, reddish pink or dirty brown color was produced.

Wagner's test

In a test tube containing 0.5 ml of alcoholic extract, 5-10 drops of dilute HCl and a small piece of ZnCl2 or Mg were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink or yellow color was produced in the chloroform layer.

Phytosterols

To 2 ml of chloroform extract, 1ml of concentrated sulphuric acid was added carefully along the sides of the test tube. In the presence of phytosterols, a golden yellow color was produced in the chloroform layer.

Glycosides

A small amount of alcoholic extract was dissolved in 1 ml of water and the aqueous NaOH solution was done occasionally. After seven days, the liquid was added. Formation of yellow color indicated the presence of glycosides.

MATERIALS AND METHODS

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Published online: January 31, 2013
Absence of carbohydrates (-)

Table 1. Phytochemical constituents aqueous extract of C. melo

<table>
<thead>
<tr>
<th>Tests</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Orange red ppt was observed</td>
<td>Presence of alkaloids (+)</td>
</tr>
<tr>
<td>Drenadroff’s test</td>
<td>Pale white ppt was observed</td>
<td>Presence of alkaloids (+)</td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>Dirty brown ppt was observed</td>
<td>Presence of Flavonoids (+)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Dark golden colour was observed</td>
<td>Presence of phytosterols (+)</td>
</tr>
<tr>
<td>Glycosides</td>
<td>No characteristic change was observed</td>
<td>Absence of glycosides (-)</td>
</tr>
<tr>
<td>Tannins</td>
<td>No characteristic change was observed</td>
<td>Absence of glycosides (-)</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>No characteristic change was observed</td>
<td>Absence of carbohydrates (-)</td>
</tr>
<tr>
<td>Molisch’s test</td>
<td>No characteristic change was observed</td>
<td>Absence of proteins (-)</td>
</tr>
<tr>
<td>Proteins</td>
<td>No characteristic change was observed</td>
<td>Absence of saponins (-)</td>
</tr>
</tbody>
</table>

Table 2. Cytotoxicity studies aqueous extract of C. melo using MTT assay

<table>
<thead>
<tr>
<th>Sample concentration (µg/ml)</th>
<th>OD (540 nm)</th>
<th>% viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.220</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>0.211</td>
<td>95.90</td>
</tr>
<tr>
<td>500</td>
<td>0.172</td>
<td>78.18</td>
</tr>
<tr>
<td>1000</td>
<td>0.148</td>
<td>67.27</td>
</tr>
</tbody>
</table>

167 **Tannins-Ferric chloride test**

To 1-2 ml of aqueous extract, few drops of 5% aqueous ferric chloride solution was added. A bluish black color which disappears on addition of a few ml of sulphuric acid, there is no formation of yellowish brown precipitate.

173 **Sugars-Molisch’s test**

In a test tube containing 2 ml of aqueous extract, 2 drops of freshly-prepared 20% alcoholic solution of anisnaphthol was added and mixed. To this solution, 2 ml of conc: Sulfuric acid was added so as to form a layer below the mixture. Formation of red violet ring at the junction of solution and its disappearance on the addition of an excess solution indicated the presence of carbohydrates.

182 **Proteins-Biuret’s test**

In a test tube containing 2 ml of test sample, 2 ml of 10% NaOH is added and mixed well. Then 0.1% CuSO₄ solution is added. A violet or pink colour indicated the presence of proteins.

187 **Saponins-Froth Test**

Few ml of the extract is transferred in to a test tube and shaken vigorously then is left to stand for 10 min. A thick persistent froth indicated presence of saponins.

193 **MTT assay**

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The products are then solubilized with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent. Since reduction of MTT can only occur in metabolically-active cells, the level of activity is a measure of the viability of the cells.

204 PC-3 human prostate cell lines purchased from NCCS Pune, was maintained in Dulbecco’s Modified Eagles Media (DMEM) and grown to confluence at 37°C and 5% CO₂ in a humidified atmosphere in a CO₂ incubator. The cells were trypsinized (500 µl of 0.025% Trypsin in PBS/ EDTA solution) for 2 min and passed to T flasks in complete aseptic conditions and incubated. Extracts were added to 80% confluent cells at a concentration of 100 µg, 500 µg and 1000 µg from a stock of 100 mg/volume di H₂O and incubated for 24 h. The cell culture suspension was washed with 1× PBS. Then, 200 µl MTT solutions were added to the culture (MTT: 5 mg/volume dissolved in PBS). These were incubated at 37°C for 3 hours. All MTT wash was removed with 1× PBS and 300 µl DMSO was added to each culture. The plates were incubated at room temperature for 30 min until the cell get lysed and color was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 min to precipitate cell debris. Optical density (OD) was read at 540 nm using DMSO as blank.

224 The neutral red cytotoxicity test was based on the ability of living cells to uptake and bind neutral red (NR). NR was a positively-charged dye that easily diffuses through the cellular membrane of the cells, accumulates in the cellular cytoplasm and stores in the acidic environment of lysosomes. The principle of the test consists in the fact that NR are able to absorb and bind only with live cells while this ability declines in damaged or dead cells. The amount of accumulated NR was thus directly proportional to the amount of live cells in the cell culture. The pH of the neutral red solution was thus directly proportional to the amount of live cells.
Effect of *cucumis* on prostate cancer

RESULTS

**Phytochemical analysis**

The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [8]. The Phytochemical screening of the aqueous extracts of plant sample revealed the presence of alkaloids and flavonoids and phytosterols (Table 1) [9].

**Cytotoxic studies**

MTT results showed that 1000 μg/ml aqueous extract of *Cucumis melo* showed 67.27% (Table 2, Fig 1) while the neutral red uptake assay showed 66.27% viability (Table 3, Fig 2). The photograph of PC-3 cell lines had shown a dose-dependent anti proliferative effect. The IC50 values of MTT and Neutral red assays were found to be 1470 and 1860 μg/ml respectively (Figs 4 and 5).

**DISCUSSION**

Metastatic prostate carcinoma is associated with a high morbidity and mortality rate with a median survival of approximately, 12–15 months. Available treatment alternatives include radiotherapy after radical retropubic prostatectomy, radical prostatectomy, external beam radiation, prostate brachy therapy, and androgen ablation of the prostate. Until recently, despite androgen suppression, no cytotoxic agent has been able to change the progression of metastatic prostate cancer. Androgen ablation therapy remains the main course of treatment with advanced disease. However, it has no effect on hormone-independent cancer cells. Chemotherapeutic agents result in less than a 10% response in advanced prostate carcinoma, in part due to increased resistance of androgen-independent cells to apoptosis. However, the severe side effects of chemotherapy have remained a major problem.

In recent years considerable efforts have been made to identify naturally-occurring compounds and related synthetic agents can prevent the development and recurrence of cancer. A wide variety of natural food and food products can induce apoptosis in various tumor cells. There is strong evidence supporting the positive role of some natural materials and medicinal plants in oncology and their ability affect all phases of tumorogenic process. Therefore, it is important to screen the natural products either as crude extracts or as isolated components for apoptotic properties to identify potential anti-cancer compounds. Over 60% anti-cancer agents currently used are derived from natural sources, including plants, marine organisms and micro-

**Table 3. Cytotoxicity studies aqueous extract of *C.melo* using Neutral red assay**

<table>
<thead>
<tr>
<th>Sample concentration (μg/ml)</th>
<th>OD (540 nm)</th>
<th>% viability</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.086</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>0.070</td>
<td>81.39</td>
</tr>
<tr>
<td>500</td>
<td>0.065</td>
<td>75.58</td>
</tr>
<tr>
<td>1000</td>
<td>0.057</td>
<td>66.27</td>
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Fig 1. Cytotoxicity studies aqueous extract of *C.melo* using MTT assay

Fig 2. Cytotoxicity studies aqueous extract of *C.melo* using Neutral red assay
Antioxidant activity and C. melo
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C. melo Concentration of the sample (µg/ml)
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Gill NS. C. melo - vanoi
12 - Duke JA, Ayensu ES. Medicinal Plants of China Reference
oko YA, Kusuma IW, Yulia

REFERENCES

Fig 3. Photograph of PC-3 cell line: i) control received vehicle, ii) C.melo at a conc. 100µg/ml and iii) C.melo at a conc. 1000 µg/ml.

Fig 4. IC_{50} value of aqueous extract of C.melo using MTT assay.

Fig 5. IC_{50} value of aqu. extract of C.melo using Neutral red assay.

Organisms and they offer an opportunity to study the molecular mechanisms of tumorgenesis.
Cucurbitaceae plants are highly useful as they have good potential against many health ailments. In the present study, the phytochemical screening of the aqueous extracts of plant sample revealed the presence of alkaloids and flavonoids and phytoestrogens [9]. These phytoconstituents may be responsible for various activities. Flavonoids are diverse family of compounds commonly found in fruits, vegetables and honey. Flavonoids are generally safe and associated with low toxicity, making them ideal candidates for cancer chemopreventive agents. MTT results and neutral red uptake assay confirms dose-dependent anti-proliferative effect of crude aqueous extract of Cucumis melo on prostate cancer cell lines. As the dose of the extract increases, number of viable cells decreases and confirms the cytotoxic activity.
It is concluded that the aqueous extract of C. melo was found to possess dose-dependent cytotoxic activity on metastatic human prostate cancer cell lines PC-3. Further studies are warranted to explore the anticancer effect of C. melo and also the active principles could be isolated and investigated.
Effect of *cucumis* on prostate cancer

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