

ORIGINAL ARTICLE

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

For author affiliations, see end of text.

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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 when diagnosed by the presence of symptoms. The risk of developing prostate cancer is affected by racial and environmental factors. The magnitude of familial risk increases with number of first degree relatives who are affected, and also if the affected relatives diagnosed with prostate cancer at an early age. Prostate cancer risk has been inversely associated with several dietary components including the essential non-metallic trace element selenium. Chromosomal alterations are associated with an inherited predisposition to prostate cancer and prostate cancer and its development. Two prostate cancer susceptibility genes that have been identified are the RNASEL and MSR1 genes both of which are associated with response to infections. Carbohydrates, Proteins, fibre, citric acid, vitamin K, vitamin A and folate. Traditionally, it is used for treatment of Kidney stones, cancer, cardiovascular disorders and stroke. Three components found in melons are Cucurbitacin- β , Lithium and Zinc which exhibit promise in cancer prevention, fighting depression, dandruff, and ulcers and stimulates the immune system. *C. melo* is also rich in antioxidants, flavonoids such as β - carotene, lentin, xanthin and cryptoxanthin. These antioxidants have the ability to protect cells and structures in the body from oxygen free radicals, hence offer protection against prostate, colon, breast, lungs, endometrial and pancreatic cancer. The cucurbitacea family includes several species of cultivated plants that has great economic importance like water melon (*Citrullus lanatus*.L), squash (*Cucurbita maxima*.L), cucumber (*Cucumis sativus*.L) and cantaloupe(*Cucumis melo*.L) [1]. Earlier studies on cucurbitacea family showed that cantaloupe pulp extract possesses high antioxidant and anti-inflammatory 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

antioxidant molecule. Most of these plant extracts¹¹⁷ was mixed with strained liquid, filtered to make a clear¹¹⁸ liquid and concentrated.

contain various amounts of vitamin E and C, Carotenes,¹¹⁹ triterpenoids and other flavanoids [3]. For this, these¹²⁰

were used as potential antioxidant prophylactic agents¹²¹ **Phytochemical analysis**

for both health and diseases management [3,4]. The¹²² The following tests were carried out to analyze the¹²³ methanolic seed extract (MECM) of *Cucumis melo*. Var¹²⁴ possible phytochemicals present in the aqueous extract¹²⁵ possess significant antioxidant, anti-inflammatory and¹²⁶ of *C. melo*.¹²⁷

analgesic properties [5], while the fruit extract *C. melo*¹²⁸

fruit exhibited immunomodulatory activity [6]. Even¹²⁹ **Alkaloids-Dragendroff's test**

though a large number of compounds were screened for¹³⁰ Eight gram of bismuth nitrate was dissolved in 20¹³¹ cytotoxicity and anticancer studies, hardly a few lead¹³² ml nitric acid and 2.72 g of potassium iodide in 50 ml¹³³ compounds had shown promising results. Hence, it was¹³⁴ water. These were mixed and allowed to stand. When¹³⁵ thought to identify potential compounds from our¹³⁶ potassium nitrate crystals out, the supernatant was¹³⁷ traditional ethno-medicinal knowledge for treatment of¹³⁸ discarded off and made up to 100 ml with distilled¹³⁹ kidney, urinary and prostate cancer. In the present¹⁴⁰ water. The alkaloids were regenerated from the¹⁴¹ study, an initial attempt has made for to scientifically¹⁴² precipitate by treating with sodium carbonate followed¹⁴³ evaluate its anticancer effects. The main aim of the¹⁴⁴ by extraction of the liberated base with ether. To 0.5ml¹⁴⁵ study is to evaluate the cytotoxic effects of aqueous fruit¹⁴⁶ of alcoholic solution of extract was added 2.0 ml of¹⁴⁷ extract of *C. melo* in human prostate cancer cell line¹⁴⁸ hydrochloric acid. To this acidic medium, 1.0 ml of¹⁴⁹ (PC-3) using MTT and neutral red assays.¹⁵⁰ reagent was added. An orange red precipitate was¹⁵¹ produced immediately indicated the presence of¹⁵² alkaloids.

MATERIALS AND METHODS

Plant material

C. melo fruits were collected from local fruit stall¹⁵³ Cherthala, Alappuzha District in the month of¹⁵⁴ November 2012 and authenticated at Department of¹⁵⁵ Environment Sciences, Mahatma Gandhi University¹⁵⁶ Kottayam, Kerala, India. *In vitro* methods were used for¹⁵⁷ assessing the cytotoxic activity and they were in¹⁵⁸ accordance with the guidelines of Institutional Animal¹⁵⁹ Ethical Committee (IAEC).

Reagents for phyto-chemical analysis

Bismuth nitrate, Nitric acid, Potassium iodide,¹⁶⁰ Sodium carbonate, Mercuric chloride, Sulphuric acid,¹⁶¹ Hydrochloric acid, Sodium hydroxide, Ferric chloride,¹⁶² Alpha naphthol, Copper sulphate, Zinc chloride 3-(4,5,¹⁶³ dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium¹⁶⁴ bromide(MTT), Isopropanol, Phosphate buffer¹⁶⁵

saline(PBS), Dimethyl sulfoxide (DMSO), Calorimeter,¹⁶⁶ 1M Potassium dihydrogen phosphate , CO₂ incubator,¹⁶⁷ PBS, Elution medium (ethanol/acetic acid),¹⁶⁸ Spectrophotometer.

MTT assay and neutral red assay

MTT assay and neutral red assay

PC-3 cell line-PC-3 prostate cell lines purchased¹⁶⁹ from National centre for Cell Sciences (NCCS), Pune,¹⁷⁰ Maharashtra, India. 1M Potassium dihydrogen¹⁷¹ phosphate, CO₂ Incubator, PBS, Elution medium¹⁷² (ethanol/acetic acid).

Preparation of extract: cold maceration

The fruit was washed and the outer skin was peeled¹⁷³ off. The remaining fleshy part was cut in to small¹⁷⁴ pieces. Then it was soaked in water for seven days and¹⁷⁵ was kept in a dark place. During this period shaking was¹⁷⁶ done occasionally. After seven days, the liquid was¹⁷⁷ strained and marc was pressed. The expressed liquid¹⁷⁸

Mayer's test

The amount of 1.36 g mercuric chloride was¹⁷⁹ dissolved in 60 ml of distilled water and 5 g of¹⁸⁰ potassium iodide in 10 ml of water. The two solutions¹⁸¹ were mixed and diluted to 100 ml with distilled water.¹⁸² To 1 ml of acidic aqueous solution of extracts, a few¹⁸³ drops of reagent was added. Formation of white or pale¹⁸⁴ precipitate showed the presence of alkaloids.

Wagner's test

The amount of 1.2 g Iodide and 2.0 g of H₂SO₄ and¹⁸⁵ the solution was diluted to 100 ml .Ten ml of alcoholic¹⁸⁶ extract was identified by adding 1.5% v/v of HCl and a¹⁸⁷ few drops of wagner's reagent. Formation of yellow or¹⁸⁸ brown precipitate confirmed the presence of alkaloid.

Flavanoids

In a test tube containing 0.5 ml of alcoholic extract,¹⁸⁹ 5-10 drops of dilute HCl and a small piece of ZnCl₂ or¹⁹⁰ Mg were added and the solution was boiled for few¹⁹¹ minutes. In the presence of flavonoids, reddish pink or¹⁹² dirty brown color was produced.

Phytosterols

To 2 ml of chloroform extract, 1ml of concentrated¹⁹³ sulphuric acid was added carefully along the sides of the¹⁹⁴ test tube. In the presence of phytosterols, a golden¹⁹⁵ yellow color was produced in the chloroform layer.

Glycosides

A small amount of alcoholic extract was dissolved in¹⁹⁶ 1 ml of water and the aqueous NaOH solution was¹⁹⁷ added. Formation of yellow colour indicated the¹⁹⁸ presence of glycosides.

Table 1. Phytochemical constituents aqueous extract of *C. melo*

Tests	Observation	Inference
Alkaloids		
Dragendorff's test	Orange red ppt was observed	Presence of alkaloids (+)
Mayer's test	Pale white ppt was observed	Presence of alkaloids (+)
Flavanoids	Dirty brown ppt was observed	Presence of Flavanoids (+)
Phytosterols	Dark golden colour was observed	Presence of phytosterols (+)
Glycosides	No characteristic change was observe	Absence of glycosides (-)
<i>Tannins</i>	No characteristic change was observed	Absence of glycosides (-)
Carbohydrates		
Molisch's test	No characteristic change was observed	Absence of carbohydrates (-)
Proteins		
Biurett's test	No characteristic change was observed	Absence of proteins (-)
Saponins	No characteristic change was observed	Absence of saponins (-)

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

Sample concentration (µg/ml)	OD (540 nm)	% viability
Control	0.220	100
100	0.211	95.90
500	0.172	78.18
1000	0.148	67.27

167 Tannins-Ferric chloride test

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

173 Sugars-Molish's test

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

182 Proteins-Biurett's test

183 In a test tube containing 2 ml of test sample, 2 ml of
184 10% NaOH is added and mixed well. Then 0.1% CuSO₄
185 solution is added. A violet or pink colour indicated the
186 presence of proteins.

187 Saponins-Froth Test

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

191 Cytotoxicity studies [7]**192 MTT assay**

193 MTT is a colorimetric assay that measures the
194 reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-

195 diphenyl tetrazolium bromide (MTT) by mitochondrial
196 succinate dehydrogenase. The MTT enters the cells and
197 passes into the mitochondria where it is reduced to an
198 insoluble, colored (dark purple) formazan product. The
199 products are then solubilized with an organic solvent
200 (eg. isopropanol) and the released, solubilized formazan
201 reagent. Since reduction of MTT can only occur in
202 metabolically-active cells, the level of activity is a
203 measure of the viability of the cells

204 PC-3 human prostate cell lines purchased from
205 NCCS Pune, was maintained in Dulbecco's Modified
206 Eagles Media (DMEM) and grown to confluency at
207 37°C and 5% CO₂ in a humidified atmosphere in a CO₂
208 incubator. The cells were trypsinized (500 µl of 0.025%
209 Trypsin in PBS/ EDTA solution) for 2 min and
210 passaged to T flasks in complete aseptic conditions and
211 incubated. Extracts were added to 80% confluent cells
212 at a concentration of 100 µg, 500 µg and 1000 µg from a
213 stock of 100 mg/ml and incubated for 24 h. The cell
214 culture suspension was washed with 1× PBS. Then, 200
215 µl MTT solutions were added to the culture (MTT: 5
216 mg/volume dissolved in PBS). These were incubated at
217 37°C for 3 hours. All MTT wash was removed with 1×
218 PBS and 300 µl DMSO was added to each culture. The
219 plates were incubated at room temperature for 30 min
220 until the cell get lysed and color was obtained. The
221 solution was transferred to centrifuge tubes and
222 centrifuged at top speed for 2 min to precipitate cell
223 debris. Optical density (OD) was read at 540 nm using
224 DMSO as blank.

225 Neutral red assay

226 The neutral red cytotoxicity test was based on the
227 ability of living cells to uptake and bind neutral red
228 (NR). NR was a positively-charged dye that easily
229 diffuses through the cellular membrane of the cells,
230 accumulates in the cellular cytoplasm and stores in the
231 acidic environment of lysosomes. The principle of the
232 test consists in the fact that NR are able to absorb and
233 bind only with live cells while this ability declines in
234 damaged or dead cells. The amount of accumulated NR
235 was thus directly proportional to the amount of live cells
236 in the cell culture. The pH of the neutral red solution

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

Sample concentration ($\mu\text{g/ml}$)	OD (540 nm)	% viability
Control	0.086	100
100	0.070	81.39
500	0.065	75.58
1000	0.057	66.27

was adjusted in all the experiments to 6.35 with the addition of potassium dihydrogen phosphate (1M), 10 μl of neutral red solution was added to plates and incubated for 3 h in CO_2 incubator at 37°C . Cells were then washed with phosphate buffer saline (PBS) and fixed with 200 μl of fixing solution. One ml of the elution medium (ethanol/ acetic acid, 50%/1%) was added followed by gentle shaking for 10 min, so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to cuvettes and the absorbance at 540 nm was recorded using the spectrophotometer.

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 $\mu\text{g/ml}$ 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

line is taken using inverted illuminating microscope while the control received vehicle and treatment groups were received *C.melo* at a conc. 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ (Fig 3). The results indicate that the crude aqueous extract of *C. melo* on PC-3 cell lines had shown a dose-dependent anti proliferative effect. The IC_{50} values of MTT and Neutral red assays were found to be 1470 and 1860 $\mu\text{g/ml}$ respectively (Figs 4 and 5).

DISCUSSION

Metastatic prostate carcinoma is associated with a high morbidity and mortality rate with a medium 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 tumorigenic 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-

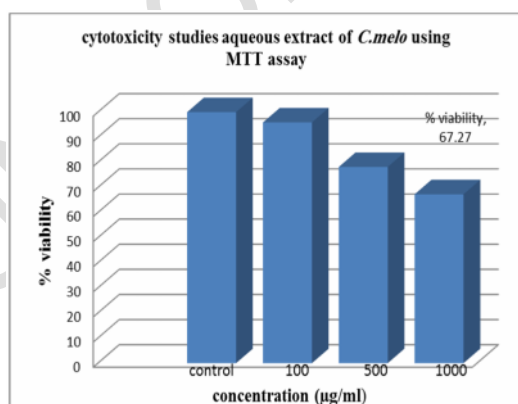


Fig 1. Cytotoxicity studies aqueous extract of *C.melo* using MTT assay

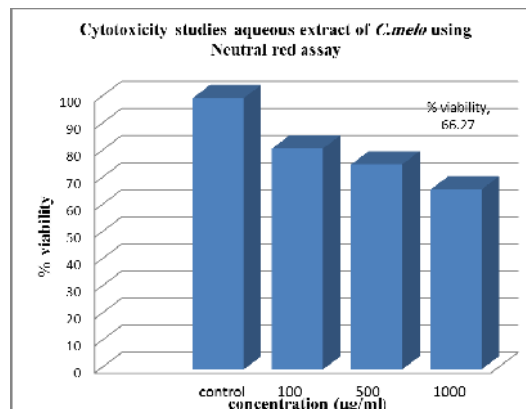


Fig 2. Cytotoxicity studies aqueous extract of *C.melo* using Neutral red assay

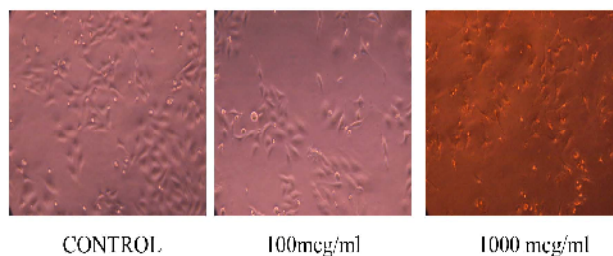


Fig 3. Photograph of PC-3 cell line: i) control received vehicle, ii) *C.melo* at a conc.100µg/ml and iii) *C.melo* at a conc.1000 µg/ml

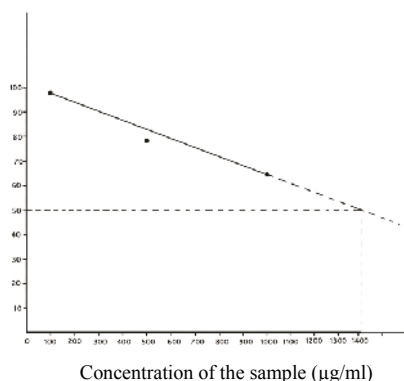


Fig 4. IC₅₀ value of aqueous extract of *C.melo* using MTT assay

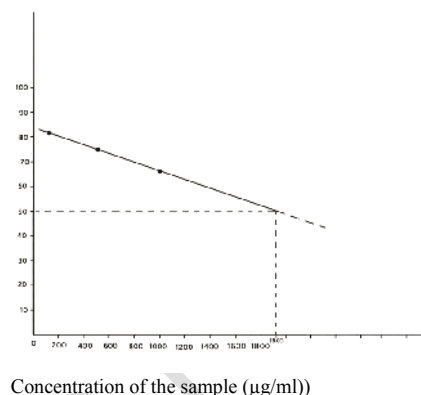


Fig 5. IC₅₀ value of aqu. extract of *C.melo* using Neutral red assay

organisms and they offer an opportunity to study the molecular mechanisms of tumorigenesis. 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 phytosterols [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.

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359 CURRENT AUTHOR ADDRESSES

Sibi P Ittiyavirah, Department of Pharmacology, University College of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam, Kerala Pin-686691, India. E-mail: sibitho@gmail.com (Corresponding author)

Ann George, Department of Pharmacology, University College of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam, Kerala Pin-686691, India.

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367 Anju M Santhosh, Department of Pharmacology, University College
368 of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
369 Kerala Pin-686691, India.

370 Sudhi T Kurian, Department of Pharmacology, University College of
371 Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
372 Kerala Pin-686691, India.

373 Prinsy Pappachan, Department of Pharmacology, University College
374 of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
375 Kerala Pin-686691, India.

376 Gifty Jacob, Department of Pharmacology, University College of
377 Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
378 Kerala Pin-686691, India.