

1 ORIGINAL ARTICLE

2 **Studies of Cytotoxic Potential of *Cucumis melo*. Linn**
3 **Fruit Aqueous Extract in Prostate Cancer Cell lines**
4 **PC-3 Using MTT and Neutral Red Assay**5 SIBI P ITTIYAVIRAH, ANN GEORGE, ANJU M SANTHOSH, SUDHI T KURIAN,
6 PRINSY PAPPACHAN and GIFTY JACOB

7 For author affiliations, see end of text.

8 Received May 12, 2012; Revised October 29, 2012; Accepted December 8, 2012

9 This paper is available online at <http://ijpt.tums.ac.ir>10 **ABSTRACT**11 The objective of the study was to evaluate the cytotoxic effects of aqueous fruit extract of *Cucumis melo*
12 in human prostate cancer cell line (PC-3) using MTT and neutral red assays. The crude aqueous extract
13 of *C. melo* was prepared by cold maceration method, filtered, concentrated and tested on PC-3 cell line.
14 Dose-dependent cytotoxic activities were exhibited by human prostate carcinoma PC-3 cell line. As the
15 dose of the extract increased, the number of viable cells decreased. This confirms the anti-cancer and
16 cytotoxic potential of the fruit of *C. melo*.17 **Keywords:** Cytotoxicity, MTT assay, Neutral red assay, Human prostate carcinoma cell lines (PC-3),
18 *Cucumis melo*19 Carcinoma of the prostate gland is the malignancy of 41 carbohydrates, Proteins, fibre, citric acid, vitamin K,
20 the male genitourinary tract and is a disorder in older 42 vitamin A and folate. Traditionally, it is used for
21 men, with mean age at presentation about 70 years 43 treatment of Kidney stones, cancer, cardiovascular
22 when diagnosed by the presence of symptoms. The risk 44 disorders and stroke. Three components found in
23 of developing prostate cancer is affected by racial and 45 melons are Cucurbitacin- β , Lithium and Zinc which
24 environmental factors. The magnitude of familial risk 46 exhibit promise in cancer prevention, fighting
25 increases with number of first degree relatives who are 47 depression, dandruff, and ulcers and stimulates the
26 affected, and also if the affected relatives diagnosed 48 immune system. *C. melo* is also rich in antioxidants,
27 with prostate cancer at an early age. Prostate cancer risk 49 flavonoids such as β - carotene, lentin, xanthin and
28 has been inversely associated with several dietary 50 cryptoxanthin. These antioxidants have the ability to
29 components including the essential non-metallic trace 51 protect cells and structures in the body from oxygen free
30 element selenium. Chromosomal alterations are 52 radicals, hence offer protection against prostate, colon,
31 associated with an inherited predisposition to prostate 53 breast, lungs, endometrial and pancreatic cancer. The
32 cancer and prostate cancer and its development. Two 54 cucurbitacea family includes several species of
33 prostate cancer susceptibility genes that have been 55 cultivated plants that has great economic importance
34 identified are the RNASEL and MSR1 genes both of 56 like water melon (*Citrullus lanatus*.L), squash
35 which are associated with response to infections. 57 (*Cucurbita maxima*.L), cucumber (*Cucumis sativus*.L)
36 *Cucumis melo* fruit is round in shape, tan to greenish 58 and cantaloupe(*Cucumis melo*.L) [1]. Earlier studies on
37 tan with a rough texture and orange pink flesh. It is well 59 cucurbitacea family showed that cantaloupe pulp extract
38 known for its sweet taste and fragrance. It is native to 60 possesses high antioxidant and anti-inflammatory
39 Persia, Armenia, etc. Many phytochemicals having 61 properties [2]. The active principles in the vegetable
40 potential benefits are present in *C. melo*. It is rich in 62 extracts are principally water soluble or lipophilic

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

triterpenoids and other flavanoids [3]. For this, these were used as potential antioxidant prophylactic agents *Phytochemical analysis*

for both health and diseases management [3,4]. The following tests were carried out to analyze the methanolic seed extract (MECM) of *Cucumis melo*. Var possible phytochemicals present in the aqueous extract possess significant antioxidant, anti-inflammatory and analgesic properties [5], while the fruit extract *C. melo* of *C. melo*.

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

though a large number of compounds were screened for cytotoxicity and anticancer studies, hardly a few lead compounds had shown promising results. Hence, it was thought to identify potential compounds from our traditional ethno-medicinal knowledge for treatment of kidney, urinary and prostate cancer. In the present study, an initial attempt has made for to scientifically evaluate its anticancer effects. The main aim of the study is to evaluate the cytotoxic effects of aqueous fruit extract of *C. melo* in human prostate cancer cell line (PC-3) using MTT and neutral red assays.

MATERIALS AND METHODS

Plant material

C. melo fruits were collected from local fruit stall 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 Ethical 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-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Isopropanol, Phosphate buffer saline (PBS), Dimethyl sulfoxide (DMSO), Calorimeter, 1M Potassium dihydrogen phosphate, CO₂ incubator, PBS, Elution medium (ethanol/acetic acid), Spectrophotometer.

MTT 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).

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 strained and marc was pressed. The expressed liquid

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 0.5ml of alcoholic solution of extract was added 2.0 ml of hydrochloric acid. To this acidic 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 precipitate showed the presence of alkaloids.

Wagner's test

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

Flavonoids

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 dirty brown color was produced.

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 added. Formation of yellow colour indicated the presence of glycosides.

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

Tests	Observation	Inference
Alkaloids		
Dragendroff'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		
Biuret'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-Biuret'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 (µg/ml)	OD (540 nm)	% viability
Control	0.086	100
100	0.070	81.39
500	0.065	75.58
1000	0.057	66.27

237 was adjusted in all the experiments to 6.35 with the
238 addition of potassium dihydrogen phosphate (1M), 10 µl
239 of neutral red solution was added to plates and
240 incubated for 3 h in CO₂ incubator at 37°C. Cells were
241 then washed with phosphate buffer saline (PBS) and
242 fixed with 200 ul of fixing solution. One ml of the
243 elution medium (ethanol/ acetic acid, 50%/1%) was
244 added followed by gentle shaking for 10 min, so that
245 complete dissolution was achieved. Aliquots of the
246 resulting solutions were transferred to cuvettes and the
247 absorbance at 540 nm was recorded using the
248 spectrophotometer.

249

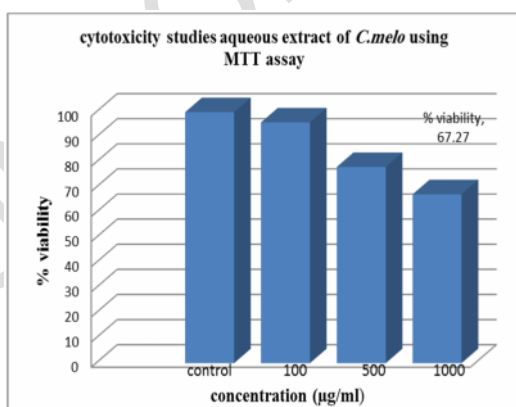
RESULTS

250 Phytochemical analysis

251 The medicinal value of these plants lies in some
252 chemical active substances that produce a definite
253 physiological action on the human body. The most
254 important of these bioactive constituents of plants are
255 alkaloids, tannins, flavonoids and phenolic compounds
256 [8]. The Phytochemical screening of the aqueous
257 extracts of plant sample revealed the presence of
258 alkaloids and flavonoids and phytosterols (Table 1) [9].

259 Cytotoxic studies

260 MTT results showed that 1000 µg/ml aqueous
261 extract of *Cucumis melo* showed 67.27% (Table 2, Fig
262 1) while the neutral red uptake assay showed 66.27%
263 viability (Table 3, Fig 2). The photograph of PC-3 cell

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

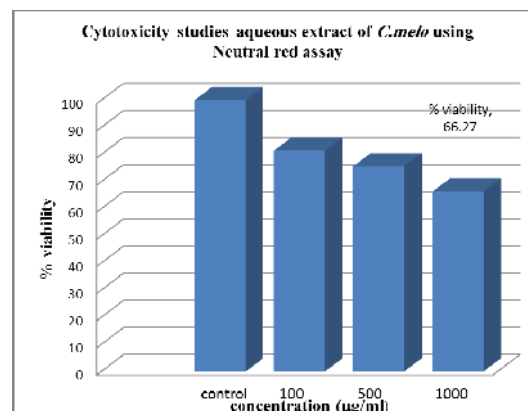
264 line is taken using inverted illuminating microscope
265 while the control received vehicle and treatment groups
266 were received *C.melo* at a conc.100µg/ml and 1000
267 µg/ml (Fig 3). The results indicate that the crude
268 aqueous extract of *C. melo* on PC-3 cell lines had shown
269 a dose-dependent anti proliferative effect. The IC₅₀
270 values of MTT and Neutral red assays were found to be
271 1470 and 1860 µg/ml respectively (Figs 4 and 5).

272

DISCUSSION

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

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

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

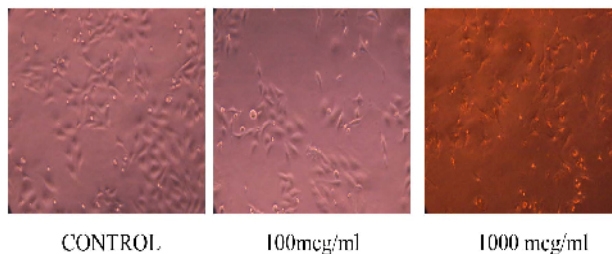


Fig 3. Photograph of PC-3 cell line: i) control received vehicle, ii) *C.melo* at a conc.100 μ g/ml and iii) *C.melo* at a conc.1000 μ g/ml

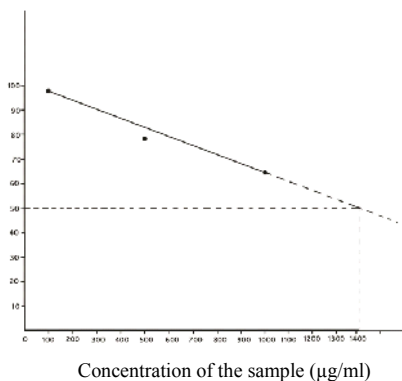


Fig 4. IC₅₀ value of aqueous extract of *C.melo* using MTT assay

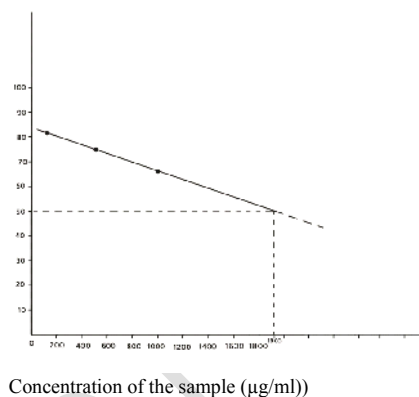


Fig 5. IC₅₀ value of aqu. extract of *C.melo* using Neutral red assay

304 organisms and they offer an opportunity to study the 333 2.
 305 molecular mechanisms of tumorigenesis. 334
 306 Cucurbitaceae plants are highly useful as they have 335
 307 good potential against many health ailments. In the 336
 308 present study, the phytochemical screening of the 337 3.
 309 aqueous extracts of plant sample revealed the presence 338
 310 of alkaloids and flavonoids and phytosterols [9]. These 339
 311 phytoconstituents may be responsible for various 340 4.
 312 activities. Flavonoids are diverse family of compounds 341
 313 commonly found in fruits, vegetables and honey. 342
 314 Flavonoids are generally safe and associated with low 343 5.
 315 toxicity, making them ideal candidates for cancer 344
 316 chemopreventive agents. MTT results and neutral red 345
 317 uptake assay confirms dose-dependent anti-proliferative 346 6.
 318 effect of crude aqueous extract of *Cucumis melo* on 347
 319 prostate cancer cell lines. As the dose of the extract 348 7.
 320 increases, number of viable cells decreases and 349
 321 confirms the cytotoxic activity. 350
 322 It is concluded that the aqueous extract of *C. melo* 351
 323 was found to possess dose-dependent cytotoxic activity 352 8.
 324 on metastatic human prostate cancer cell lines PC-3 353
 325 Further studies are warranted to explore the anticancer 354
 326 effect of *C. melo* and also the active principles could be 355 9.
 327 isolated and investigated. 356
 357
 358

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359 CURRENT AUTHOR ADDRESSES

- 360 Sibi P Ittiyavirah, Department of Pharmacology, University College
 361 of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
 362 Kerala Pin-686691, India. E-mail: sibitho@gmail.com
 363 (Corresponding author)
 364 Ann George, Department of Pharmacology, University College of
 365 Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam,
 366 Kerala Pin-686691, India.

Effect of *cucumis* on prostate cancer

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.

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