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1 ORIGINAL ARTICLE

2 Studies of Cytotoxic Potential of Cucumis melo. Linn Fruit Aqueous Extract in Prostate Cancer Cell lines ⁴PC-3 Using MTT and Neutral Red Assay

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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

Carcinoma of the prostate gland is the malignancy of 41 carbohydrates, Proteins, fibre, citric acid, vitamin K, 35 which are associated with response to infections.

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 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 57 (Cucurbita maxima.L), cucumber (Cucumis sativus.L) 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

64 contain various amounts of vitamin E and C, Carotenes, 118 liquid and concentrated. 65 triterpenoids and other flavanoids [3]. For this, these 66 were used as potential antioxidant prophylactic agents 119 Phytochemical analysis 67 for both health and diseases management [3,4]. The 68 methanolic seed extract (MECM) of Cucumis melo. Var 69 possess significant antioxidant, anti-inflammatory and 70 analgesic properties [5], while the fruit extract C. melo 71 fruit exhibited immunomodulatory activity [6]. Even₁₂₃ Alkaloids-Dragendroff's test 72 though a large number of compounds were screened for 73 cytotoxicity and anticancer studies, hardly a few lead 74 compounds had shown promising results. Hence, it was 75 thought to identify potential compounds from our 76 traditional ethno-medicinal knowledge for treatment of 77 kidney, urinary and prostate cancer. In the present 78 study, an initial attempt has made for to scientifically 79 evaluate its anticancer effects. The main aim of the 80 study is to evaluate the cytotoxic effects of aqueous fruit 81 extract of C. melo in human prostate cancer cell line

MATERIALS AND METHODS

84 Plant material

83

C. melo fruits were collected from local fruit stall 86 Cherthala, Alappuzha District in the month of 87 November 2012 and authenticated at Department of 88 Environment Sciences, Mahatma Gandi University 89 Kottayam, Kerala, India. In vitro methods were used for 90 assessing the cytotoxic activity and they were in 91 accordance with the guidelines of Institutional Animal 92 Ethical Committee (IAEC).

93 Reagents for phyto-chemical analysis

82 (PC-3) using MTT and neutral red assays.

Bismuth nitrate, Nitric acid, Potassium iodide, 146 95 Sodium carbonate, Mercuric chloride, Sulphuric acid,147 the solution was diluted to 100 ml .Ten ml of alcoholic 96 Hydrochloric acid, Sodium hydroxide, Ferric chloride,148 extract was identified by adding 1.5% v/v of HCl and a 97 Alpha naphthol, Copper sulphate, Zinc chloride 3-(4,5149 few drops of wagner's reagent. Formation of yellow or thiazole-2-yl)-2,5-diphenyl 98 dimethyl 99 bromide(MTT), Isopropanol, Phosphate buffer 100 saline(PBS), Dimethyl sulfoxide (DMSO), Calorimeter, 151 Flavanoids 101 1M Potassium dihydrogen phosphate, CO₂ incubator, 102 PBS, Elution medium (ethanol/acetic acid), 103 Spectrophotometer.

104 MTT assay and neutral red assay

PC-3 cell line-PC-3 prostate cell lines purchased 106 from National centre for Cell Sciences (NCCS), Pune, Maharashtra, India. 1M Potassium dihydrogen 108 phosphate, CO₂ Incubator, PBS, Elution medium 158 109 (ethanol/acetic acid).

Preparation of extract: cold maceration

The fruit was washed and the outer skin was peeled 112 off. The remaining fleshy part was cut in to small 113 pieces. Then it was soaked in water for seven days and 163 114 was kept in a dark place. During this period shaking was 1641 ml of water and the aqueous NaOH solution was 115 done occasionally. After seven days, the liquid was 165 added. Formation of yellow colour indicated the 116 strained and marc was pressed. The expressed liquid166 presence of glycosides.

63 antioxidant molecule. Most of these plant extracts 117 was mixed with strained liquid, filtered to make a clear

The following tests were carried out to analyze the 21 possible phytochemicals present in the aqueous extract 2 of *C. melo*.

Eight gram of bismuth nitrate was dissolved in 20 25 ml nitric acid and 2.72 g of potassium iodide in 50 ml 6 water. These were mixed and allowed to stand. When potassium nitrate crystals out, the supernatant was 8 discarded off and made up to 100 ml with distilled 9 water. The alkaloids were regenerated from the 30 precipitate by treating with sodium carbonate followed 31 by extraction of the liberated base with ether. To 0.5ml 32 of alcoholic solution of extract was added 2.0 ml of 133 hydrochloric acid. To this acidic medium, 1.0 ml of 134 reagent was added. An orange red precipitate was 135 produced immediately indicated the presence of 136 alkaloids.

137 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.

145 Wagner's test

The amount of 1.2 g Iodide and 2.0 g of H₂SO₄ and tetrazolium₁₅₀ brown precipitate confirmed the presence of alkaloid.

In a test tube containing 0.5 ml of alcoholic extract, 153 5-10 drops of dilute HCl and a small piece of ZnCl₂ or 154Mg were added and the solution was boiled for few 155 minutes. In the presence of flavonoids, reddish pink or 56 dirty brown color was produced.

57 Phytosterols

To 2 ml of chloroform extract, 1ml of concentrated 159 sulphuric acid was added carefully along the sides of the 160 test tube. In the presence of phytosterols, a golden 161 yellow color was produced in the chloroform layer.

62 Glycosides

A small amount of alcoholic extract was dissolved in

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Table 1. Phytochemical constituents agueous 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 (-)
Tannins	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 (-)

assav

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

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

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 220 until the cell get lysed and color was obtained. The 181 carbohydrates.

182 Proteins-Biurett's test

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 225 Neutral red assay 186 presence of proteins.

187 Saponins-Froth Test

189 and shaken vigorously then is left to stand for 10 min. A_{230} accumulates in the cellular cytoplasm and stores in the 190 thick persistent froth indicated presence of saponins.

191 Cytotoxicity studies [7] 192 MTT assay

MTT is a colorimetric assay that measures the 235 was thus directly proportional to the amount of live cells 194 reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-236 in the cell culture. The pH of the neutral red solution

Table 2. Cytotoxicity studies aqueous extract of C. melo using MTT195 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

> PC-3 human prostate cell lines purchased from 205 NCCS Pune, was maintained in Dulbecco's Modified Eagles Media (DMEM) and grown to confluency 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 passaged 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/ml 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 19 plates were incubated at room temperature for 30 min 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 4 DMSO as blank.

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 Few ml of the extract is transferred in to a test tube 229 diffuses through the cellular membrane of the cells, 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

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Sample concentration	OD	% viability
(µg/ml)	(540 nm)	
Control	0.086	100
100	0.070	81.39
500	0.065	75.58
1000	0.057	66.27

264 line is taken using inverted illuminating microscope Table 3. Cytotoxicity studies aqueous extract of *C.melo* using Neutral₂₆₅ 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).

DISCUSSION

237 was adjusted in all the experiments to 6.35 with the 273 238 addition of potassium dihydrogen phosphate (1M), 10 µl₂₇₄ high morbidity and mortality rate with a medium 239 of neutral red solution was added to plates and 275 survival of approximately, 12-15 months. Available 240 incubated for 3 h in CO₂ incubator at 37°C. Cells were 276 treatment alternatives include radiotherapy after radical 241 then washed with phosphate buffer saline (PBS) and 277 retropubic prostatectomy, radical prostatectomy, 242 fixed with 200 ul of fixing solution. One ml of the 278 external beam radiation, prostate brachy therapy, and 243 elution medium (ethanol/ acetic acid, 50%/1%) was₂₇₉ androgen ablation of the prostate. Until recently, despite 244 added followed by gentle shaking for 10 min, so that 280 androgen suppression, no cytotoxic agent has been able 245 complete dissolution was achieved. Aliquots of the 281 to change the progression of metastatic prostate cancer. 246 resulting solutions were transferred to cuvettes and the 282 Androgen ablation therapy remains the main course of 247 absorbance at 540 nm was recorded using the 283 treatment with advanced disease. However, it has no 248 spectrophotometer.

RESULTS

250 Phytochemical analysis

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

261 extract of Cucumis melo showed 67.27% (Table 2, Fig301 potential anti-cancer compounds. Over 60% anti-cancer 262 1) while the neutral red uptake assay showed 66.27%302 agents currently used are derived from natural sources, 263 viability (Table 3, Fig 2). The photograph of PC-3 cell303 including plants, marine organisms and micro-

Metastatic prostate carcinoma is associated with a on hormone-independent cancer 284 effect 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 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 296 role of some natural materials and medicinal plants in 297 oncology and their ability affect all phases of 298 tumerogenic process. Therefore, it is important to screen 299 the natural products either as crude extracts or as MTT results showed that 1000 µg/ml aqueous300 isolated components for apoptotic properties to identify

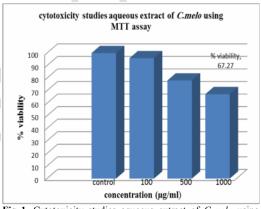


Fig 1. Cytotoxicity studies aqueous extract of C.melo using MTT assay

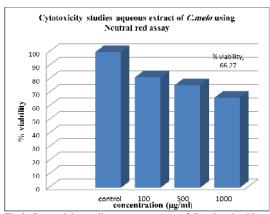


Fig 2. Cytotoxicity studies aqueous extract of C.melo using Neutral red assav

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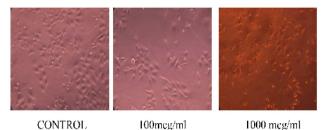


Fig 3. Photograph of PC-3 cell line: i) control received vehicle, ii) C.melo at a conc.100μg/ml and iii) C.melo at a conc.1000 μg/ml

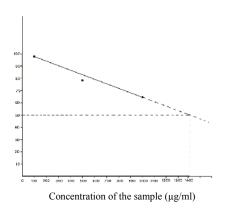
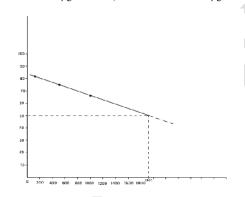


Fig 4. IC₅₀ value of aqueous extract of *C.melo* using MTT assay



Concentration of the sample (µg/ml))

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 tumerogenesis. 306 Cucurbitaceae plants are highly useful as they have 307 good potential against many health ailments. In the 3373 308 present study, the phytochemical screening of the 338 309 aqueous extracts of plant sample revealed the presence 310 of alkaloids and flavonoids and phytosterols [9]. These 340 4. 311 phytoconstituents may be responsible for various³⁴¹ 312 activities. Flavanoids are diverse family of compounds 313 commonly found in fruits, vegetables and honey. 314 Flavanoids are generally safe and associated with low345 315 toxicity, making them ideal candidates for cancer 346 6. 316 chemopreventive agents. MTT results and neutral red347 317 uptake assay confirms dose-dependent anti-proliferative₃₄₈₇. 318 effect of crude aqueous extract of Cucumis melo on349 319 prostate cancer cell lines. As the dose of the extract³⁵⁰ 320 increases, number of viable cells decreases and

It is concluded that the aqueous extract of C. melo₃₅₄ 323 was found to possess dose-dependent cytotoxic activity_{355 9} 324 on metastatic human prostate cancer cell lines PC-3.356 325 Further studies are warranted to explore the anticancer357 326 effect of *C. melo* and also the active principles could be³⁵⁸ 327 isolated and investigated.

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321 confirms the cytotoxic activity.

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Effect of cucumis on prostate cancer

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