IJPT
1735-2657/13/121-24-29
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 12: 24-29, 2013

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

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

This paper is available online at *http://ijpt.tums.ac.ir*

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.

Cucumis melo fruit is round in shape, tan to greenish 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 potential benefits are present in *C. melo*. It is rich in 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 extracts are principally water soluble or lipophilic

antioxidant molecule. Most of these plant extracts contain various amounts of vitamin E and C, Carotenes, triterpenoids and other flavanoids [3]. For this, these were used as potential antioxidant prophylactic agents for both health and diseases management [3,4]. The methanolic seed extract (MECM) of *Cucumis melo*. Var possess significant antioxidant, anti-inflammatory and analgesic properties [5], while the fruit extract *C. melo* fruit exhibited immunomodulatory activity [6]. Even 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 Gandi 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 , CO_2 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, CO_2 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 was mixed with strained liquid, filtered to make a clear liquid and concentrated.

Phytochemical analysis

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

Alkaloids-Dragendroff'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 0.5ml 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 H_2SO_4 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.

Flavanoids

In a test tube containing 0.5 ml of alcoholic extract, 5-10 drops of dilute HCl and a small piece of $ZnCl_2$ 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.

Published online: January 31, 2013

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 (-)
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 (-)

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

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

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.

Sugars-Molish's test

In a test tube containing 2 ml of aqueous extract, 2 drops of freshly-prepared 20% alcoholic solution of α-naphthol 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.

Proteins-Biurett'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% $CuSO_4$ solution is added. A violet or pink colour indicated the presence of proteins.

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.

Cytotoxicity studies [7]
MTT assay

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-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

PC-3 human prostate cell lines purchased from NCCS Pune, was maintained in Dulbecco's Modified Eagles Media (DMEM) and grown to confluency at 37°C and 5% CO_2 in a humidified atmosphere in a CO_2 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 of100 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 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.

Neutral red assay

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

Published online: January 31, 2013

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

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 ul 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 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 line is taken using inverted illuminating microscope while the control received vehicle and treatment groups were received *C.melo* at a conc.100µg/ml and 1000 µg/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 µg/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 tumerogenic 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-

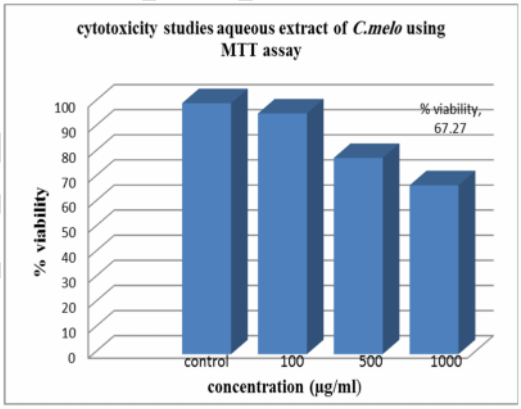

Fig 1. Cytotoxicity studies aqueous extract of *C.melo* using MTT assay

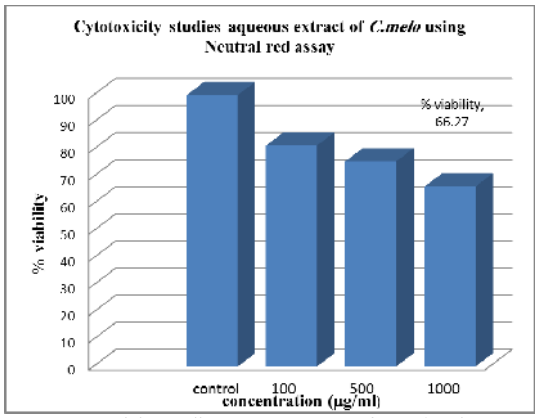

Fig 2. Cytotoxicity studies aqueous extract of *C.melo* using Neutral red assay

Published online: January 31, 2013

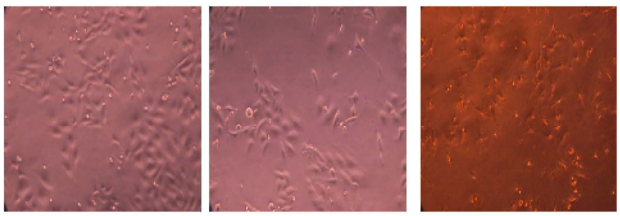

Fig 3. Photograph of PC-3 cell line: i) control received vehicle, ii)*C.melo* at a conc.100µg/ml and iii) *C.melo* at a conc.1000 µg/ml

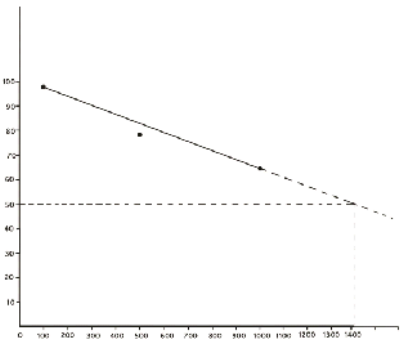

Fig 4. IC_{50} value of aqueous extract of *C.melo* using MTT assay

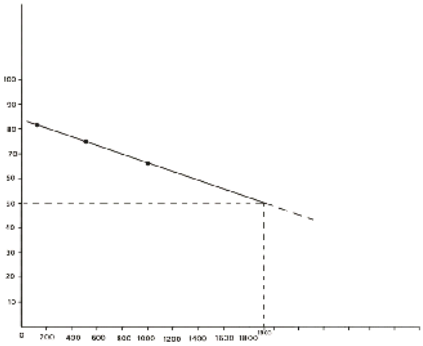

Fig 5. IC_{50} value of aqu. extract of *C.melo* using Neutral red assay

organisms and they offer an opportunity to study the molecular mechanisms of tumerogenesis.

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. Flavanoids are diverse family of compounds commonly found in fruits, vegetables and honey. Flavanoids 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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Published online: January 31, 2013

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Published online: January 31, 2013