

***In vitro* Evaluation of Cytotoxic Properties of Piperic Acid**

**NITYA RUDRARAJU^{1,2#}, ANIMISHA MOKKAPATI^{1#}, RADHAKRISHNA NAGUMANTRI¹,
CHINNA BABU PYDI¹, RAMAKRISHNA CHINTALA³ and SATYANARAYANA RENTALA^{1*}**

¹Department of Biotechnology, Institute of Technology, GITAM University,
Gandhi Nagar, Visakhapatnam 530 045 India.

²School of Engineering, Boston University, Massachusetts USA.

³GITAM Institute of Science, GITAM University, Gandhi Nagar, Visakhapatnam 530 045 India.

*Corresponding author E-mail: mail.dr.satya@gmail.com

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ABSTRACT

A daily diet rich in spices may offer protection against cancer and other illnesses. This may be reason, why Indians suffer lower cases of many cancers. One of the most widely traded spices in the world, Piperine, the main alkaloid from black pepper has been shown to substantially increase the bioavailability of the nutrients in foods and supplements. Piperic acid is a chemical obtained by the hydrolysis of the alkaloid piperine from black pepper, followed by acidification of the corresponding salt. The cytotoxic effects of piperic acid are hitherto unknown. In this paper, the cytotoxic effects of piperic acid in prostate cancer cells (PC-3) and breast cancer cells (MDA-MB-231) were studied. The drug treatment experiments clearly indicated that maximum cytotoxicity was achieved at 48 hours and at 100 μ M concentration of piperic acid in both the cell lines.

Keywords: Piperic acid, Cytotoxicity, Prostate cancer cell lines,
Breast cancer cell lines and Mononuclear cells.

INTRODUCTION

Ayurvedic medicine is a system of diagnosis and treatments that has been practiced in India for more than 2500 years. The term "*ayurveda*" comes from Sanskrit. It means "knowledge of life". Ayurvedic theory holds that the human body represents the entire universe in microcosmic form, and that we come to know how we function as organisms only by observing and understanding the world around us. Modern pharmaceutical research is concerned with all aspects of identifying new chemical substances with new modes of action.

The fruit of the Black Pepper plant, Black Pepper is useful both as a spice and an Ayurvedic medicine¹. The spiciness in Black Pepper is due

to the chemical Piperine. One of the most widely traded spices in the world, Piperine, the main alkaloid from black pepper has been shown to substantially increase the bioavailability of the nutrients in foods and supplements^{2,3}. Piperic acid is a chemical often obtained by the base-hydrolysis of the alkaloid piperine from black pepper, followed by acidification of the corresponding salt^{4,5,6,7}. Piperic acid is an intermediate in the synthesis of other compounds such as piperonal, and as-such may be used to produce fragrances, perfumes flavorants and drugs as well as other useful compounds^{8,9,10,11}. The structure of piperic acid was given in Figure 1.

The cytotoxic effects of piperine, an alkaloid found in black pepper, have been studied extensively^{12,13,14,15}. However the cytotoxic activity

of its derivative, piperic acid has not been properly investigated. The present paper is focused on the research works to fill the gap and examine the cytotoxic effects of piperic acid.

MATERIALS AND METHODS

Materials used

DMEM (Dulbecco's Modified Eagle Medium), MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) FBS (Fetal Bovine serum), DMSO (DiMethyl Sulphoxide), 96 well micro plate, Inverted Microscope, Micropipettes, Micro tips, Ficoll Histopaque 1077, Piperic acid procured from Sigma-Aldrich, India, Trypsin-EDTA solution 1X w/ 0.025% Trypsin and 0.01% EDTA in Dulbecco's Phosphate Buffered Saline Sterile filtered.

Cell lines used

PC-3 (Prostate cancer) and MDA-MB-231 (Metastatic Breast Cancer) Cell lines was obtained from NCCS (National centre for cell science), Pune, India. The PC-3 and MDA-MB -231 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The cell lines were maintained at 37°C in a 5% CO₂ incubator.

Culturing of Mononuclear cells from peripheral blood

5ml peripheral blood was collected in heparinized blood collection tubes.

The sample was carefully added to the layer of the Ficoll Histopaque 1077 (Lymphocyte separating medium whose density is 1.077 times higher than peripheral blood) (1:1) in 15ml tubes. The tubes were centrifuged at 7000 rpm for 30 min without brake. The buffy coat was carefully aspirated and the mononuclear dense ring was collected in fresh sterile 15ml centrifuge tubes. The collected cells were washed two times using 10ml DMEM. The cell pellet was suspended DMEM enriched with 10% heat- inactivated fetal bovine serum and antibiotics. The cells were maintained in 5% CO₂ incubator at 37°C.

Drug treatment and Cell viability / cytotoxicity analysis using MTT assay

Mononuclear cells were obtained as mentioned previously and cultured in DMEM serum

containing medium. The trypsinised cancer cells from T-25 flask were incubated in a 96 well plate and allowed to adhere to the wells overnight in CO₂ incubator. Every time 5000 cells per well were taken. Cell lines were treated with different concentrations of piperic acid (1,10,100 µM solutions). After treatment at various time intervals (24, 48 and 72 hours) , 20µl of MTT (5mg/ml in PBS) was added into each well and incubated in a CO₂ Incubator until purple precipitate was visible. Then the supernatant was discarded and 200 µl of DMSO was added to each well to dissolve formazan crystals. The absorbance was read at a wavelength of 492nm on microplate reader

The following formula was used to calculate cell viability

$$\% \text{ Cell viability: } (\text{Test OD}_{492} / \text{Control OD}_{492}) \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \% \text{ Cell viability}$$

RESULTS AND DISCUSSIONS

MDA-MB-231, PC-3 and Mononuclear cells were cultured in 96 well plates. Figure 2 shows the cultured MDA-MB-231 (Breast cancer), PC-3(Prostate cancer) and Mononuclear cells.

To test the cytotoxicity. Prostate cancer cells (PC-3 cell line) and breast cancer cells (MDA-MB-231 cell line) and Mononuclear cells were cultured.

Once the MTT assay was conducted on the MNCs and MDA-MB-231, after the incubation period the cell viability was calculated and the following graph was obtained.

After the cells were treated with 1 micromolar concentrations of piperic acid (as shown in Table 1 and Figure 3), it was found that the cell death was

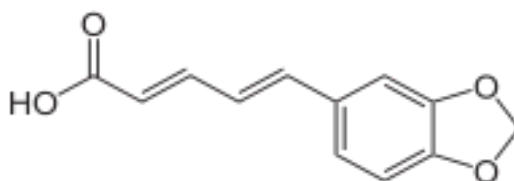


Fig. 1: Piperic Acid

IUPAC name: (2E,4E)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid

much higher in the metastatic breast cancer cell lines than the mononuclear cells. This is certainly ideal as this indicates the drug can probably be safely injected into the body without causing harm to healthy cells. From the graph it was understood that piperic acid interfere with the DNA replication process hence halting the cell cycle and replication. The cytotoxic properties of 10 μM Piperic acid on MDA-MB 231 and mononuclear cells were given in Table 2 and Figure 4.

The results obtained after treatment of 10 μM piperic acid were given in Table 2 and Figure 4. In both types of cells cytotoxicity appeared to have peaked after 48 hours of incubation. This seemed to be the point where the cells had completely utilized the entire drug that was added. It is important to note that a decrease in cytotoxicity does not indicate that cell death has been reversed in any way, since these values were calculated relative to control. The cytotoxic properties of 100 μM Piperic acid on

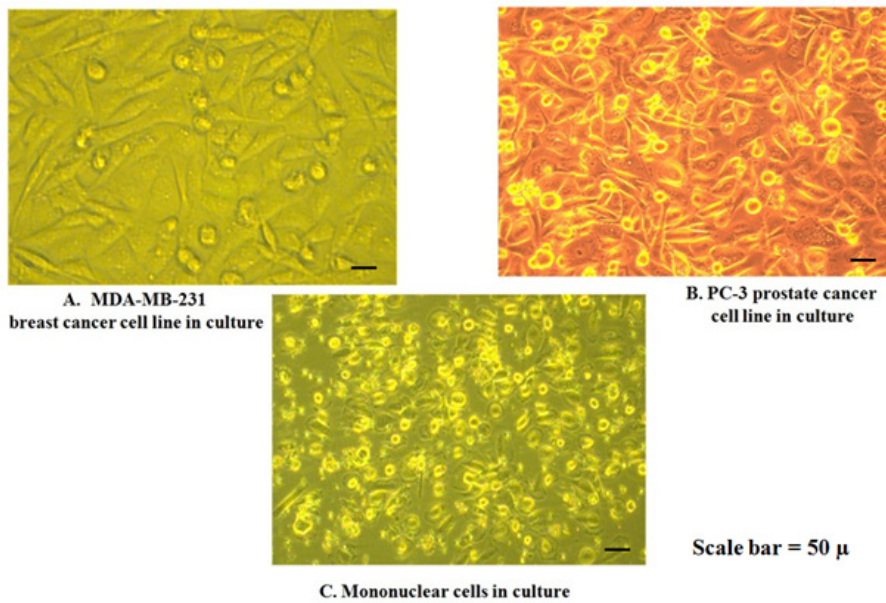


Fig. 2: Mononuclear cells, Breast and prostate cancer cells in culture

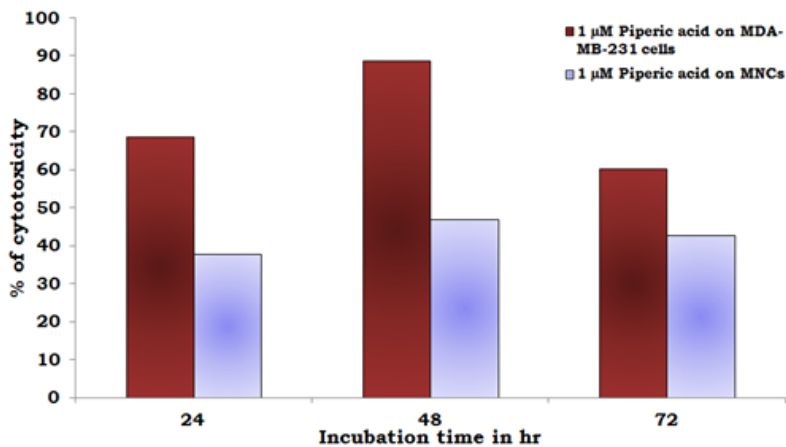


Fig. 3: Cytotoxicity of 1 μM piperic acid in MDA-MB231 and MNCs

MDA-MB 231 and mononuclear cells were given in Table 3 and Figure 5.

The results obtained at 100 micromolars also exhibits a similar trend to the previous concentrations. The percentage of cytotoxicity was maximum at this particular concentration of piperic acid in the MDA-MB-231 cells.

Once the MTT assay was conducted on the MNCs and PC-3 cells, after the incubation period the cell viability was calculated and the following graph was obtained.

After the cells were treated with 1 micromolar concentrations of piperic acid (Table 4 and Figure 6), it was found that the cell death was

much higher in the metastatic breast cancer cell lines than the mononuclear cells. This is certainly ideal as this indicates the drug can probably be safely injected into the body without causing harm to healthy cells. From the graph it was understood that piperic acid interfere with the DNA replication process hence halting the cell cycle and replication. The cytotoxic properties of 10 μ M Piperic acid on PC-3 and mononuclear cells were given in Table 5 and Figure 7.

The results obtained after treatment of 10 μ M piperic acid were given in Table 5 and Figure 7. In both types of cells cytotoxicity appeared to have peaked after 48 hours of incubation. This seemed to be the point where the cells had completely utilized the entire drug that was added. It is important to

Table 1: % of Cytotoxicity effects of 1 μ M piperic acid on MDA-MB-231 and MNCs

% of Cytotoxicity effects of 1 μ M Piperic acid on MDA-MB 231 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on MDA-MB 231 cells Piperic acid	% of Cytotoxicity on MNCs Piperic acid
24	68.71 \pm 0.01	37.9 \pm 0.01
48	88.76 \pm 0.03	47.0 \pm 0.01
72	60.26 \pm 0.36	42.72 \pm 0.03

Table 2: % of Cytotoxicity effects of 10 μ M piperic acid on MDA- MB 231 and MNCs

% of Cytotoxicity effects of 10 μ M Piperic acid on MDA-MB 231 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on MDA-MB 231 cells Piperic acid	% of Cytotoxicity on MNCs Piperic acid
24	72.59 \pm 0.06	27.41 \pm 0.02
48	85.04 \pm 0.19	24.69 \pm 0.15
72	58.7 \pm 0.49	45.34 \pm 0.00

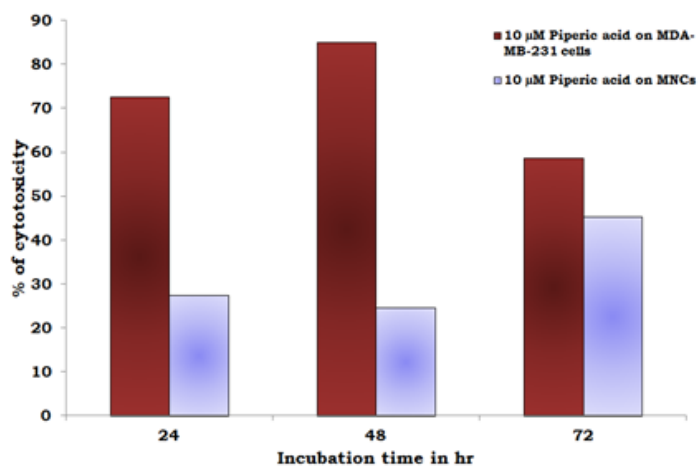


Fig. 4: Cytotoxicity of 10 μ M piperic acid in MDA-MB231 and MNCs

Table 3: % of Cytotoxicity effects of 100 µM piperic acid on MDA-MB 231 and MNCs

% of Cytotoxicity effects of 100 µM Piperic acid on MDA-MB 231 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on MDA-MB 231 cells Piperic acid	% of Cytotoxicity on MNCs Piperic acid
24	68.79±0.02	11.4±0.11
48	87.58±0.02	37.43±0.08
72	64.7±0.40	27.99±0.13

Table 4: % of Cytotoxicity effects of 1 µM piperic acid on PC-3 and MNCs

% of Cytotoxicity effects of 1µM Piperic acid on PC-3 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on PC-3 cells Piperic acid	% of Cytotoxicity on MNCs Piperic acid
24	9.8±0.01	37.9±0.01
48	50.29±0.05	47.0±0.01
72	0	42.72±0.03

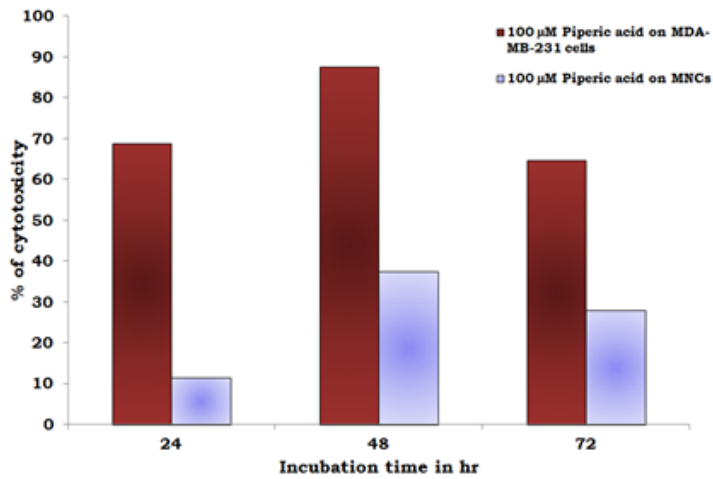


Fig. 5: Cytotoxicity of 100 µM piperic acid in MDA-MB-231 and MNCs

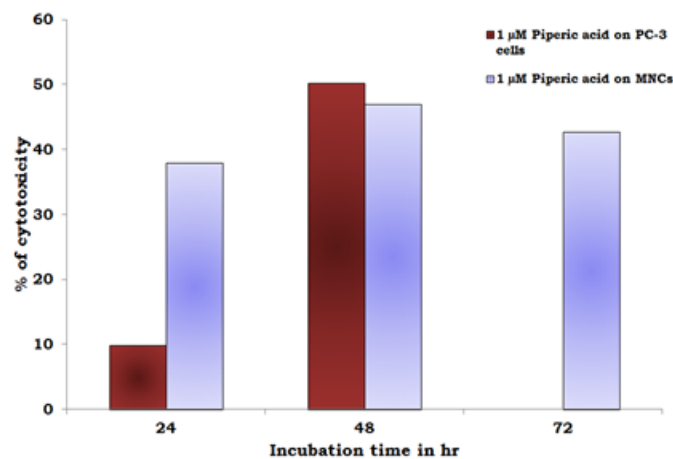


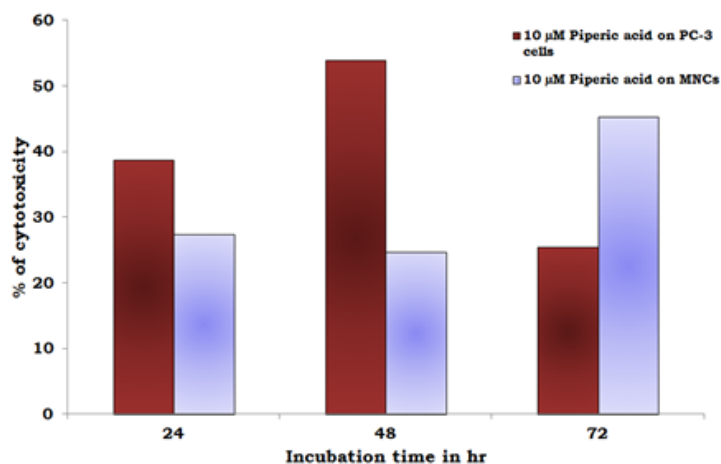
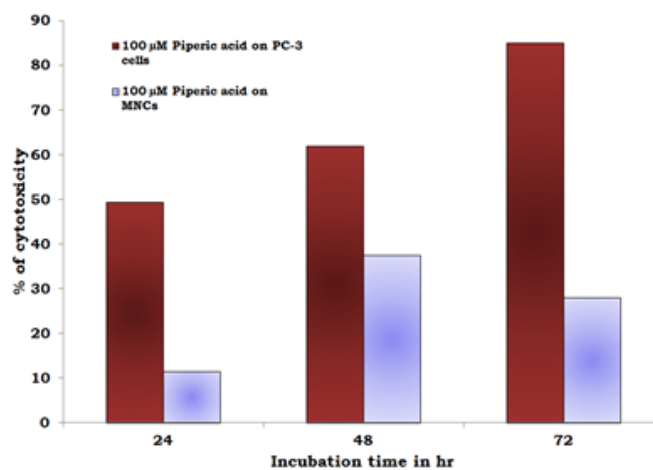
Fig. 6: Cytotoxicity of 1 µM piperic acid in PC-3 and MNCs

Table 5: % of Cytotoxicity effects of 10 μ M piperinic acid on PC-3 and MNCs

% of Cytotoxicity effects of 10 μ M Piperinic acid on PC-3 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on PC-3 cells Piperinic acid	% of Cytotoxicity on MNCs Piperinic acid
24	38.7 \pm 0.08	27.41 \pm 0.02
48	53.9 \pm 0.03	24.69 \pm 0.15
72	25.42 \pm 0.01	45.34 \pm 0.00

Table 6: % of Cytotoxicity effects of 100 μ M piperinic acid on MDA- MB 231 and MNCs

% of Cytotoxicity effects of 100 μ M Piperinic acid on PC-3 and mononuclear cells		
Time in Hrs	% of Cytotoxicity on MDA-MB 231 cells Piperinic acid	% of Cytotoxicity on MNCs Piperinic acid
24	68.79 \pm 0.02	49.45 \pm 0.01
48	87.58 \pm 0.02	62 \pm 0.02
72	64.7 \pm 0.40	84.98 \pm 0.06

**Fig. 7: Cytotoxicity of 10 μ M piperinic acid in PC-3 and MNCs****Fig. 8: Cytotoxicity of 100 μ M piperinic acid in PC-3 and MNCs**

note that a decrease in cytotoxicity does not indicate that cell death has been reversed in any way, since these values were calculated relative to control. The cytotoxic properties of 100 μ M Piperic acid on PC-3 and mononuclear cells were given in Table 6 and Figure 8.

The results obtained at 100 μ M also exhibits a similar trend to the previous concentrations. The percentage of cytotoxicity was maximum at this particular concentration of piperic acid in the PC-3 cells.

In the prostate cancer (PC-3) cells when treated with piperic acid at different time periods of incubation, it was estimated that the optimal concentration was 100 μ M.

CONCLUSIONS

It is important to understand the mechanism of cell death in MDA-MB231 and PC-3 cells when treated with piperic acid. Both the cell lines were treated with piperic acid in different concentrations (1,10 and 100 micromolars), at different time periods of incubation (24,48,72 hours.) The mononuclear

cells were isolated from healthy human volunteers peripheral blood. These MNCs were also treated as mentioned above. From these experiments it was estimated that 100 micromolar concentration was optimal to obtain the maximum cytotoxicity in cancer cells. This concentration did not affect the cell viability of the mononuclear cells. At all the concentrations the optimal time of incubation was measured to be 48 hours. After this time period the cytotoxicity was reduced when measured at 72 hours. Perhaps the cytotoxicity would have been maintained if a second dose of the drug were administered. This decrease in cell death indicates that after the drug had been completely utilized by the cells and shown its effect, the few cells which were still alive after 48 hours of incubation began to multiply thereby decreasing the percentage of cytotoxicity.

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