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A COMPARATIVE STUDY OF THE CYTOTOXIC EFFECT OF CHLOROGENIC ACID ON BM-12 CELLS FROM *BOMBYX MORI* (INSECT) AND MAMMALIAN CELL LINE

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ABSTRACT

Mammalian cell lines are routinely used to study the effect of various drugs However working on human cell lines is expensive and is associated with problems like slow growth rate and contamination. Using insect cells as an option for cancer research is more cost effective, easy to handle, and the culture conditions are relatively facile. The main objective of this study was to check the effect of chlorogenic acid, a phytochemical, on Bm12 cell line and PA-1 cell line and compare its cytotoxicity on both cell lines. For this Bm12 cells were treated with 100, 300 and 500µM CGA and cell viability was assessed at every 2 hr interval for 10 hrs using trypan blue dye exclusion assay. PA-1 cells were treated with different concentrations of CGA ranging from 50-500µM and cytotoxicity was determined using MTT assay. Cisplatin, a standard drug for ovarian cancer, was used as standard for comparing cytotoxicity. It was observed that the cell viability decreases with increasing concentration of CGA. Transformed cells were found to be more sensitive to CGA as compared to Bm12 cells, which suggests that cytotoxicity of CGA is more specific to transformed or cancer cells as compared to normal, non-transformed cells. Around 50% cytotoxicity of PA-1 cells is observed using CGA of higher concentrations (500µM), which indicates that CGA is a better alternative to Cisplatin. Results indicate that, at higher concentration of CGA, cytotoxic effect was much faster in Bm12 cells than PA-1 cells. Thus, it can be concluded that Bm12 cells can be used to determine cytotoxicity of any anticancer compound, much readily and easily.

KEYWORDS: *Chlorogenic acid, Cisplatin, PA-1 (human ovarian teratocarcinoma cell line), MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Assay, Bm-12, Bombyx mori*

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INTRODUCTION

Cell culture is one of the major tools used in cellular and molecular biology. The major advantage of using cell culture is the consistency and reproducibility of results that can be obtained. Normal cells usually divide only a limited number of times & undergo senescence but few cell lines become immortal through a process called transformation. Continuous cell lines may be utilized for the production of biopharmaceuticals, vaccines and gene therapy vectors or as fusion partners with other cell type[1]. Mammalian cell culture has played an important part in the advancement of the life sciences and more recently has been a key factor in the development of new therapeutic strategies. Tumor cell lines are an invaluable resource not only for understanding disease mechanisms, but also for drug design and discovery[2]. The benefits of mammalian cell culture have become increasingly important for the production of new protein biopharmaceuticals, including monoclonal antibodies and cytokines, as well as providing new strategies for vaccine production[3]. However it is often found to get contaminated with mycoplasma & cross contamination also occurs. Costs for mammalian cell culture process are drastically higher.[4] Insect cells closely resemble mammalian cells and have better chances to produce soluble proteins with correct folding and high order posttranslational modifications compared to bacteria and yeast[5]. Insect cell culture is a mature technology and is being applied having major advantages compared with mammalian systems like more stress resistant, easier to culture, more productive and they have the capability to grow in suspension in defined serum free culture media.[6,7]. Lepidopteran insect cell lines have potential application in the fields of agriculture and medicine[8] and *Bombyx mori* (Silkworm) is an ideal reference for the Lepidoptera[9]. Cancer chemoprevention with natural phytochemical compounds is an emerging strategy to prevent, impede, delay or cure cancer[10]. Dietary phytochemicals may also improve the effectiveness of chemotherapeutics[11]. Chlorogenic acid (CGA)[12] the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols in the human diet with coffee, fruits and vegetables as its major sources.[13] Its antioxidant and anti-carcinogenic properties have been well established[14,15,16,17]. It has been reported that peaches and plums which contains higher amounts of chlorogenic acid are capable of killing breast cancer cells, not only the cancerous cells were killed, but also normal cells were rescued[18]. Studies also indicate that it inhibits DNA methylation in cultured cancer cells[19]. The main objective of this study was to check the effect of chlorogenic acid, a phytochemical, on Bm12 cell line[20,21] (ovarian cell line of silkworm) and PA-1 cell line (human ovarian teratocarcinoma cells line)[22,23] and compare its cytotoxicity on both cell lines.

MATERIALS AND METHODS

Larval ovarian cell line of *Bombyx mori* (silkworm), Bm12, was generously provided by Dr. M. M. Rai, Head of the Department of Sericulture, Nagpur. Human ovarian teratocarcinoma cell line, PA-1 was procured from National Centre for Cell Sciences, (NCCS), Pune. Chlorogenic acid (CGA) was purchased from Himedia.

In vitro cell culturing of Bm12

The cells were cultured in TNM-FH medium (Himedia) supplemented with 10% FBS (Himedia). Cells were maintained at 25°C in non-humidified incubator. On reaching confluency, proliferated cells were subcultured or just split into two plastic flasks (Orange Scientific, Belgium) . Cells were seeded at a concentration of 2×10^5 cells/ml[24]. Final volume of each flask was maintained at 2ml.

Treatment with CGA on Bm12 cells

100, 300 and 500μM of CGA solution was prepared in autoclaved distilled water. Cells were treated with 100μl of each of these concentrations and were incubated for 10hrs. Cell counting using Neubauer chamber was performed initially at 0 min . Cell viability test using trypan blue dye exclusion method was done at the interval of 2-2 hrs for 10hrs.

Microscopic images

Microscopic images of cells were taken using a camera (digital microscope camera; Axiocam), focused on the optic lenses of an inverted microscope with 10X magnification.

Cell Viability-Trypan Blue Dye Exclusion Assay

In trypan blue dye exclusion assay, the viable cells with an intact cellular membrane do not take up the blue dye and maintain a clear appearance whereas the damaged nonviable cells take up the dye and stain blue due to damaged membrane. . The cell suspension was carefully mixed with 0.4% trypan blue reagent (Himedia) in 1: 1 ratio and the mixture was transferred to a Neubauer hemocytometer to determine the number of viable cells. Cells were counted by using an inverted light microscope.

In vitro cell culturing of PA-1

Culture was grown in DMEM medium supplemented with 10% FBS (Himedia), 1% antibiotic and antimycotic solution (Himedia). Culture was maintained at 37°C with 5% CO_2 and 85% humidity. Upon 80–90% confluency, they were, trypsinized for the removal of the attached cells, harvested and seeded into another sterile culture flask (25 ml) with 5 ml culture media.

Treatment with CGA on PA-1 cell line

Series of concentrations of CGA was prepared using cell culture medium, (DMEM), ranging from 50uM, 100uM, 150uM to 450uM, and 500uM. In a 96 well plate, cells were seeded at a density of 10,000 cells/well. The plate was incubated for 24 hrs to allow adhering of the cells to the walls of the wells. After 24 hr, spent media was discarded and cells were treated with 200μl of different concentrations of CGA. . Each concentration was replicated as a quadruplicate.

Treatment with standard drug (Cisplatin) on PA-1 cell line

Various concentrations of standard drug cisplatin were prepared from 5ug/ml to 50ug/ml in DMEM medium. In a 96 well plate, cells were seeded at a density of 10,000

cells/well and treated in the same way as described above with different concentrations of cisplatin solution.

Cytotoxicity assay- (MTT assay) on PA-1 cell line

In MTT assay, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means which measures the cell proliferation rate. This was performed after 4 days of treatment with CGA and Cisplatin. Spent media was discarded and fresh DMEM medium added. 25ul of MTT reagent is added and the plate was wrapped using silver foil and incubated at 37°C for 4 hrs. After 4 hrs, MTT dye from the wells was removed and 100ul DMSO and 12.5ul glycine buffer was added and the plate was read at 570nm on a plate reader.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM (Standard Error of the Mean). The Analysis Tool Pack software was used to construct graphs and statistical analysis. Statistical significance was determined using t-test: two sample assuming unequal variances. p value ≤ 0.05 was considered 'probably significant', p value ≤ 0.01 was considered 'significant', p value ≤ 0.001 was considered 'highly significant'.

RESULTS AND DISCUSSION

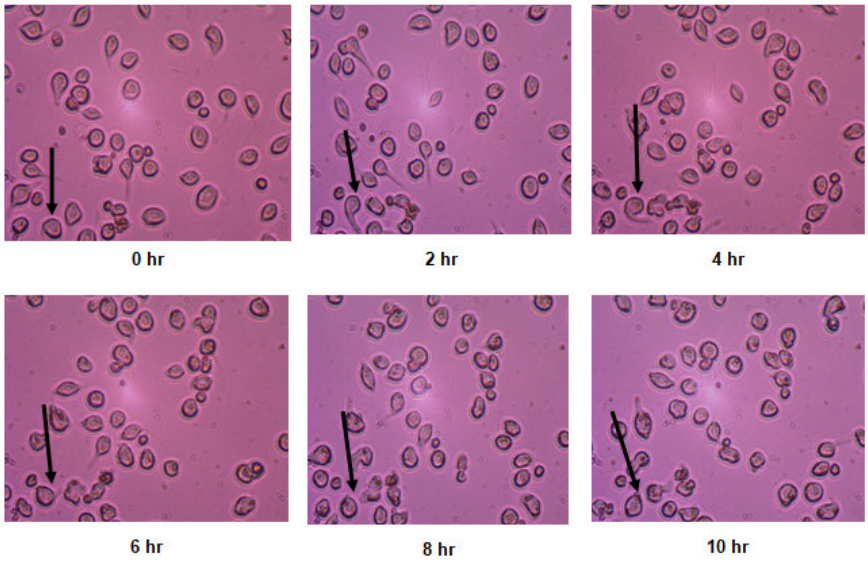


Figure 1
Bm12 cells treated with 100µM CGA observed under inverted microscope at various time intervals

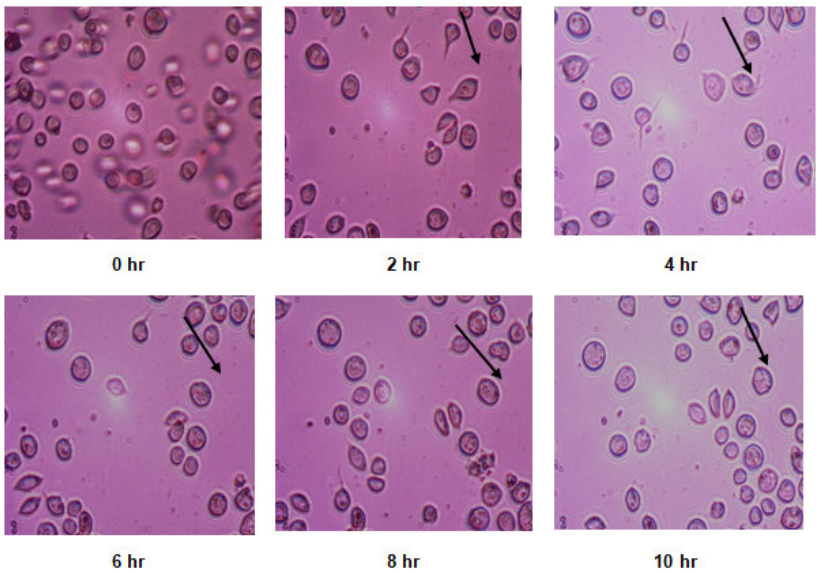


Figure 2
Bm12 cells treated with 300µM CGA observed under inverted microscope at various time intervals

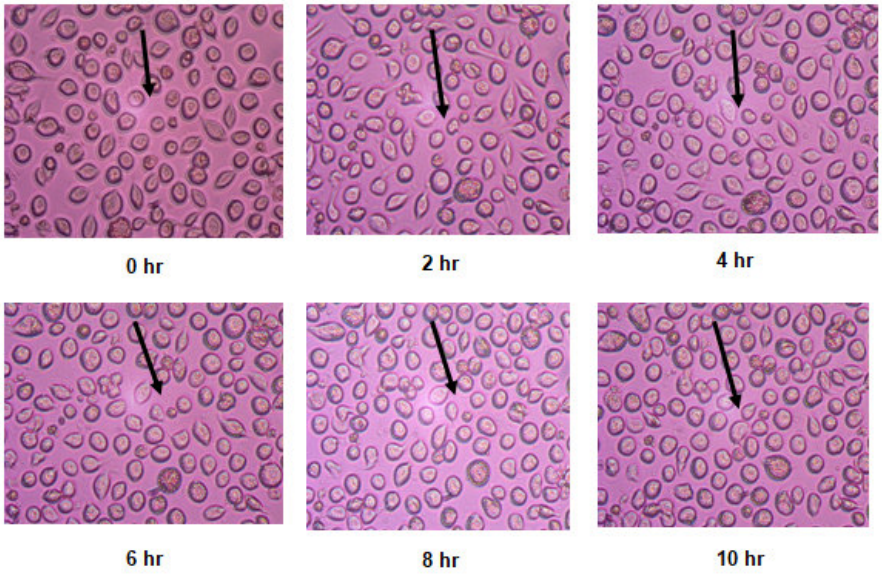
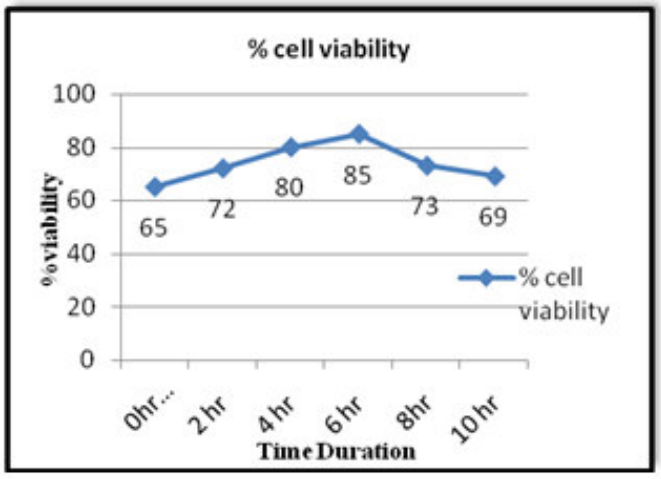


Figure 3
Bm12 cells treated with 500μM CGA observed under inverted microscope at various time interval

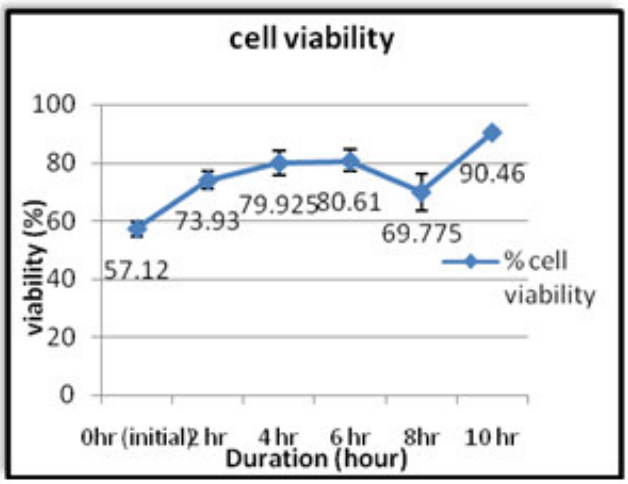
Figure 1, 2 and 3 indicates that cells died using similar mechanisms irrespective of different concentrations of CGA treatment given to it. Spherical shape of cell was first distorted, approximately 2 hr after treatment and then it burst out completely in one or two hours. Chromatin material oozed out and the cell membrane dissolved. . In some cells it was observed that a nick is formed in the cell wall at one site and the intracellular material oozes out forming a tube like passage (figure 1). In some cells nick was formed in more than one site and thus more than one tube like passages were formed (figure 3). In some cells nick became wider and eventually complete intracellular material came out. However, , after 6 hrs the break in the cell wall is repaired and the cell regained its spherical shape (Figure 1, 2 and 3 with arrowheads). The cells appear faint after joining of the nick. Thus, there must be a mechanism through which cell membrane reforms. . There might be some stress protein synthesized, which helped in formation of cell membrane. This proves that Bm12 cells are stress-resistant.

Viability of Bm12 cells treated with chlorogenic acid (Trypan Blue dye exclusion assay)

Trypan blue dye exclusion assay was performed to determine the number of viable cells after treatment with chlorogenic acid. Percent viability obtained was plotted against time duration (from 0hr to 10hrs). As observed from graphs (1-4) cell viability in control (Graph 1) increases with time starting from 0 hr. It attains a peak at 6 hr and then start declining afterwards. In cells treated with 100uM CGA (Graph 2) viability increases gradually, gets constant at 4hr-6hr and then declines after 6 hr, as in control. But, an increase in cell viability was observed after 8hrs. It was observed that viability decreases in cells treated with 300uM CGA (Graph 3). But, the decrease did not persist for long and the viability again increases after 2hrs of treatment. There was a sharp decline in viability of Bm12 cells, when treated with 500uM CGA (Graph 4). Viable cell count increase after 2hr, but then viability remains constant after 4hrs duration.



Graph 1
Viability of Bm12 cells control at different time interval

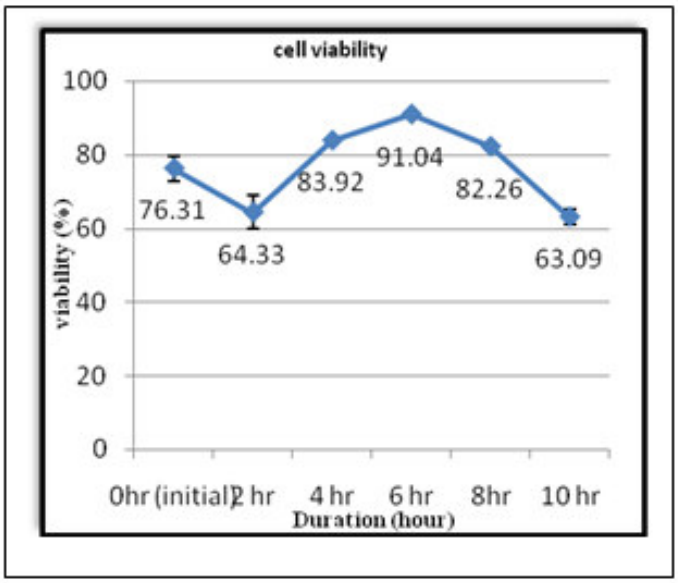


Graph 2
Viability of Bm12 cells on treatment with 100uM CGA (±SE) at different time duration

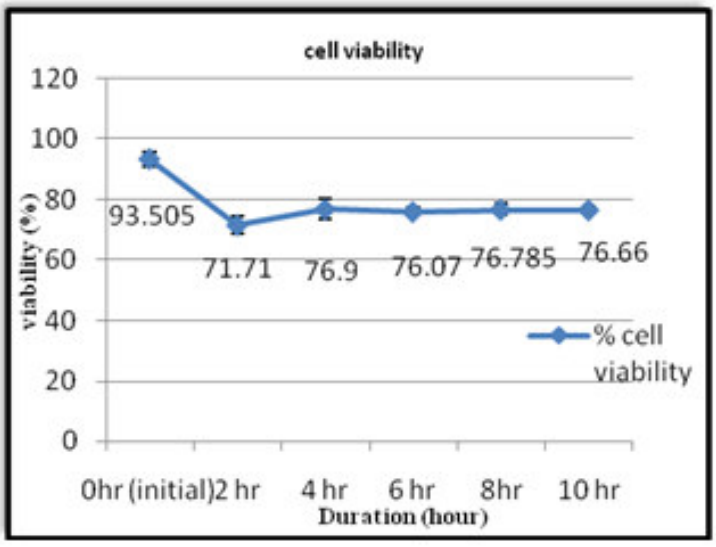
Graph 1
Proliferation rate of cells in control decreases after some time which may be due to depletion of nutrients, as the doubling time for insect cell line is less i.e. 24hr. As per the results, viability rate decreases after 6hr. Graph 1 and Graph 2 shows plateau like region.

Graph 2
In case of cells treated with 100µM CGA, increase in viability was not as observed in control, may be due to the addition of CGA. As it was a new and foreign compound for the cells, may be cells were trying to adapt in the new environment and hence the rate of cell division was not as fast as in control. Viability decreased after 6hr as in control, however, it was observed that they regain their cell division rate after 8hr. This may be due to 100µM CGA had some proliferative effect on the Bm12 cells once they got adapted to it.

Graph 3
When the concentration of CGA was increased to 300µM Bm12 cell viability decreased during initial two hours, may be due to anti-proliferative effect of 300µM CGA on Bm12 cells. Increase in viability after 2hr which may be by some unknown mechanisms, evaded the anti-proliferative effect of CGA. Viability again depleted after 6hr as in case of *above two.*



Graph 3
Viability of Bm12 cells on treatment with 300uM CGA (±SE) at different time duration

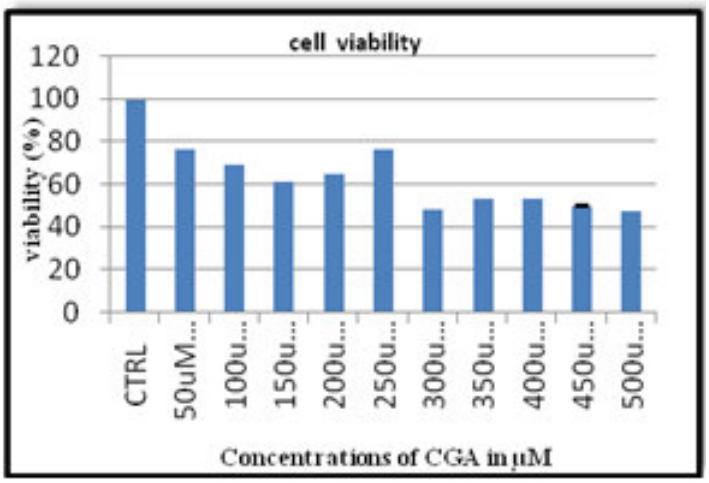


Graph 4
Viability of Bm12 cells on treatment with 500uM CGA (±SE) at different time duration

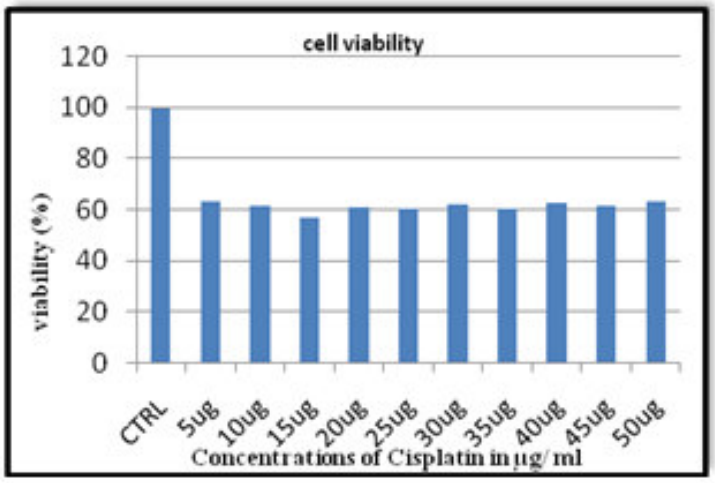
Graph 4
On addition of 500µM CGA, viability decreases sharply. It increases after 2hr as observed with 300µM CGA, but the increase is not as good as above and viability remained similar thereafter. This may be due to cessation of cell division or may be the number of cells divided was compensated by number of cell death. It showed the anti-proliferative effect of 500µM CGA which was better than 300uM. Thus it may be concluded that higher the concentration of CGA, higher the anti-proliferative effect.

Cytotoxicity of chlorogenic acid on PA-1 cell line (mammalian cell line): (Cytotoxicity assay using MTT)

Graph 5
Cytotoxicity of CGA was investigated by MTT assay. Absorbance was measured at 570nm, which is a measure of cell viability. It was observed that viability of cancer cells decreases with increasing concentration of CGA. Highest cytotoxicity was observed in wells treated with 500uM CGA, which is the highest concentration used in the experiment. Standard treatment of cisplatin showed similar effect at different concentrations used in this experiment. Difference in percent cell viability was less.



Graph 5
Percent cell viability of PA-1 cells (±SE) treated with different concentrations of CGA (P≤0.001)



Graph 6
Percent cell viability of PA-1 cells (±SE) treated with different concentrations of Cisplatin (P≤0.001)

Graph 6
It was observed that as the concentration of CGA increases, viability of PA-1 cells decreases. Lowest viability was observed at the highest concentration of CGA used in the experiment. By assessing the cytotoxicity of cisplatin, a standard drug used in ovarian cancer, it was observed that CGA is comparably more effective than cisplatin in its anti-proliferative action. It may be possible that cells would have generated resistance to cisplatin, as there are many reports showing induction of cisplatin resistance [25,26,27, 28].

Comparison of viability between Bm12 and PA-1
The highest concentration i.e. 500µM CGA resulted 35% cytotoxicity in Bm12 cells within 2 hours only whereas 50% cytotoxicity was observed in 96 hours in PA-1 cells. This indicates that Bm12 cells are more sensitive to CGA as compared to PA-1 and effect observed much faster in Bm12 as compared to PA-1. Viability of Bm12 increases with exposure and duration initially and remains all most constant after some time which shows the stress-resistant capacity of insect cells, which never observed in mammalian cells.

CONCLUSION

Bm12 cell line is a continuous, suspension and fast growing cell culture having 36-48 hr doubling time. Cell viability assay using trypan blue dye showed that 100µM CGA has no anti-proliferative action in Bm12 cell. However it shows proliferative activity after some time of treatment. Further investigation is required to suggest its specific proliferative effect. Similarly 300µM and 500µM of CGA was found to have anti-proliferative action on Bm12 cells. Surprisingly by some unknown mechanism, Bm12 cells may evade the anti-proliferative action of 300µM CGA and regain its cell division. Cell division is kept at a suppressed and constant level in cells treated with 500µM CGA. Microscopic images of Bm12 cells treated with CGA showed that the cells are stress-resistant. The resistance mechanism, the mechanism of action of CGA on Bm12 cells and mechanism of cell death needs to be investigated. On the other hand in PA-1 cells, CGA seems to be a better alternative to standard drug Cisplatin. It was found that viability of PA-1 cells decreases with increase in concentration of CGA and around 50% cytotoxicity in PA-1 cells was obtained using higher concentrations of CGA (500µM). It has been observed that mammalian cancer cells are more sensitive to CGA as compared to Bm12, i.e. lower concentrations of CGA can kill transformed or cancerous cells (PA-1) but not normal cells (Bm12). Thus CGA cytotoxicity is more specific towards cancer cells. Higher doses of CGA were found to be comparable between Bm12 (insect) and PA-1 cells (mammalian). It was found that the effect of CGA was much faster in Bm12 as compared to PA-1 cells. Thus cytotoxicity of compound may be evaluated rapidly using Bm12 cells. Results suggested that Bm12 cells may also be used to assess the effect of anticancerous compound on normal cells. The resistance of PA-1cells to Cisplatin and mechanism of action of CGA on PA-1 cells needs to be investigated. Further studies are required using some other phytochemicals to check the comparable effect on both insect and mammalian cells.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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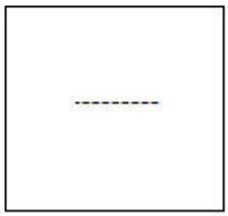

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