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Evaluation of cytotoxic effects of *Costus igneus* and Metformin Hydrochloride in Vero Cell Line using MTT and Trypan Blue assay

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Abstract

Cytotoxicity assessment is essential for determining the safety profile of natural products and therapeutic agents prior to further pharmacological applications. The present study was conducted to evaluate the cytotoxic effects of *Costus igneus* crude powder (CLP) and Metformin hydrochloride (MTF) on Vero cell lines using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay and trypan blue dye exclusion assay. Vero cells were treated with different concentrations of CLP and MTF ranging from 100 µg to 1000 µg and cell viability was evaluated after 24 hours of incubation. In the MTT assay, lower concentrations of CLP (100 µg and 250 µg) maintained high cell viability comparable to the control group, whereas higher concentrations caused a significant reduction in viability. CLP at 1000 µg showed 53.88% viability, while MTF at the same concentration reduced viability to 16.08%. Similar findings were observed in the trypan blue dye exclusion assay, where CLP at 1000 µg demonstrated 68.19% viability and MTF showed 48.96% viability. The IC50 value of CLP was calculated as 1042.64 µg The findings indicate that both CLP and MTF induce concentration-dependent cytotoxicity in Vero cells, with MTF exhibiting comparatively stronger cytotoxic effects at elevated concentrations.

Keywords: Cytotoxicity, Vero cell line, MTT assay, Trypan blue assay, *Costus igneus*, Metformin hydrochloride

Introduction

Medicinal plants have gained increasing attention owing to their therapeutic potential and relatively lower adverse effects compared to synthetic drugs. Among these plants, *Costus igneus*, commonly known as the insulin plant, has been traditionally used for the management of diabetes mellitus. The plant is widely recognized for its hypoglycemic properties and contains several bioactive constituents including flavonoids, alkaloids and terpenoids that contribute to its pharmacological activities. The insulin plant is native to Southeast Asia and has become popular in India as a medicinal and ornamental plant (Shinde *et al.,* 2022) [6]. Despite the growing utilization of herbal preparations, scientific validation of their safety profile remains essential before therapeutic application. Cytotoxicity testing is an important preliminary approach used to evaluate the potential toxic effects of natural products and drugs on living cells. Such studies help identify safe dosage ranges and determine whether a substance interferes with cellular metabolism, membrane integrity or cell survival.

Vero cell lines derived from kidney epithelial cells of the African green monkey are widely employed in toxicological and pharmacological research due to their stable growth characteristics and sensitivity to toxic compounds. These cells provide a reliable *in vitro* model for evaluating the cytotoxic potential of therapeutic agents (Freshney, 2015) [3]. The MTT assay is a colorimetric assay that measures cellular metabolic activity through mitochondrial dehydrogenase-mediated reduction of tetrazolium salts into purple formazan crystals, thereby reflecting viable cell populations (Bahuguna *et al.,* 2017) [1]. Similarly, the trypan blue dye exclusion assay is a widely used method for assessing membrane integrity, where viable cells exclude the dye while dead cells absorb it due to compromised membranes (Strober, 2001) [7].

Therefore, the present study was undertaken to comparatively evaluate the cytotoxic effects of *Costus igneus* crude powder and Metformin hydrochloride on Vero cell lines using MTT assay and trypan blue dye exclusion assay.

Materials and Methods

Cell culture and maintenance

The cells are typically grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic-antimycotic solution to inhibit bacterial and fungal growth. Cultures are kept at 37°C in a humidified atmosphere with 5% CO_2. Regular monitoring and medias are changed every 2-3 days and are sub cultured once 80-90% confluence monolayer is formed. The sub culture is used for further assays to be performed.

Treatment

The cells were treated according to the experimental design as follows in table 1.

Table 1: Experimental design for cytotoxicity of *Costus igneus* in Vero cell line

Groups	Treatment	Dose
I	Blank control	Only Medium
II	Negative control	Only cells
III	*Costus igneus* crude powder	100 µg
IV	*Costus igneus* crude powder	250 µg
V	*Costus igneus* crude powder	500 µg
VI	*Costus igneus* crude powder	750 µg
VII	*Costus igneus* crude powder	1000 µg
VIII	Standard control- Metformin Hydrochloride	100 µg
IX	Standard control- Metformin Hydrochloride	1000 µg

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] cell viability assay is a widely used colorimetric technique to assess the metabolic activity of cells, which is directly proportional to the number of viable cells. This method relies on the conversion of the yellow tetrazolium salt MTT into insoluble purple formazan crystals by the action of mitochondrial dehydrogenases in living cells (Bahuguna *et al.,* 2017) [1]. After subculture, the cells were suspended in the medium and 100 µL of cells were seeded in each well of 96 well cell culture plate and kept for incubation at 37°C with 5% CO2 for 24 hours. To blank control, 200 µL of medium and to negative control only cells are added. Treatment was done on the following day after cells have attached and complete monolayer formation is seen, with the doses diluted in 100 µL to respective wells and incubated again for 24 hours. MTT reagent is diluted in PBS at 5 mg/mL concentration and 10 µL is added to each well and incubated for 4 hours at 37°C to allow the MTT to be metabolized by viable cells. After incubation, the medium was carefully aspirated from each well without disturbing the formazan crystals. Then 100 µL of DMSO was added to each well to dissolve the formazan. The absorbance of each well was measured using a microplate reader at a wavelength of 570 nm. The cell viability was then determined by:

Cell Viability % = [(sample OD- Blank OD) / (Control OD- Blank OD)] x 100

Trypan blue dye exclusion assay

The trypan blue dye exclusion assay is a widely utilized technique for assessing cell viability and membrane integrity, providing a reliable means to quantify the proportion of living and dead cells within a given sample. This method indicates that viable cells with intact cell membranes were capable of excluding the trypan blue dye, while non-viable or dead cells, with compromised membranes, will readily absorb the dye (Strober, 2001) [7]. After treatment, 100 µL cells was resuspended in a micro centrifuge tube and 100 µL of 0.4 % trypan blue solution was added. After 5 mins, 20 µL of the suspension was taken and was charged in the hemocytometer and cells were counted in the four corner primary squares that lie on the top and left-hand lines of each square but not those on the bottom or right-hand lines to avoid counting the same cells twice under 10x objective. The number of cells were then determined as follows:

No. of live cells/ mL = Average no. of cells x 10^4 x Dilution factor

Data analysis

The experimental data were statistically analyzed using a completely randomized design (CRD) and one-way ANOVA with SPSS software (version 20). Post-hoc analysis was performed using Duncan's multiple range test to identify significant differences between groups, with statistical significance set at $p < 0.05$.

Result

MTT assay

The effect on Vero cell lines by CLP and MTF as analyzed through MTT assay is presented in table 2 and figure 1. The control group exhibited cell viability percentage of 99.29 ± 0.18. Treatment with CLP at 100 µg resulted in 99.02 ± 0.19 and at 250 µg yielded 98.56 ± 0.24, both not significantly different from the control. In contrast, treatment with CLP at 500 µg, 750 µg and 1000 µg produced viability of 89.07 ± 0.49, 79.37 ± 0.47 and 53.88 ± 0.66, all demonstrating significant reductions ($p < 0.05$). MTF treatment at 100 µg showed viability of 87.44 ± 0.37, while treatment at 1000 µg resulted in 16.08 ± 0.28, both significantly different from the control ($p < 0.05$). The IC50 was also found to be 1042.64 µg.

Trypan blue dye exclusion assay

The results of trypan blue dye exclusion assay demonstrating the effects of CLP and MTF on Vero cell lines are displayed in the table 2 and figure 2. The control group exhibited viability percentage of 99.40 ± 0.12. Treatment with CLP at 100 µg resulted in 99.07 ± 0.18 and at 250 µg produced 98.28 ± 0.18, both showing no significant difference from the control. However, treatment with CLP at 500 µg yielded 91.60 ± 0.48, while 750 µg showed 82.69 ± 0.52, and 1000 µg resulted in 68.19 ± 0.38 cell viability, all demonstrating significant reductions ($p < 0.05$). MTF treatment at 100 µg produced viability of 91.46 ± 0.50 and at 1000 µg, viability significantly decreased to 48.96 ± 0.59 ($p < 0.05$).

Table 2: Detection of Cell Viability by MTT and trypan blue dye exclusion assay

Treatment Groups	Cell Viability (%)	
	MTT assay	Trypan blue dye exclusion assay
Control	$99.29^a \pm 0.18$	$99.40^a \pm 0.12$
Treated with CLP - 100 µg	$99.02^a \pm 0.19$	$99.07^a \pm 0.18$
Treated with CLP - 250 µg	$98.56^a \pm 0.24$	$98.28^a \pm 0.18$

Treated with CLP - 500 µg	$89.07^{b} \pm 0.49$	$91.60^{b} \pm 0.48$
Treated with CLP - 750 µg	$79.37^{d} \pm 0.47$	$82.69^{c} \pm 0.52$
Treated with CLP - 1000 µg	$53.88^{e} \pm 0.66$	$68.19^{d} \pm 0.38$
Treated with MTF - 100 µg	$87.44^{c} \pm 0.37$	$91.46^{b} \pm 0.50$
Treated with MTF - 1000 µg	$16.08^{f} \pm 0.28$	$48.96^{e} \pm 0.59$

Data were expressed as Mean ± SE. Means bearing common superscripts between rows do not differ significantly at 5 % ($p<0.05$) level based on Duncan's multiple range test.

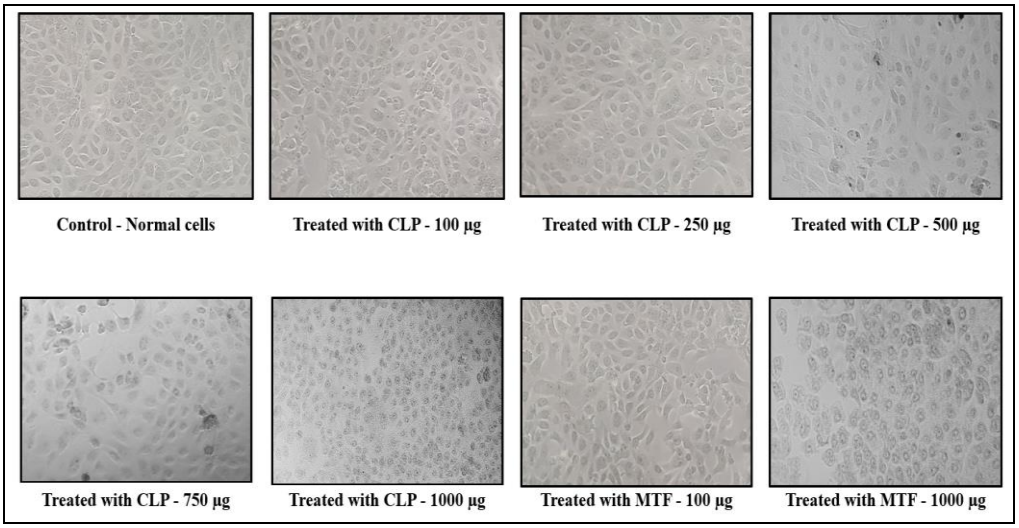

Fig 1: Effect of *Costus igneus* and Metformin hydrochloride on Vero cell line by MTT assay

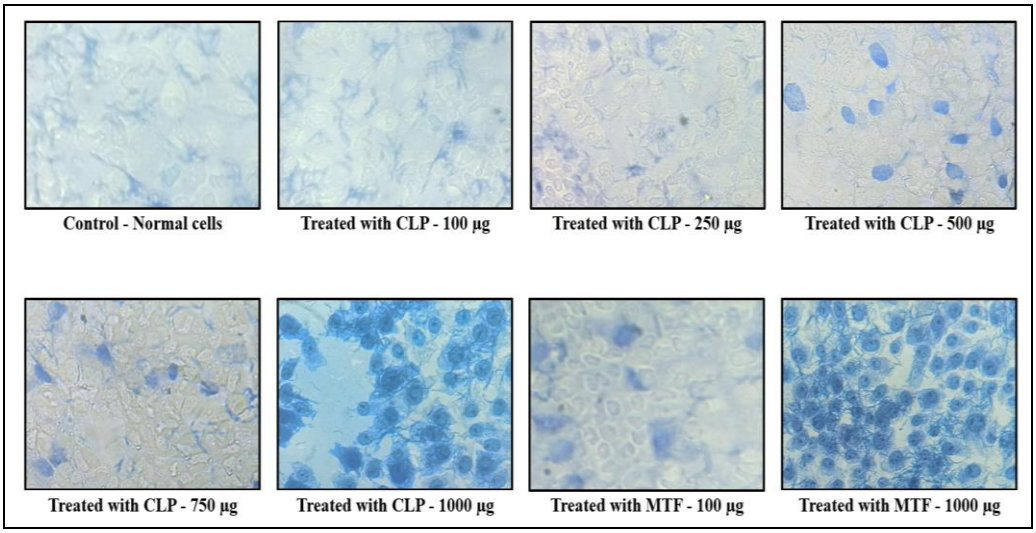

Fig 2: Effect of *Costus igneus* and Metformin hydrochloride on Vero cell line by trypan blue dye exclusion assay

Discussion

The results from the present study align with the findings of Samarakoon *et al.* (2014) [5] and Prasanna *et al.* (2019) [4], demonstrating a dose-dependent decrease in cell viability with increasing concentrations of plant extracts. Samarakoon *et al.* (2014) [5] reported over 98% cell survivability for ethyl and aqueous fractions of *C. speciosus* at concentrations of 2.5, 5.0 and 10.0 µg/mL, indicating the non-cytotoxic nature of the plant in Vero cells. Consistent with this, the current study showed high cell viability at lower concentrations of CLP (100 µg and 250 µg), which were comparable to the control, suggesting minimal cytotoxic effects.

However, as observed in the present study, cell viability declined significantly at higher concentrations, particularly from 500 µg onwards. For CLP, a noticeable reduction in cell viability was detected at 750 µg and 1000 µg, dropping to 53.88% and 68.19% in the MTT and trypan blue assays, respectively. The more substantial cytotoxicity of MTF was evident at 1000 µg, where cell viability dramatically decreased to 16.08% (MTT assay) and 48.96% (trypan blue assay). This dose-dependent trend supports Prasanna *et al.* (2019) [4] and their observation showed that higher concentrations impair metabolic activity, leading to significant cytotoxic effects. Overall, the findings indicate that while lower concentrations of the treatments preserve cell viability, higher doses notably compromise cell survival, with MTF exhibiting a more pronounced cytotoxic effect than CLP at the highest tested concentration.

The results of the trypan blue dye exclusion assay showed a dose-dependent decrease in cell viability, consistent with the observations from the MTT assay for the plant extract. Similarly, the standard drug metformin also reduced cell viability at the highest tested concentration of 1000 µg/mL.

In this assay, live cells with intact membranes were able to exclude the dye, while dead cells, with compromised membranes, allowed the dye to enter. A comparable cytotoxicity study conducted by kalidoses *et al.* (2017) [2] investigated the anticancer activity of *Costus igneus* in Ehrlich Ascites carcinoma cell lines and Dalton's lymphoma Ascites cells, using both trypan blue exclusion and MTT assays, and found that the ethanol extract of *Costus igneus* exhibited significant cytotoxicity against cancer cells.

Conclusion

This study confirms that both CLP and MTF treatments induce cytotoxicity in a dose-dependent manner, as demonstrated by the MTT assay and trypan blue dye exclusion. At lower concentrations (100 µg and 250 µg), cell viability remained high, indicating minimal cytotoxic effects. However, as the dosage increased, a significant decline in cell viability was observed, especially at 500 µg and above. The cytotoxic impact was more pronounced with MTF, particularly at 1000 µg. These findings highlight the importance of careful dose selection to minimize cytotoxic risks and suggest further investigation into optimizing safe and effective use of these compounds.

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