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IN VITRO EVALUATION OF THE FORCHLORFENURON IN MCF-7 CELLS

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ABSTRACT

Forchlorfenuron [N-(2-chloro-4-pyridyl)-N′-phenylurea] or CPPU is a synthetic cytokinin which is normally used to increase the berry size of grapes and kiwifruit. CPPU was assessed for their cytotoxicity in MCF7 cell line by 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay at concentrations of 320, 160, 80, 40, 20, 10 and 5 µg/mL and the half maximal inhibitory concentration (IC50) was calculated using Graph Pad Prism 5.0. The cells were subjected to Acridine Orange/ Ethidium bromide to find out the possible mechanism of toxicity. The results of MTT assay indicated a dose dependent cytotoxicity to MCF-7 cells with the dose 320 µg/mL. The IC50 was found to be 92.6 µg/mL. The staining of cells with AO/EB showed cells with orange to red fluorescence when treated with IC50 and double IC50 concentrations with chromatin condensation indicating apoptosis. Hence it could be concluded that the cytokinin causes apoptosis in MCF-7 cells.

INTRODUCTION

Plant bioregulators, also known as plant growth regulators, are a key breakthrough for optimizing crop management for more efficient and sustainable food production. The term Plant growth regulators refer to natural or manmade substances that mimic natural hormones or affect plant metabolism and biosynthesis. Plant growth regulators are commonly used in perennial fruits to improve bud break, flower development, fruit set, disease resistance, yield, and quality. In addition, they can improve post-harvest storage. Forchlorfenuron (1-(2-chloropyridin-4-yl)-3-phenylurea), commonly known as CPPU, is a synthetic cytokinin-based plant growth regulator that is frequently used to increase fruit size and quality in apple, cherry, grape, and kiwifruit.

In kiwifruit, CPPU treatment increased gibberellin and cytokinin production and signalling, while suppressing auxins and the ABA (abscisic acid) pathway. Subepidermal cell division is promoted by the expansion of epidermal and parenchymal cells (Mian *et al.,* 2024). However, excessive usage of CPPU can be harmful to both the environment and human health. Its residues in agricultural goods can induce health issues, and excessive use can result in the development of CPPU-resistant pests and diseases. To maintain food safety and environmental health, CPPU must be used in an appropriate and controlled manner, also the residues in agricultural goods should be monitored. According to the US Environmental Protection Agency (EPA), continuous exposure to CPPU in humans can have negative health consequences. The EPA states that excessive amounts of CPPU can damage the endocrine system. It is also harmful to the nerves. Studies have indicated that CPPU can harm heart muscle cells, resulting in contractile dysfunction. It has also been demonstrated to have an estrogenic effect, which may alter the endocrine system and severely impact reproduction and development (Lei *et al*., 2023).

Breast cancer is the most common malignancy in females. Because of its significant influence on the population, this disease is a crucial public health issue. Cell lines play a crucial role in the molecular diagnosis of breast cancer due to their versatility in laboratory research and *in vitro* models. MCF-7 is a widely utilized breast cancer cell line that has been raised for more than 40 years by multiple research groups (Comşa *et al*., 2015). The present study was carried out to investigate the effect of Forchlorfenuron in MCF-7 cell line.

MATERIALS AND METHODS

Cell line

Cells were maintained in T25 flasks with Rosewell Park Memorial Institute Medium (RPMI), 10% foetal bovine serum and 1% of gentamicin (50mg/mL) and incubated for 24 hrs. at 37^{o}C in $CO_{2,}$ incubator throughout the experiment.

Cytotoxicity studies: 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

The *in vitro* evaluation of Forchlorfenuron was carried out in MCF-7 cell line using MTT reduction Assay. Microtiter plates (96 wells) were seeded with 5×104 cells/mL and incubated for 24 hours to ensure cell adhesion. Then the cells were treated with different concentrations of 5, 10, 20, 40, 80, 160 and 320 µg/mL of Forchlorfenuron and incubated for 24

hours. Doxorubicin, a well-established chemotherapeutic agent, was employed as the positive control to validate the experimental conditions. After 24 hours of incubation with Forchlorfenuron, MTT (5mg/mL) was applied to each well at 10 µL and incubated for 4 hours in 100 µL serum-free medium. To stop the reaction, 100 µL of DMSO was added to each well to dissolve the formazan crystals that formed. The absorbance was measured using a microplate reader at 594 nm. Cell inhibition was assed and Graph Pad Prism. 5.0 was used to evaluate IC50 values for the compound.

Acridine orange / Ethidium bromide (AO/EB) staining

The cells were cultured in 6 well plates at a concentration of 1×105 cells/mL and incubated for 24 hours to ensure cell attachment. Then, the cells were exposed to Forchlorfenuron at t IC50 concentration for 24 hours. The AO/EB staining method was used to distinguish between living, apoptotic, and necrotic cells. Twenty-five microliters of treated or untreated cells were stained with five microliters of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analyzed using a Trinocular Research fluorescent microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 10X magnification (Sandesh *et al*., 2022).

RESULT

Cytotoxic assessment of Forchlorfenuron in MCF-7 Cell line

MTT Assay was used to evaluate the cytotoxicity Forchlorfenuron. The cells were treated with different concentrations of 5, 10, 20, 40, 80, 160 and 320 µg/mL. The maximum inhibition of MCF-7 cell after 24 hours treatment with Forchlorfenuron was seen in 320 µg/mL. The IC50 was found to be 92.6 µg/mL. The per cent cell inhibition MCF-7 cell after 24 hours of treatment with Forchlorfenuron was shown in Table. 1. The results were comparable to that of doxorubicin.

Concentration	Inhibition
320	44.9
160	39.6
80	31
40	18.5
20	14.5
10	29.4

Acridine orange / Ethidium bromide (AO/EB) staining

Following the treatment with Forchlorfenuron, cells were stained with acridine orange/ethidium bromide (AO/EB) to discriminate between living, apoptotic, and necrotic cells. Figure 1 depicts cell images after various treatments. Early apoptotic cells had crescent-shaped or granular nuclei that were stained yellow or green.

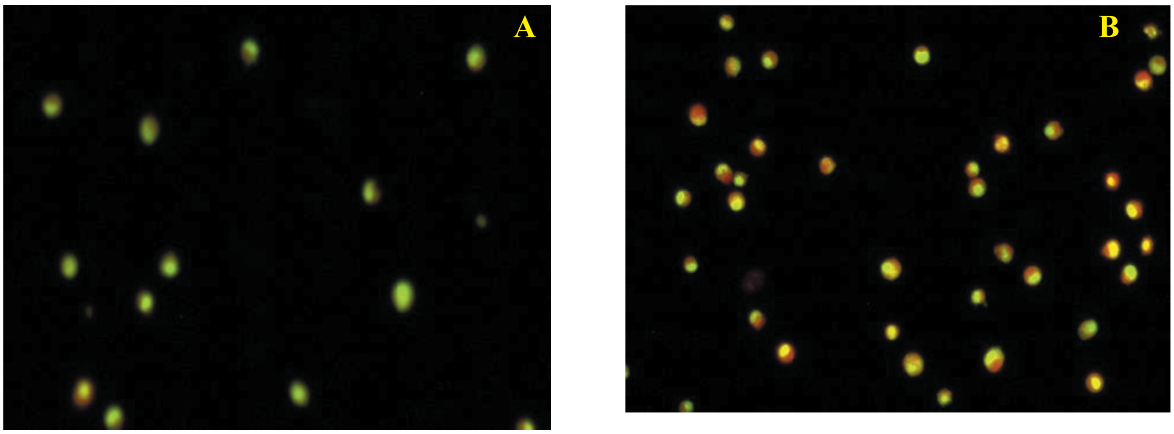

Figure 1. (**A**) Normal cells showing green fluorescence (**B**) Treated cells showing orange to red fluorescence indicating apoptosis.

DISCUSSION

Forchlorfenuron, commonly known as CPPU belongs to the phenylurea synthetic cytokinin group and is a popular plant growth regulator (PGR) used to improve fruit size. FCF may boost plant cell division and lateral development by interacting with endogenous auxins, leading to parthenocarpy and increased fruit size, set, and cluster weight. This method is commonly used to increase the size of fruits including grapes, apples, and kiwis (Kocaman and Yakar, 2023). Kim *et al*. (2020) used MTS assay to evaluate the cytotoxicity of Forchlorfenuron. The study examined the impact of FCF on cell viability in gynaecologic cancer cell lines, including endometrial (ECC-1 and KLE) and ovarian (HCH-1, OVCAR-3, and SKOV-3). They discovered that FCF treatment reduced the cell viability in a time and dose-dependent way. FCF shown cytotoxic effect against these cell lines. Gong *et al*. (2019) examined the cardiotoxic effects of CPPU exposure in zebrafish larvae/adults and H9c2 cardiomyocytes. CPPU treatment drastically reduced the cell number and vitality of H9c2 cells. Meanwhile, the CPPU treatment groups enhanced red fluorescence showed skeletal actin fiber rearrangement and aggregation. In addition, they found reduced cell size and abnormal cell morphologies following CPPU exposure, which occurred dose-dependently. These data indicate that CPPU caused cytoskeleton damage and cardiotoxicity in H9c2 cardiomyocytes in a concentration-dependent manner.

Apoptosis was initially defined as a distinct type of cell death. Apoptosis is a natural process of cell death that does not typically cause inflammation. Apoptosis causes biochemical and physical changes to the cytoplasm, nucleus, and plasma membrane. During apoptosis, cells round

up, lose contact with their neighbours, and shrink (Lawen., 2003). Physiological cell death typically involves apoptosis rather than necrosis. Defects in apoptotic cell death regulation can cause a variety of diseases, such as cancer, restenosis, stroke, heart failure, neurodegeneration, and AIDS. Recent research has identified caspases, a family of intracellular proteases that cause morphological and biochemical changes during apoptosis. Caspase regulators, such as activators and inhibitors, have been discovered to control cell death (Reed, 2000). The present study line up with the study of (Sun *et al*., 2024) that Forchlorfenuron caused cellular death and elevated intracellular reactive oxygen species (ROS), indicating the involvement of oxidative stress.

CONCLUSION

The present study demonstrates that Forchlorfenuron (CPPU), a synthetic cytokinin widely applied in agriculture, exhibits significant cytotoxic effects on MCF-7 breast cancer cells in a dose-dependent manner. The calculated IC_{50} value of 92.6 µg/mL, along with the observation of apoptotic features such as chromatin condensation and orange-red fluorescence under AO/EB staining, confirms apoptosis as the primary mechanism of cell death. These findings suggest that CPPU, beyond its role as a plant growth regulator, may possess potential anticancer properties, warranting further investigation into its molecular mechanisms and therapeutic relevance.

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