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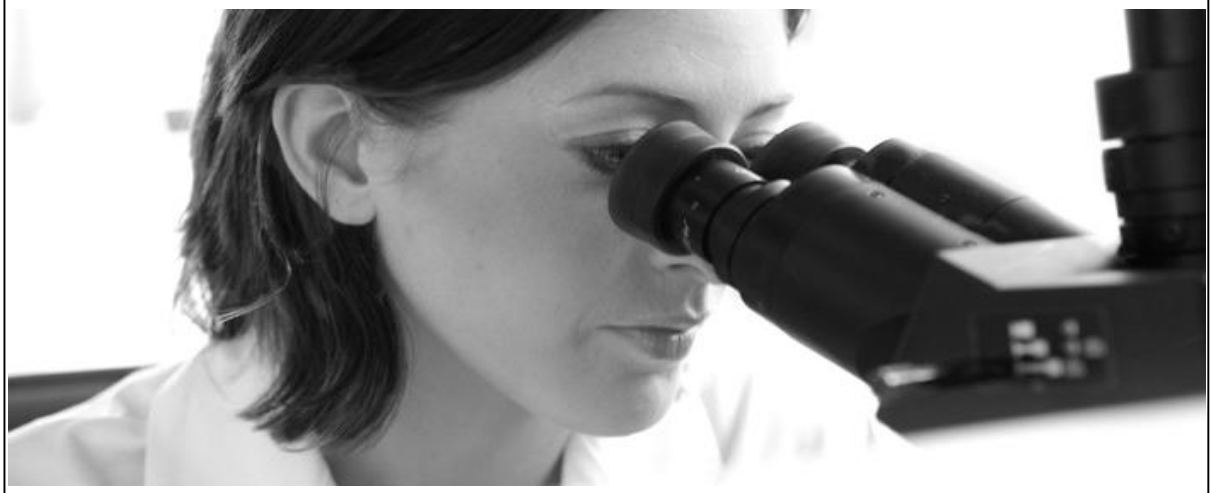

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RESEARCH ARTICLE

IN VITRO TOXICITY SCREENING OF TRIAZOLE FUNGICIDE PROPICONAZOLE

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ABSTRACT

Propiconazole, a triazole group of fungicide, broadly used against powdery mildew, rust, leaf spot of cereals and coffee. In the present study, *in vitro* toxicity of propiconazole was examined with the hepatocellular carcinoma (Hep G2) and mouse embryonic fibroblast (NIH 3T3) cell lines. The viability assays of the cell lines were examined with exposure to the propiconazole in different concentrations (20 to 100µg/ml) and IC 50 values are determined. The results indicate the evidence of propiconazole toxicity, the compound found lethal to Hep G2 cell line, which resulted in 98.66% of cell death and IC 50 values were found to be 41.025 µg/ml. In contrast, NIH 3T3 cells remain viable even in the higher concentration (100 µg/ml). The results would draw attention on propiconazole which found cytotoxic to human cancer cell line and exhibits an anticancer properties. These results suggest that it is necessary to take precaution while using propiconazole.

INTRODUCTION

The anxiety about environmental toxicology is more related to the pesticide associated problems (Carson, 1962). Toxic effects of many pesticides are studied from the last few years (Kovach, *et al*., 1992; Mileson *et al*., 1998; Shetti and Kaliwal, 2012; Kulkarni and Kaliwal, 2014). However, the concerns of toxicity from the pesticides were not reduced because of their distinctive mode of action. In the recent past few years, the usage of agrochemicals has been increased because of their availability and different formulation to control the pests and diseases. Unfortunately, most of the pesticides are found toxic to non-target organisms as well human too. Toxicity of pesticide can be experienced by individuals or the formulators, in the other way, the toxicity can be measured by low and high level exposure to sources (Yassi, 2001; Hamilton and Crossley, 2004; Owen and Pickering, 2006; Gupta, 2011). Pesticides are the well-known significant source for the pollution of water, air and soil because of their extensive and inappropriate use in agricultural fields.

Propiconazole (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole) is a foliar triazole group of fungicide also called as demethylation inhibiting fungicide. Because of it broad range of activity it is routinely used against many fungal diseases. Toxicity of triazole fungicides reported as they sold under signal name caution or danger, and this kind of fungicides popularly known for the causing skin irritation and redness by oral route of interaction, dust inhalation can cause irritation of nose, lung and throat (Nesheim *et al*., 2005; Butzen *et al*., 2005; Reigart and Roberts 2013). In addition, propiconazole is toxic to fish and bees. It is considered to be more mobile in the soil with less organic matter (Toxipedia, propiconazole). In spite of their usage concern, the cytotoxic research has not been studied much (Filipov and Lawrence, 2001). Hence, the present study would throw the light on the toxic effect of propiconazole by MTT assay using **the** hepatocellular carcinoma (Hep G2) and mouse embryonic fibroblast (NIH 3T3) cell lines under the *in vitro* conditions.

MATERIALS AND METHODS

Chemicals and Reagents

The technical gradePropiconazole was obtained from the Nagarjuna Agrichem Co., Ltd (srikakullam, India). The cell lineshepatocellular carcinoma (Hep G2)and mouse embryonic fibroblast (NIH 3T3) were procured from the NCCS, Pune. Cells in appropriate medium: DMEM-High glucose and D-PBS were purchased from Invitrogen. MTT reagent and DMSO were obtained from himedia**.**

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Preparation of standard solution

The stock solution of propiconazole was prepared with concentration of 1mg/ml. Further, for the viability test stock solution was diluted to require concentrations.

Determining Cell Cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals (Fig. 1). Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm **(**Berridge*et al*., 2005).

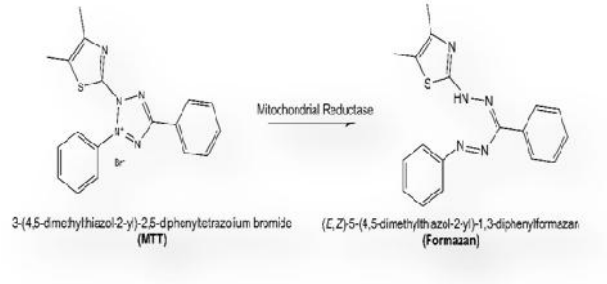

Figure 1 Mechanism of the MTT assay, explaining the reduction of tetrazolium dye MTT to formazan crystals (Berridge*et al*., 2005)

Assay Controls

For the cytotoxicity assay three controls were maintained throughout the experiment.**(i)** Medium control (medium without cells) (ii) Negative control (medium with cells but without the experimental drug/compound) and(iii) Positive control (medium with cells treated with a known drug, Metformin; 5mM). All experimental controls were studied in the different concentration of propiconazole i.e. 20, 40, 60, 80 and 100 µg/ml. it is important to use the same medium in control as well as test wells because extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. To account for this reduction**,** it is important to use the same medium in control as well as test wells.

Cell seeding

The cell suspension of Hep G2 and NIH 3T3 was seeded in a 96-well plate at required cell density (25,000 cells per well), without the test agent. Allowing the cells to adhere to the culture plate for about 24 hours with the different concentration of propiconazole and all plates were incubated for 24 hours at **37°C**in a 5% carbon dioxide atmosphere. All culture plates were removed from the incubator after24 hours and MTT reagent was added to final concentration of 10% of total volume and the same volume should be used while determining optimum cell density. For avoiding the light, aluminium foil was used for the wrapping the plates, after plates were return to the incubator for three hours. Without disturbing the monolayer of culture medium the adherent cells were aspirated than solubilisation solution (DMSO) is added in an amount equal to the culture volume. For the enhanced dissolution gyratory shaker was used and occasional pipetting up and down may completely dissolve the MTT formazan crystals especially in dense cultures.

Determination of IC 50 value

The absorbance of medium was recorded by using**96-well ELISA plate reader(Biotek)** at 570nm. The IC50 value was determined by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values are derived from the viability graph and x value represents IC 50 value of the test drug.

RESULTS

Several pesticides are very hazardous to the soil, crop yielding, non-target microorganisms and human than their stated active principles and it is also reported that triazole fungicides are oncogenic, mutagenic and long-lasting toxic possessions on the nervous system, immune system and reproductive system (Mesnage *et al*., 2014).

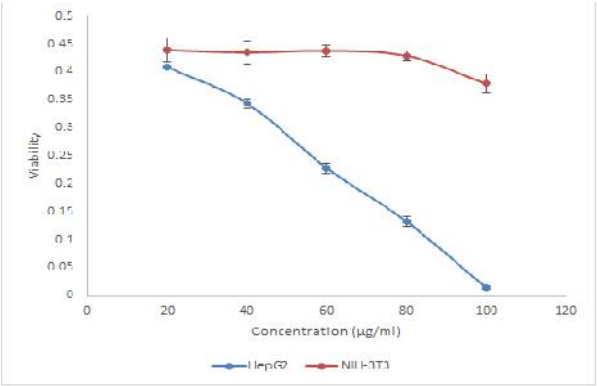

Figure 2 The effect of propiconazole on Hep G2 and NIH-3T3 cell line indicating the cell viability.

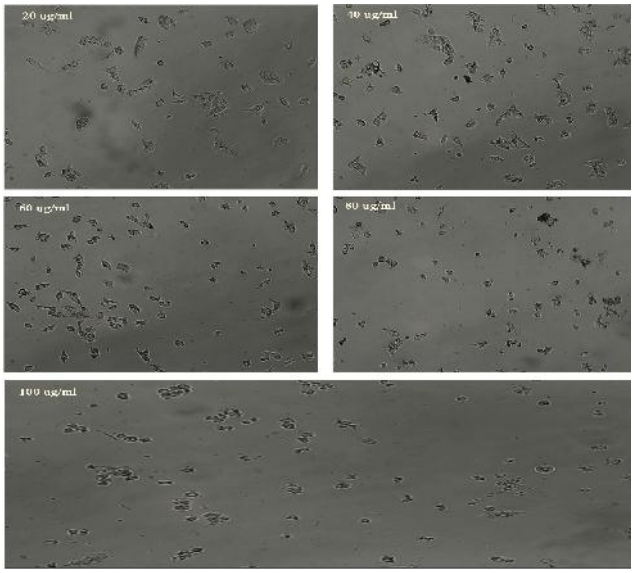

Figure 3 Hep G2 cell line treated with different concentrations of propiconazole showing toxicity.

In the present study, We observed the cytotoxicity of propiconazole on the Hep G2 cell line which cause the rapid decrease in viable cells (Fig. 2), resulting 98.66% (Fig. 3) cells were killed in the concentration of 100 µg/ml and highest viability (64.01%) were found at the initial concentration, i.e. 20 µg/ml. conversely, cells treated with metformin (20mM) successfully retain the good rate of viable cells (76%) and IC 50 values of propiconazole were found at 41.025 µg/ml. In contrast to the Hep G2 cell mortality, NIH-3T3 cells were sustained against the propiconazole and least viability (84.31%) was found to be at the concentration of 100 µg/ml (Fig. 4). IC 50 values for the propiconazole against NIH-3T3 cells lines were not determined because of their viability over the propiconazole at the all given concentration.

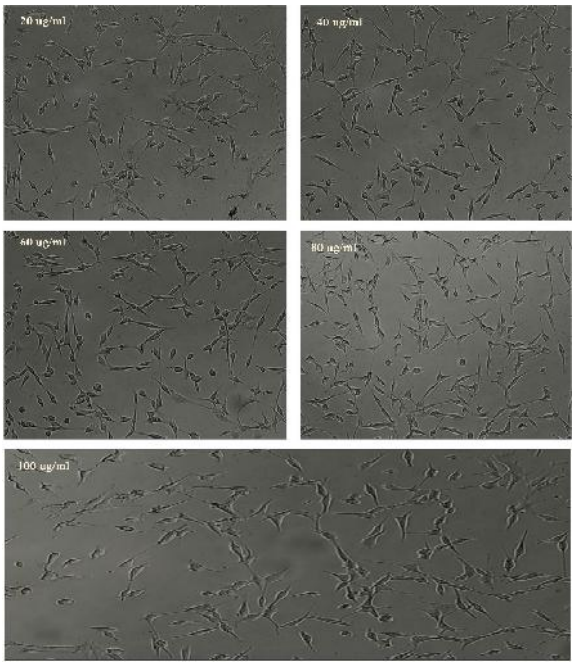

Figure 4 Exposure of propiconazole to NIH 3T3 cell line with different concentrations showing the viability of the cells

DISCUSSION

In supporting to our present work, earlier experiments on the toxicity of triazole fungicides have shown that, hepatotoxicity and associated hepatic tumors have been reported (Juberg *et al*., 2006 and Sun *et al*., 2005) because of their increased use of azole antifungal in large scales in association with this issue it has been described Concentrations of ketoconazole and itraconazole in liver tissue showed 22- and 10-fold greater than plasma levels (Prentice and Glasmacher, 2005). It indicates the toxicity of triazole more acute in liver considering the other tissues. Conversely, Moser *et al*., (2001) conducted the complete toxic exposure with one of the triazole products, tubeconazole. It was addressed that chemical exposure has the adverse effect. Kjaerstad and Andersen, (2007) have described that propiconazole treated with MCF-7 cell line not showed the cytotoxicity at the highest concentration of 30 µM. In contrast, it is evident that, in our study propiconazole is toxic for cancerous cell compare to normal embryonic fibroblast cells, results indicate it may be toxic for other cells also if taken in higher concentration. We clarify that, the cytotoxicity of propiconazole against any cell lines is definitely a significant concentration and duration dependent. Apart from the cell lines viability assay, the study conducted through pregnant rats with exposure to epoxiconazole or tebuconazole showed an increase of progesterone in parental serum was observed (Taxvig, 2007). However, epoxiconazole in a similar strain of rats described a decrease in serum progesterone levels of dams (Stinchcombe, 2013). In addition, Rieke *et al*., (2014) have demonstrated the toxicity assay with Jeg-3 cell's treatment with prochloraz and triflusulfuron-methyl, and toxicity was found quite high to respected cell line. In contrast, triazole fungicide prochloraz previously has been shown no effect on the human adrenal cell line H295R (Sanderson *et al*., 2002; Vinggaard *et al*., 2006). It has been also reported that mixture of triazole with the imidazole prochloraz also exhibited additive effects. Accordingly, pesticides with independent profile of action will not exhibit accumulative effects. Hence, typically all azole fungicides share a same mode of action (Li *et al*., 2013). Therefore, propiconazole can also affect the serum progesterone levels, and it can also may toxic to Jeg-3 cells and human adrenal cell line H295R.

In strong support to our work, Bulbul and Ozhan, (2012) have investigated that cytotoxic effects of triazole fungicides with the cervical cancer cell line (HeLa). Interestingly, all fungicides exhibit the cytotoxicity to HeLa cell line except Tubeconazole. It indicates the anticancer property of fungicides, as is the case in our study. As mentioned earlier about the mode of action of azole fungicides, propiconazole can also have toxic effect on the HeLa cell line. It is also important to known that fungicide toxicity has the several consequences, they might be of concern for the human health, animals, adverse effect on non-target microorganisms and mainly soil fertility. Further, the research should be carried for the detoxification of toxic compounds by microorganisms. Because microorganisms are thought to be a key reservoir for the biological activity and significantly enhance environmental cleanup (Satapute *et al*., 2012).

CONCLUSION

The toxic effect of propiconazole on cell lines indicates that, propiconazole is toxic to the human cell lines and exhibits anti-cancer property. The longer exposure to propiconazole may lead to adverse effect on the environmental healthiness, agricultural soil and also human. In addition, more research would take place to understand the toxicity mechanism and also its detoxification study. It is also essential to take all precautions while handling propiconazole.

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