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CYTOTOXICITY OF IMIDACLOPRID AND MYCLOBUTANIL PESTICIDES ON THREE CANCER CELL LINES

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ABSTRACT

Three cancer cell lines, i.e. HEpG-2 (human liver carcinoma), MCF-7 (human breast adenocarcinoma), and PC3 (Prostatic Small Cell Carcinoma) were used to determine the cytotoxic effects of the neonicotinoid insecticide (imidacloprid) and conazole fungicide (myclobutanil). Cytotoxicity was measured by neutral red incorporation (NRI) assay. The lowest concentration of the tested pesticides (0.5 µg/ml) was toxic. With the increase of the concentration up to 80 µg/ml, the Department of plant protection, Faculty of Agric., Ain Shams University, shoubra Elkheima, Cairo, Egypt

Department of Genetics, Faculty of Agric., Ain Shams University, Cairo, Egypt damage degree of the cellular form and size was more serious. The midpoint cytotoxicity value, (NRI_{50}) for imidacloprid and myclobutanil for HEpG-2, MCF-7, and PC3 cancer cell lines were 110.5, 67.7 and 67.6 µg/ml and 38.12, 41 and 27.5 µg/ml, respectively. In general, myclobutanil was very toxic in the three cancer cell lines compared with imidacloprid.

INTRODUCTION

The use of pesticides is one of the most effective tactics used for controlling agricultural pests. Pesticides significantly contribute in increasing of crop productivity. Environmental contamination by pesticides can occur by means of volatilization, leaching, adsorption, absorption in soil, and runoff. (**SIGRH, 2005**).

Since the mid of 1990s, the class of neonicotinoids has become the most widely used and fastest growing family of insecticides worldwide (**Buckingham et al 1997; Tomizawa & Casida, 2005; Brown et al 2006 and Jeschke et al 2011**). Development of new pesticides, such as neonicotinoid family includes imidacloprid, are used against severe pests with the lowest negative environmental impact (**Ambrose, 2003**). Imidacloprid (IMI), [1-(6-chloronicotinyl)-2-nitroimino-imidazolidine], is selective pesticide against the target species and show less toxic effects to non target organisms (NTO) compared to other insecticide groups (**Casida & Quistad, 2004 and Jeschke et al 2011**). The lower toxicity of IMI, may be due to its interact with the nicotinic acetylcholine receptors (nAChRs) of the central nervous system. They target and bind to postsynaptic nAChRs of insects, hence they induce a neuronal hyper-excitation and accumulation of acetylcholine, leading to the insect's death within minutes (**Buckingham et al 1997; Matsuda et al 2005 and Tomizawa & Casida, 2005**).

Myclobutanil is a Systemic conazole class fungicide widely used as an agrochemical, which was introduced in the 1980s, for controlling several diseases such as powdery mildew, *Erysiphe necator*; (*Uncinula necator*) downey mildew, *Plasmopara viticola* on fruit, vegetables, and seed commodities in the EU and elsewhere to control fungi such as Ascomycetes, Fungi Imperfecti and Basidiomycetes. Its widespread use has raised the issue of possible health risks for agrarian communities and the general population, which can be exposed to residues present in food and drinking water. The toxicities identified include adverse effects on liver and kidney and on the development of male repro-

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ductive organs, Also it has a suspected endocrine disrupting properties (**Antonietta et al 2016 and Maarke et al 2014 & Fishel, 2005).**

The neutral red incorporation (NRI) assay, the method as described by **Borenfreund and Puerner (1985)** measures inhibition of cell growth, which is based on the absorbance of the vital dye NR by lysosomes of living, but not by dead cells (**Hongyan and Shicui., 2001**). This assay based on the ability of viable cells to incorporate and bind neutral red dye, is widely used as a cell survival/viability assay (**Lee et al 2008**).

The use of cell lines along with bio-monitoring data could enable a proper understanding of environmental metal/chemical toxicity, holistically **(Nakadai et al 2006)**. Several toxicology test animal systems are undependable, as the exemplary mouse or rabbit or guinea pig systems are not true representative of human body. Therefore, there are large gaps in the mirror of toxicity models with animals and humans **(Tzimas et al 1997)**. Nonetheless, all popular animal models used in toxicology are mammals. Further, the requirement of a high number of animals in toxicity studies is so much that, for myriad of test chemicals like 30,000 or more in number, discourages the use of whole animals in assay systems **(Gilbert et al 2010).** Therefore, the use of cell lines in toxicology had been well recognized **(Rosler et al 2004).** It is consensus that the animal system has a holistic approach in toxicity studies that is unavailable in cell cultures. a fact that supports the use of whole animal models, which have physiological homeostasis that would suppress an expression of toxicity, on the other hand. When the toxic event crosses a threshold concentration of the chemical in focus only, the toxicity is expressed in animal models. But, in cell line systems, each toxicity level at and beyond the minimum inhibitory concentration (MIC) level is detected. However, whole animal tests can never be adequately standardized like human cellular systems, as the experiment should have growing cells under controlled conditions, for example, in vitro-cultured human cell lines.

The present study aims to assess the acute cytotoxicity of the insecticide imidacloprid and the fungicide myclobutanil which used in Egypt to control of the major pests specially on grape crop. Cytotoxicity test against three cancer cell lines, like HEpG-2 (human liver carcinoma), MCF-7 (human breast adeno-carcinoma), and PC3 (Prostatic Small Cell Carcinoma) by neutral red incorporation assay were done.

MATERIAL AND METHODS

Chemicals used

Active Ingredient of imidacloprid (1-((6-chloro-3-pyridinyl) methyl)-4,5-dihydro-N-nitro-1Himidazol -2-amine, (CAS: 105827-78-9) and myclobutanil (α-butyl-α-(4-chlorophenyl)-1H-1,2,4-triazole-1-propanenitrile), (CAS: 88671-89-0), neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) were purchased from Sigma-Aldrich (California,USA).

Cell Lines

Three cancer lines were used in this study, i.e. HEpG-2 (human liver carcinoma), MCF-7 (human breast adeno-carcinoma), and PC3 (Prostatic Small Cell Carcinoma). The cell lines were obtained from Egyptian Company for Production of Vaccines, Sera and Drugs (VACSERA) Cairo Egypt. To maintain and sub culturing such cell lines, 10^4 cells/cm^2 was plated in monolayers in 22-cm^2 polystyrene tissue culture dishes with DMEM medium (Sigma Chemical Co.) supplement with 25 mM hepes buffer (PH 7.4), 10% heat-inactivated FCS, and gentamicin (30 µg/ml). the routinely subculture procedure and maintain cells at 37^o C in humidified incubators in 85% R.H. twice a week to keep the number of subpassages at a minimum and thus genetic homogeneity. Cells were counted on an improved Neubauer hemocytometer and cell viability will determined by exclusion intake of trypan blue dye as described by Freshney, (1987). Cells will be allowed to reach 65% confluence (day 3, mid log-phase) and then exposed to different concentrations of the tested compounds.

Neutral red incorporation (NRI) assay

To evaluate the cytotoxicity of the tested pesticides the NRI assay **(Borenfruend and Puerner, 1985)** was followed. Such assay monitors the lysosomal function in viable cells in the presence of the tested substances. In such method individual wells of 96-well tissue culture microtiter plate was inoculated with 200 µl medium containing 65% confluence of the cultured cells. The plate was incubated for 24h and the medium was replaced with medium amended with varied concentrations of tested agents, i.e. 0.5, 5, 15, 25, 50 and 80 µg/ml. Such medium, is serum free medium. After 24 h of exposure periods. The medium was replaced with other medium containing neutral red dye at 40 mg/ml,

which prepared and pre-incubated overnight at 37°c. the plate is then reincubated for another 3 h to allow the target cells for the uptake of the supravital dye into the lysosomes of viable cells. The medium was removed and the cells washed twice with 0.5 % formaldehyde in 1 % $CaCl_2$, followed by the addition on 200 µl of 1 % acetic acid in 50 % ethanol to extract the dye from the cells. After the rapid agitation of the plate for 20 min. on a microtiter plate shaker, the absorbance of the resulted colour was measured at 570 nm using ELISA reader.

Estimation of cytotoxicity values

To evaluate the cytotoxicity of the tested compounds, the prepared series of concentrations of each pesticide were tested and the percentage of cellular viability which corresponds to the resulted absorbance was used to plot the toxicity regression lines **(Bayoumi, 1998)**. To estimate LC_{50} computer program (Sigma Plot® version 2.0 for windows) was used. The relative cytotoxic potencies of the tested agents was evaluated by comparing their midpoint cytotoxicity value, (NRI_{50}), i.e. the concentration of tested agent needed to reduce the absorbance of extracted dye by 50% as compared to control value.

Statistical analysis

Statistical analysis was performed to compare the obtained cytotoxicity values using the student t-test using sigma plot program version 2.0 software.

RESULTS AND DISCUSSION

1. Cytotoxicity of the two tested pesticides on cancer cell lines.

Imidacloprid and myclobutanil pesticides were tested for determination the cellular toxicity on three cancer cell lines; HEpG-2(human liver carcinoma), MCF-7(human breast adeno-carcinoma), and PC3 (Prostatic Small Cell Carcinoma) in comparison to doxorubicin, as positive control, by using Neutral Red incorporation assay (NRI Assay). Three cell lines: HEpG-2, MCF-7, and PC3were exposed to Six concentrations of tested pesticides ranged from 0.5-80 µg/ml and incubated for 24 hours.

The results presented in **Table 1 and Figure 1** displayed a marked decrease in cell viability of all the three cancer cell lines; HEpG-2, MCF-7, and PC3 in a concentration dependent manner after treatment with imidacloprid pesticides.

Table 1. Viability percentage and NRI_{50} values (µg/ml) of imidacloprid pesticides on HepG-2, MCF-7 and PC3 cell lines using Neutral Red incorporation Assay.

Concentrations/ (µg/ml)	% cell viability		
	HepG-2	MCF-7	PC3
0.5	83.8	98.6	76.3
5	77.9	94.5	75.0
15	72.0	94.5	72.3
25	69.1	82.2	72.3
50	64.7	63.0	55.2
80	60.3	39.7	44.7
NRI_{50}	110.5	67.7	67.6

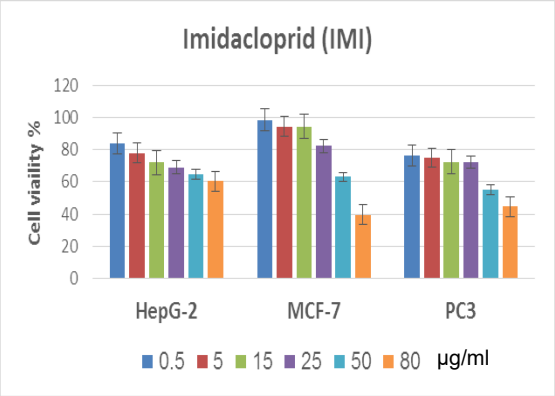

Fig. 1. Cytotoxicity of imidacloprid to HepG-2, MCF-7 and PC3 cells as determined by Neutral Red Incorporation Assay.

The obtained results showed that there was different response with different cell lines where, cell line PC3 found to be more affected and sensitive at low concentrations of IMI (0.5, 5 µg/ml) in comparison with HEpG-2, MCF-7 cell lines (Figure 1). HEpG-2 cell line exhibited the highest affected on cell viability at concentrations 15 µg/ml, 25 µg/ml with cell viability 72% and 69.1%, respectively. While, HEpG-2, PC3 and MCF-7 exhibited the most affected on cell viability at concentrations 25, 50, 80 µg/ml, respectively. Maximum reduction in cell viability at 50 and 80 µg/ml were found to be 55.2 % and 39.7% at 50 and 80 µg imidacloprid / ml for PC3 and MCF-7 cells, respectively (**Fig. 1).**

The NRI_{50} values for imidacloprid were 110.5, 67.7 and 67.6 µg/ml for HEpG-2, MCF-7, and PC3 cancer cell lines respectively **(Table 1).**

The lowest concentration of myclobutanil pesticides tested (0.5 µg/ml) was toxic to the all tested cell lines, and toxicity increased as the concentration of myclobutanil was progressively increased. In cell lines treated with myclobutanil pesticides, the PC3 cell was found to be the highest toxicity and has lowest viability than HEpG-2 and MCF-7 cells at lower concentrations (0.5, 5,15 and 25 µg/ml) while HEpG-2 cells showed the lowest cell viability at concentrations 50, 80 µg/ml **(Table 2 and Fig. 2).**

Table 2. Viability percentage and NRI_{50} values **(**µg/ml) of myclobutanil pesticides on HepG-2, MCF-7 and PC3 cell lines using Neutral Red Incorporation Assay.

Concentrations/ µg/ml	% cell viability		
	HepG-2	MCF-7	PC3
0.5	97.0	97.3	77.6
5	97.0	79.5	68.4
15	91.2	76.7	60.5
25	58.8	71.2	51.3
50	26.5	52.0	31.6
80	00.0	00.0	1.3
NRI_{50}	**38.1**	**41**	**27.5**

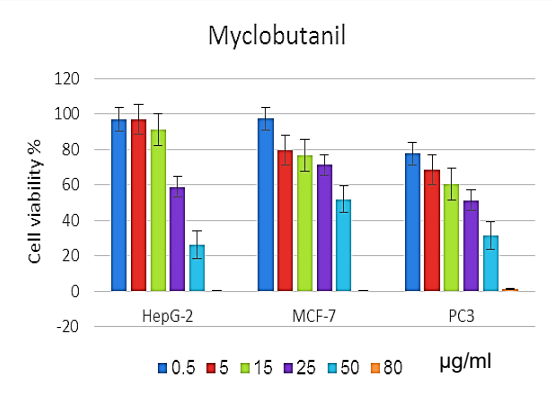

Fig. 2, Cytotoxicity of myclobutanil to HepG-2, MCF-7 and PC3 cells as determined by Neutral Red Incorporation Assay.

The NRI_{50} value, the concentration of myclobutanil resulting in 50% inhibition of cell viability after 24 h exposure to myclobutanil, was calculated from concentration–response curves **(Fig. 3).** The NRI_{50} values were 38.12, 41 and 27.5 µg/ml for HEpG-2, MCF-7, and PC3 cancer cell lines, respectively.

Depicts the cytotoxic curves from NRI assay showing the survival of HEpG-2, MCF-7, and PC3 treated with imidacloprid and myclobutanil pesticides. Comparing the cytotoxic efficacy of imidacloprid and myclobutanil pesticides in different cell lines **(Fig. 3).**

In liver cancer cell line, the higher cytotoxic effect was observed with myclobutanil at concentrations (0.5, 5, 15 µg/ml), conversely in higher concentrations, imidacloprid at 25, 50 and 80 µg/ml showed the highest cytotoxic effect with cell viability percentage 58.8, 26.47 and 00.0 respectively.

Comparing the cytotoxicity of imidacloprid and myclobutanil pesticides in breast cancer cell the result showed that the highest cytotoxicity was observed with myclobutanil pesticides in all concentrations.

The same result was found in Prostatic Small Cell Carcinoma, myclobutanil pesticides was found to be more toxic than imidacloprid.

The results clarify the concentration dependent inhibitory effect of cell viability and induction of cytotoxicity. Maximum cytotoxic effects were observed at 80ppm with complete reduction of cell count **(Fig. 3).**

The NRI_{50} values found for imidacloprid were 110.5, 67.7 and 67.6 µg/ml for HEpG-2, MCF-7, and PC3 cancer cell lines, respectively. The NRI_{50} values were 38.12, 41and 27.5 µg/ml for HEpG-2, MCF-7, and PC3 cancer cell lines, respectively.

As a general trend, the cell line PC3 inhibition was highly observed in myclobutanil with $INRI_{50}$ value 27.5 µg/ml compared to IMI with NRI_{50} values was 67.6 µg/ml.

PC3 cell line was the most sensitive cell line, the NRI_{50} being on average, respectively, 27.5 µg/ml, 38.12 µg/ml lower than for HepG2 and MCF-7, the least sensitive with NRI_{50} value 41µg/ml. myclobutanil pesticides induced highest cytotoxicity because it gave lowest NRI_{50} value in all tested cell lines than imidacloprid and consider more toxic. Data showed that myclobutanil pesticides was the most toxic in all cell types.

Also, PC3 cell line exhibited higher sensitivity toward the tested pesticides after 48 hrs. In addition, it was observed that the responses of the targeted cell lines (MCF-7 and PC3) were equals toward imidacloprid pesticides treatment with midpoint cytotoxicity value 67.7 and 67.6 µg/ml respectively.

The utilized cell lines were suitable as biological target to evaluate the cytotoxicity of toxic agents. The differences between the determined midpoint cytotoxicity values may be due to the

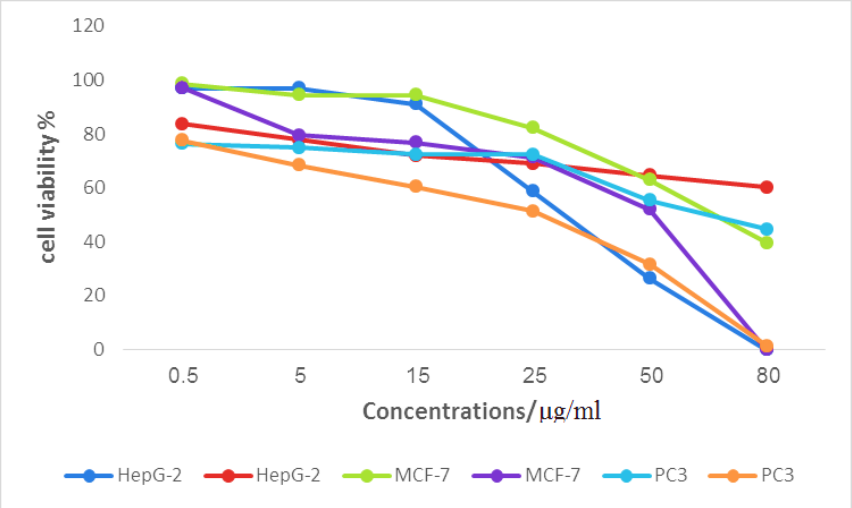

Fig. 3. Curves of viability VS. concentration of HepG-2, MCF-7 and PC3 cells affected by imidacloprid and myclobutanil.

effects of the corresponding metabolites of each compound which exhibit in turn more or less cytotoxic effect compared to the parent substance.

These results are consistent with previous reports, which demonstrated that low concentrations of Organophosphate (Ops) did not affect cell viability. However, when cells were exposed to pesticide concentrations greater than or equal to 12 µM, cell viability decreased significantly in a concentration dependent manner (**Moore et al 2010**). According to **Feng et al (2005),** high concentrations of IMI led to some toxic and genotoxic effects in human peripheral lymphocytes.

Imidacloprid is able to inhibit the growth of flounder gill (FG) cell culture causing severe injury to the mitochondria. They suggested that the mitochondria are probably the primary target of imidacloprid **(Su et al 2007).**

Four neonicotinoid pesticides, including imidacloprid, significantly induced DNA damage when measured by the comet frequency and the tail length **(Calderón-Segura et al 2012)**. Admire, the commercial product of imidacloprid, produced calf thymus DNA adducts upon activation by S9 mix **(Shah et al 1997)**. Exposure of the human peripheral blood lymphocytes to imidacloprid (0.05 to 0.5 mg/l) significantly increased the levels of sister chromatid exchange (SCE) and micronucleus(MN) and enhanced DNA strand breaks **(Feng et al 2005)**; however, imidacloprid (0.1 to 100µg/ml) did not affect the frequency of sister chromatid exchange (SCE) and micronucleus (MN) formation **(Demsia et al 2007)**. In the presence and absence of S9 mix, imidacloprid (20µM) significantly increased the frequency of MN in the peripheral blood lymphocytes and DNA strand breaks in the leukocytes **(Costa et al.2009)**. Recently, **(De Arcaute et al 2014)** demonstrated that imidacloprid can be considered a harmful agent with genotoxic effects at both DNA and chromosomal levels. The molecular mechanisms motivating the genotoxicity of the neonicotinoid insecticides are generally unknown. In vitro studies of **(Yao et al 2006)** have indicated that acetamiprid may induce reactive oxygen species (ROS) generation in bacteria. However, the incubation of imidacloprid with Yurkatcells and lymphocytes did not increase the production of reactive oxygen species **(Costa et al 2009)**. Although these results are inconsistent**, (Valko et al 2006)** suggested that the neonicotinoid insecticides are direct genotoxic compounds that could act as a source of reactive oxygen species or free radicals in the treated human cells.

According to **(Antonietta et al 2016)** myclobutanil reduced cell viability at all concentrations tested. In particular, the cytotoxic effect was partial in low concentrations (1-10 µg/ml), but in high concentrations there was near total cell death.

In Another study **(David et al 2014)** metabolic activity After incubation with myclobutanil, the readings obtained with Cell Titer-Blue TM were used to determine the IC_{50} for U-251 MG and SH-SY5Y cell line. For both cell lines, concentrations of myclobutanil from 1.953 µM to 31.25 µM, had

little impact on cell viability, whereas high concentrations, (500 µM 1 mM) were lethal.

Reviewing the obtained results, it could be concluded that, concentrations of myclobutanil pesticide tested (0.5 – 80 µg/ml) was toxic to the all tested cell lines, and toxicity increased as the concentration of myclobutanil was progressively increased. In cell lines treated with myclobutanil pesticides, the PC3 cell was found to be the highest sensitive and has lowest viability than HEpG-2 and MCF-7 cells at lower concentrations (0.5, 5,15 and 25 µg/ml) while HEpG-2 cells showed the lowest cell viability at concentrations 50, 80 µg/ml.

The NRI_{50} value, the concentration of myclobutanil resulting in 50% cellular mortality after 24 h exposure to myclobutanil were 38.12, 41 and 27.5 µg/ml for HEpG-2, MCF-7, and PC3 cancer cell lines, respectively.

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