RESEARCH ARTICLE
JIVA 23 (1) April 2025
https://doi.org/10.55296/JIVA/23.1.2025.47-54

Received on 22.04.2025
Accepted on 26.04.2025

EVALUATION OF CYTOTOXICITY *OF* CHLORANTRANILIPROLE USING *IN VIVO* AND *IN VITRO* ASSAY.

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Abstract

Chlorantraniliprole is an insecticide that has been widely used in agriculture and has been found to be toxic to aquatic organisms as well as in *in vitro* studies. The present study evaluates the *invitro* and *invivo* cytotoxicity of chlorantranilitprole in selected models. The cytotoxic potential of chlorantraniliprole was examined *invitro* using MTT reduction assay at concentrations of 18.5, 9.25, 4.62, 2.3, 1.15, 0.57, 0.28, 0.14 mg/mL. The IC50 values of the compound was calculated by plotting the concentration against per cent cell inhibition using Graph pad prism v.5.0. The IC50 value obtained in HepG2 cell line was found to be 1.517 mg/mL. The *invivo* test was conducted by Brine Shrimp (*Artemia salina*) mortality assay at concentrations of 500,100, 50, 25,and 10, 5mg/mL for 24 hours. After the treatment, the number of live, mobile and deceased shrimp were recorded. TheLC50 value of Chlorantraniliprole against Artemia was found to be 2.228 mg/mL (95% confidence interval range 1.714 to 2.918). The findings of this study demonstrate that Chlorantraniliprole 18.5 SC can adversely affect aquatic arthropods, irrespective of their concentrations as well as effecting cytotoxicity in mammalian cells.

Key words: Chlorantraniliprole 18.5 SC, HepG2 cells, MTT assay, Artemia

INTRODUCTION

Pesticides have played a significant role in modern agriculture, by enhancing crop production through control over a wide range of pests, including insects, weeds and diseases that threaten crop yields. They help to minimise the damage caused by these pests, ensuring consistent

and high-quality food supplies. The use of pesticides allow farmers to grow a greater variety of crops in regions where pests and diseases would otherwise limit agricultural productivity (Indiradevi *et al*., 2022). This in turn contributes to global food security. Even when the benefits of pesticide use are undeniable, the long-term environmental and health implications require careful management and consideration.

The overuse or improper application of these chemicals can lead to a variety of negative outcomes. Resistance among pests is a growing concern, whereas the runoff can contaminate water sources, harm non-target organisms and disrupt ecosystems (Reck *et al*., 2014). To mitigate these risks, integrated pest management (IPM) strategies are gaining popularity, focusing on sustainable pest control methods that combine biological, cultural and chemical approaches in a balanced manner. This shift aims to reduce pesticide reliance, promote biodiversity, and maintain the health of agricultural landscapes.

HepG2 cells, a human hepatocellular carcinoma cell line, are widely utilised in toxicity assays due to their ability to mimic key functions of human liver cells. These cells retain critical liver-specific functions, such as the expression of drug-metabolizing enzymes, protein synthesis, and the ability to process xenobiotics (Qiu *et al*., 2015). While they exhibit many liver-like functions, they do not fully replicate all the complexities of human liver tissue, such as the presence of a full spectrum of liver cell types and the interactions between them. As such, they are used in *invitro* assays to assess the hepatotoxicity of drugs, chemicals, and environmental pollutants, allowing researchers to evaluate potential risks to human health without the need for animal testing (Knasmuller *et al*., 1998). HepG2 cells do not undergo all the same metabolic processes as primary hepatocytes, which may influence the way certain compounds are metabolised. HepG2-based assays can also provide insights into the mechanisms of liver toxicity, including cellular stress responses, oxidative damage, and apoptotic pathways (Kanne bratt and Andersson, 2008).

Brine shrimp (*Artemia salina*) are increasingly used as a model organism in toxicity testing due to their simplicity, rapid development and sensitivity to environmental changes. Brine shrimp larvae, or nauplii, are particularly advantageous for toxicity testing because they are easy to cultivate, require minimal resources, and have a short life cycle, making them cost-effective for large-scale screenings. When exposed to toxic substances, brine shrimp exhibit a range of behavioural and physiological responses, including changes

in movement, lethality, and morphological alterations, which can be quantitatively measured to assess the level of toxicity (Harwig , 1971; Wu, 2014). To enhance the relevance of brine shrimp assays, they are often used in combination with other *invitro* or *invivo* models, providing a more comprehensive understanding of a compound's toxicological profile.

Chlorantraniliprole is an insecticide of the diamide class used to control a range of pests belonging to the orders Lepidoptera (moth), coleoptera (beetle), Diptera (fly) and Isoptera (termite). It opens muscular calcium channels, particularly the ryanodine receptor, rapidly causing paralysis and ultimately death of sensitive species. The differential selectivity of chlorantraniliprole towards insect ryanodine receptors explains the outstanding profile of low mammalian toxicity (Dutta *et al.,* 2014). However, there are certain reports on the toxicities of other drugs of class diamide insecticides like flubendiamide and cyantraniliprole on mammals.

Animal studies have suggested that chlorantraniliprole has a low likelihood of causing significant health issues such as carcinogenicity or mutagenicity (Abdel-Mobdy *et al.,* 2017). However, like many pesticides, it can cause mild to moderate effects, including liver or kidney stress at higher exposure levels. For example, studies in rodents have reported transient liver enzyme elevations, but these effects were not considered to pose significant health risks under normal conditions of exposure.

MATERIALS AND METHODS

Drugs/Chemicals

Dulbecco's Minimum Essential Medium (DMEM) (1X), antibiotic-antimycotic solution (100X) and 0.25 per cent Trypsin-EDTA (1X) were procured from Gibco, Life technologies Corporation, USA. Foetal Bovine Serum, Heat inactivated was procured from Himedia Laboratories Pvt Ltd., Mumbai. 3-(4, 5- dimethyl thiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) dye and Dimethyl sulfoxide (DMSO) were purchased from Sisco Research Laboratories (SRL), Maharashtra. Specimen tube was purchased from Tarson® and Acridine orange and Ethidium bromide (AO/EB) dye were purchased from Sigma Aldrich®.

Cell line and culture conditions

Adherent Hepatocarcinoma cell line HepG2 procured from National Centre for Cell Sciences, Pune was used for in vitro studies. Cells were adapted to grow in DMEM media supplemented with 10 % foetal bovine serum and 1% Gentamicin (50 mg/mL). The cells were maintained in

a humidified incubator at 37ºC with 5% carbon dioxide (CO2). Cell lines were sub cultured by enzymatic digestion with 0.25 per cent trypsin/1mM EDTA solution when they reached approximately 70 to 80 % confluency and the collected cells were used for the studies.

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

The cytotoxic potential of chlorantraniliprole was examined *invitro* using MTT reduction assay (Benny *et al*., 2022). Stock solution of the compound was prepared in serum free media. The stock was diluted to 18.5, 9.25, 4.62, 2.3, 1.15, 0.57, 0.28, 0.14 µg/mL using complete medium. 96 well plates were seeded with $1x10^4$ cells/mL and was allowed to attach and proliferate for 24 h. Then the extract at the desired concentrations was added to the cells, again incubated for 24 h. Then 10 µL of MTT (5 mg/mL prepared in DPBS) was added to all wells including blanks. The plates were covered with aluminium foil and incubated at 37°C for 4 h, in CO_2 incubator. After incubation, the media containing MTT was removed. Added 200 µL of DMSO to all the wells to dissolve formazan crystals formed. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 570 nm.

The per cent cell viability and per cent cell inhibition were calculated using the following formulae:

Per cent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100

Per cent cell inhibition = 100 - per cent cell viability

The net absorbance from the control wells was taken as 100 per cent viable. The IC_{50} values of extracts were calculated by plotting the concentration against per cent cell inhibition using Graph pad prism v.5.0.

***In vivo* method studies**

Commercially available chlorantraniliprole was purchased from local market. The stock solution was prepared by dissolving 2.0 µL of commercial chlorantraniliprole in 100 mL of salt water with resulting concentration of 1mg/mL . From this stock solution 500,100, 50, 25,10 and 5µg/mL were prepared by serial dilution using salt water.

The experiment was conducted under uniform condition as the stock culture was kept in the lab. Small quantities of dry cysts of Artemia were sprinkled into the dark, larger chamber. Yeast solution 0.06% was added to the hatching chamber for every litre of salt water to feed the

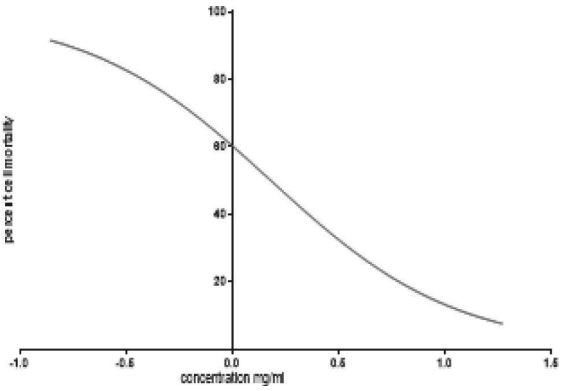
larvae after 24 hours, The experimental group comprised four replicates, with each replicate containing 10 healthy Brine Shrimp from established stocks. A total of 10 Brine Shrimps were exposed to each concentration of chlorantraniliprole, for 24 hours. After the treatment, the number of live, mobile and deceased shrimp were recorded (Harwig 1971; Wu, 2014).

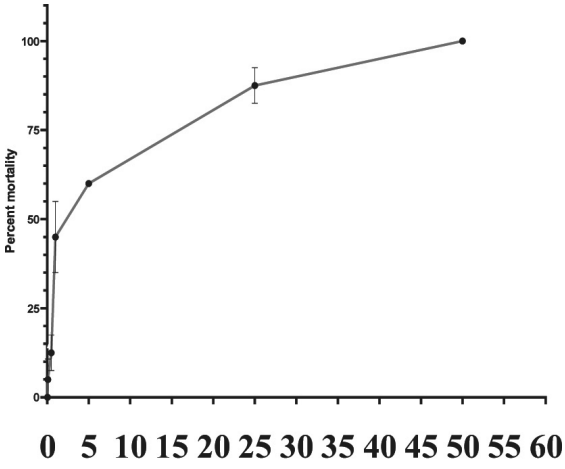
RESULTS AND DISSCUSSION

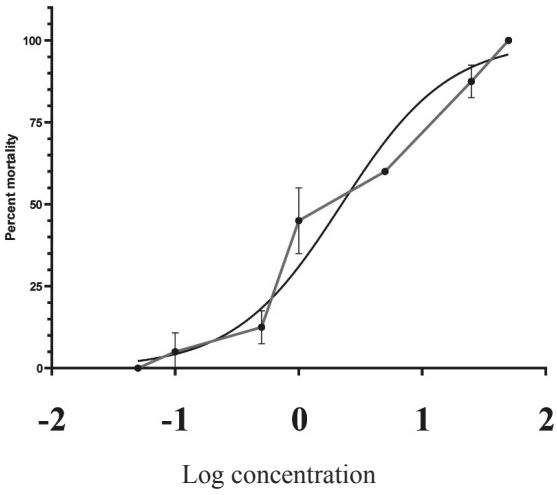
In vitro – There was no complete inhibition of proliferation in any of the concentrations used. Hence a simple linear regression analysis was conducted to find out the possible point at which the cells will be inhibited. The linear regression of the concentration versus cell viability yielded a linear regression equation of mortality = 3.98 + 0.72 with a correlation coefficient of 0.99. From this equation, the IC_{50} was calculated and found to be 1.517mg/mL in the HepG2 cells.

In vivo -

No mortality of the brine shrimp was observed in the concentration of 0.05 mg/ml and 100% mortality in the concentration of 50 mg/mL. Based on the percentage of mortality, the concentration that lead 50% lethality (LC_{50}) to the shrimp was determined and was found to be 2.228 mg/mL (95% confidence interval range 1.714 to 2.918 mg/mL). The LC50 was calculated using non-linear regression using log dose using GraphPad prism.







From both the analyses, it could be concluded that the pesticide at lower concentrations is proliferative in nature and does not cause serious cell damage.

Coragen is a branded product of chlorantraniliprole at 18.5 % SC and is used as a plant protection agent against various pests of Lepidoptera species. They belong to the anthranilic diamide class of insecticides that bind to the ryanodine receptors of the insects and cause a reduction in the calcium related neurotransmission and muscle contraction. Since the affinity of chlorantraniliprole to insect ryanodine receptors is very high, at the recommended concentrations, they are considered safe to mammals and other aquatic species. However, inadvertent use of these can cause a significant increase in the environmental levels of these pesticides and may be toxic to mammals in such higher doses. Various studies have reported the ill effects of this pesticide in mammals which included weight loss, blood disorders, liver, lung and spleen abnormalities (Dutta et al. 2014; Abdel-Mobdy et al. 2017) and elevated uric acid, abnormal hormone levels, anaemia, renal failure. It induced testicular cell apoptosis, with increased expression of proapoptotic proteins and decrease in antiapoptotic proteins with substantial testicular DNA damage (Mahmoud *et al.,* 2025).

Various studies reported the persistence of different classes of diamide insecticides in soil and agriculture products from one to four weeks (Chawla *et al*., 2011; Kale *et al*., 2012; Das *et al*., 2014). Chronic exposure of this pesticide produced apoptosis in larval imaginal discs of *Drosophila melanogaster*. Studies in aquatic animals showed that all periods of fish life i.e., egg, larva, juvenile and adults were susceptible to flubendiamide.

Brine shrimp lethality bioassay is a simple, high throughput cytotoxicity test of bioactive chemicals. The results of the study indicated that chlorantraniliprole produced a dose dependent toxicity for the brine shrimp. It was lethal within 24 hours post exposure and hence ecotoxic, especially to marine animals (Gajardo and Beardmore, 2012). Pesticides like diazinon, atrazine and endosulphan produced proliferation in a biphasic manner in treated colon cells (Greenman *et al.,* 1997). The exact mechanism by which the pesticide caused an increase in proliferation needs to be studied and the exact concentration where it causes cytotoxicity also need to be explored in detail.

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