JSIANE : Vol. 4, Issue 1, 2024, Page 13-16 ISSN (Online) 2583-2093

Journal of Science Innovations and Nature of Earth

Journal homepage : www.jsiane.com

TOXICITY OF ANTRACOL, IN SNAKE HEADED FISH, Channa punctatus (BLOCH.): AN ASSESSMENT BASED ON LDH ISOZYME GEL ELECTROPHORESIS

Rashmi Sharma[1] and Harendra Nath Sharma[2]

[1]Department of Zoology, Government Degree College, Jewar, G.B. Nagar, Uttar Pradesh, India
(Constituent College of CCS University, Meerut)

[2]Department of Zoology, S.V. College, Aligarh

Corresponding Author E-mail: dr.rashmisharma1976@gmail.com

www.doi.org/10.59436/https://jsiane.com/archives4/1/117

Abstract

The growth of industry is an important feature of civilization. Agriculture is also an important sector of the economy of any nation. It is true that the economic development in modern times has come to be associated with industrialization can follow only on the sound heels of agriculture. Indian agriculture is the backbone of Indian economy. In comparison with the other countries as regards the share of agriculture in national income we find that India contributes much more, as a vast populated country. The current strategy for agricultural development is mainly bent towards intensifying cultivation in selected areas through the spread of high yielding varieties and multi-cropping programmes which aim at producing two or more crops of short duration as against one crop under the traditional varieties. For this purpose, so many devices are used to increase in per capita income and production; such devices are in the form of fertilizers, pesticides and different mechanical tools also. Electrophoretically estimation of LDH isoformes also showed varying band pattern with respect to control, after function of time followed by antracol intoxication.

Keywords : *Channa punctatus*, LDH Isozyme, Electrophoresis, agricultural

Received 03.01.2024 **Revised 23.01.2024** **Accepted 23.03.2024**

Introduction

In this modern era, there is a competition among the nations for development in every field. For a nation to progress socially, economically as well as politically, development processes in different field are very necessary. Development leads into industrialization. Organometallic fungicide, antracol 70WP (propineb) comes under the Zn containing dithiocarbamate group of compounds. It is mostly to treatment of general crops influenced by fungal diseases. The effects of antracol on some biochemical parameters of blood enzymes and histology of liver and kidney of Channa punctatus has been studied. Among aquatic organisms, fish is a significant source of protein rich food for people and get more affected if there is any change in ecosystem due to environmental pollution. Chemicals are used extensively in form of different categories of pesticides in agriculture to improve crop yield. Such chemicals even fungicides effect on individual species, change in species abundance and production are predictable in communities exposed to chemical stress. Fishes are found to be extremely sensitive to any environmental alterations. Fungicides have been found as underground and aquatic pollutants and their bioaccumulation may cause damage to aquatic animals. Antracol, Zn containing dithiocarbamate has tendency of bioaccumulation due to the presence of heavy metal as zinc. The effect of organometallic compounds on aquatic organisms is currently attracting wide spread attention, particularly in studies related to biochemical observations and envisage the toxic effects.

The Antracol, an organometallic fungicide is introduced by Bayer (India) limited as a contact fungicide. Today the area under potato crops is steadily increasing around the world. Also on the increase are the demands made of the farmer in a fiercely competitive market. Fungal diseases constitute the main threat to the harvest. Late blight of potato (Phytophthora infestans), the most important fungal disease, spreads very quickly and can cause an untreated canopy to collapse within a few days if the weather is warm. It is widely used by peasants in treatment of potato late blight fungus in rural areas around Agra (U.P.). Antracol has remained popular due to its special mode of action, because it affects several sites in the metabolism of fungal cells. Active constituent in Antracol is propineb that belongs to the dithiocarbamates class of compounds. It is found in wettable powered form. Like organophosphates, the carbamates also interfere in cholinergic transmission. The carbamate enters the synapse and inhibits the acetylcholine-esterase as a result the actylcholine contains to depolarize the post synaptic membrane, causing prolonged stimulation resulting into the failure of the nerves or effector tissue. This experiment shows distinctive band pattern of changes in enzyme's biochemical values. Illiteracy and not proper knowledge about the use of these chemical compounds results a disturbed ecosystem. Fungicides and other undesirable chemicals constitute a sufficiently powerful cause of ecological disturbance. The whole ecosystem will be affected when any of the components of the ecosystem is affected by aquatic pollution such as fishes. Fish is a significant source of food production, and can definitely boost our production

of protein rich food compaign. India, presently is the world's seventh largest nation, with fish catches, both from coastal waters and from various inland water i.e. rivers, lakes reservoirs and irrigation ponds. Fish farming also adds substantially to our national income. Communities and ecosystems are hierarchical systems with emergent properties, stability, diversity, and the like that in the part reflect the integral effect of interactions among system components. A key factor in aquatic pollution is the adequate supply of such chemicals.

Material and Methods

Maintenance of Fish in Laboratory: *Channa punctatus*, an air breathing snake headed murrel, ranging from 12cm to 15 cm in length and 35 gm to 45 gm in weight were collected from local fresh water ponds in Aligarh and transported to laboratory during the month of September, when room temperature ranged from 300C to 350C and water temperature from 250C to 280C. The experiments were generally carried out in aquarium tanks containing 25 litre tap water and each aquaria measuring 75cmX37.5cmX37.5cm. These fishes were acclimatized to laboratory conditions and fed on small pieces of liver and boiled egg yolk according to feeding schedule. Tap water free from chlorine, underground water was used as the diluting water. It was stored in a large aquarium for dechlorination and changed every two days or week if it give foul smell.

Properties of Experimental Compound: Propineb 70WP, Zinc containing dithiocarbamate has been selected for present study.

Trade name: Antracol, Bayer 46131

Other synonyms: Methylzineb, Mezineb, Airone, Taifen and Zipromat

Chemical name: Zinc, (N, N' – Propylene-1, 2-bis (dithiocarbamate)

Action: Fungicide , Form: Wettable powder , Colour: White

Odour: Weak characteristic odour, Melting point: 150-1600C , pH value: 6.0 – 8.0

Molecular weight: 279.774 amu

Molecular formula: (C5 – H8N2 – S4 – Zn).

Field Application and Formulation: Propineb 70WP (Antracol), a contact fungicide is used as a foliar spray for the control of fungal diseases in different crops. It shows solid formulation i.e. a wettable powder and based on propineb technical containing 70% w/w. Propineb as active ingredient and balance auxillaries as inert materials.

Determination of LC_{50}: Evaluation of a chemical's hazard to aquatic life is generally done using LC50 test in which the value reported is the concentration in water lethal to 50% of the test population in a given time, usually 96hrs. To determine the mean lethal concentration, well acclimatized active Channa punctatus were chosen and divided into four groups (A, B, C & D). Each group consisting of four individuals and kept into the standard solution of compound Antracol 70WP that was prepared by different doses, 2000mg, 4000mg, 6000mg and 8000mg, in different aquarium tanks. The mortality of survival number of test fishes of each group was recorded after 96 hrs. The LC_{50} was assessed by plotting the experimental results on graph paper, taking the logarithms of the concentration on abscissa and the mortality percentage on the ordinate. Actually the probit values are plotted in place of the mortality percentages. The data were analysed statistically by log dose/probit regression line method (Finney, 1971). Regression line was drawn on the basis of two variables log dose and empirical probit on a simple graph paper and used to determine the expected probit necessary for LC50 determination.

Polyacrylamide Gel Electrophoresis (PAGE) (Siciliano *et al*., 1976)

In this study two types of PAGE were used:

1. Disc electrophoresis
2. Slab electrophoresis

Each polyacryl gel consisted two sections in discontinuous PAGE

(i)Separating or small pore gel which separates the various protein components on the basis of their molecular weight and charges.

(ii)Stacking or large pore gel for concentrating and removing the large particles. In this study, mainly discontinuous PAGE was used. The method consists of two parts:

(A)Preparation of PAGE

i. The optically plain glass plates (a rectangular and other notched) were washed properly with detergent, DW and dried finally with ethyl alcohol slab.

ii. Three plasting spacers of same thickness (1.5mm) were placed along two sides and along the bottom side leaving two mm space on other sides. The assembled plates were tightened with clamps. The notched side plate was kept up and the thickness of spacer determined the thickness of gel.

iii. The outer sides of three spacers was sealed with 2% fine agar.

iv. The assembled pair of glass plates, with agar seal was made to stand vertically on plain horizontal surface table keeping notched end of the plate up.

v. For 1.5mm spacer, 35ml of separating gel solution was required 7.5% PAGE solution was prepared in 100ml conical flask by mixing the following solutions:

30% Acrylamide bisacrylamide solution	8.76ml
Discontinuous buffer pH 8.8	4.40ml
DW	20.10ml
TEMED	0.04ml

These were mixed and then ammonium per sulphate 1.7ml was added and solution was mixed well. The gel was poured immediately into the groove of paired plates. A small volume of water saturated batanol was overlaid on the gel solution to a height of about 0.5 to 1cm. This prevented the formation of air gel minimize and ensured a flat gel surface. The gel solution was allowed to polymerize for about 30 minutes.

The large pore gel was prepared as follows:

30% Acrylamide and 0.8% Bisacrylamide solution	1.6ml
Stacking buffer, pH 7.4	2.50ml
DW	5.32ml
TEMED	0.01ml

All solutions were mixed in a 50ml conical flask. Solidification of the gel was ensured by visible interface between polymerized gel and water. The water saturated butanol was removed from the polyacrylamide gel.

Ammonium persulphage 0.5ml was added and was mixed with the stacking gel solution. The solution was poured into the assembled plates and the comb was plated immediately in such a way that end of the tooth remaining just 2mm above the surface of separating gel. The gel as allowed to solidify for 15 minutes.

Comb was removed after polymerization of stacking gel without cracking the walls of wells. The wells were washed with electrophoresis running buffer to remove the

unpolymerized solution. The well was flooded with the buffer.

Electrophoresis Method (1) Apparatus was washed with tap water and later with DW and air dried. The stock running buffer was diluted 8 times with DW and chilled. The buffer was poured into the lower chamber and after removing the lower horizontal spacer, the assembled plates with gel were mounted. Care was taken to ensure that there were no air bubbles in the bottom of the plate's sandwich. The mounted plates were clamped to the apparatus and upper buffer chamber of the apparatus was filled with buffer.

(2)The wells were washed by flushing with the buffer to remove unpolymerized solution and air bubbles.

(3)The low voltage current (10mA/50volts) was applied for 30 minutes to remove the ammonium persulphage and unpolymerized acrylamide etc. which may effect the protein separation.

(4)Loading samples were prepared by adding 200µl of loading dye into 2ml clear protein solution (5mg/ml) and then mixed.

(5)By using the micropipette the dye mixed samples were loaded to each well. This was done by keeping the tip of pipette just above the bottom of the well and slowly ejecting the required amount of samples without giving jerk to micropipette.

(6)The current (25mA, 110volts) was applied to the gel for separation. Electrophoresis was first allowed to run at low current/voltage (10mA/50volts) until the dye mark line got compacted at the top of the separating gel layer. Then the current was increased. Electrophoresis was performed below 100C.

(7)The current was stopped when marking dye approached 1cm above the bottom surface of the gel. The gel plates were removed from the apparatus.

(8)The slab gel was immediately removed by gently separating the glass plates apart with the help of spatula.

(9)The gels were used immediately for further procedures like staining and elucidation of required enzyme/protein bands.

Staining procedure for LDH activity

(1)After electrophoresis gel was taken out into a staining tray and covered with 25ml nitro blue tetrazolium solution (2.45mM). Then the gel was incubated at RT in dark for 20 minutes.

(2)The NBT solution was removed and 25ml of coomassie brilliant blue solution was added. The gel was again incubated in dark for 10 minutes.

(3)The coomassie solution was removed from the gel tray and gel was illuminated in alluminium foil covered box with 100 watt bulb till the gel become blue coloured.

(4)LDH isozymes activity bands appeared in the blue coloured gel.

(5)Then the gels were stored in the 7% acetic acid in polyethene bags.

Reagents

1. A suitable staining mixture for lactate dehydrogenase enzyme contains:

(A)L-lactate

It was used directly as substrate and kept at 40C

(B)PMS (Phenazine methosulphate) solution

0.0030gm of phenazine methosulphate was dissolved in 50ml of distilled water and was stored in a dark coloured container at 40C; used as intermediate.

(C)NBT (Nitro blue tetrazolium) solution

NBT (0.0025gm) was dissolved in 25ml of distilled water and was stored in the dark coloured container at 40C. The solution was used within 1week. Fresh solution was prepared and used as hydrogen acceptor.

(D)NAD solution

0.0028gm of NAD was dissolved in 5ml distilled water and the solution was prepared in a dark coloured container with special care during handling as it is very hydroscopic. Fresh solution was prepared before use.

2. The following reagents and buffers were used for PAGE

(A)Acrylamide and Bis-Acrylamide (30 : 0.8) solution

The acrylamide (30%) and bis-acrylamide (0.8%) was prepared by dissolving 30gm of acerylamide (Sigma/SRL) and 0.8gm of bis-acrylamide (Sigma/SRL) in 70ml of distilled water and then the volume was made upto 100ml, insoluble particles were removed was stored at 40C in a amber coloured bottle. Automatic pipette and hazard gloves were used while handling unpolymerized acrylamide because it is carcinogenic in nature.

(B)Ammonium per sulphate solution (1.5%)

0.75gm of ammonium persulphate in 50ml of distilled water and was dispensed in oliquotes of 0.5ml into 1.5ml plastic storage vials. The solution was stored at -200C until use but it was used within one month.

(C)TEMED

It was used directly and kept in an amber coloured bottle at 40C.

(D)Stacking gel buffer (Tris-HCl pH 7.4)

It was prepared by dissolving 8.0gm of Tris in 48ml of 1N-HCl and pH was adjusted to 7.4 with 1N-HCl. The final volume was made to 100ml with distilled water. The solution was stored in amber coloured bottle at 40C.

(E)Electrode buffer (Tris glycine buffer, pH 8.3)

Tris and glycine were dissolved in 800ml of distilled water and pH was adjusted to 8.3. Then the volume was made upto 1000ml with distilled water. It was stored at 40C and used as stock buffer. For one time use, it was diluted to 8 times with DW and was used within a fortnight.

(F)Loading dye

1gm Bromophenol blue was dissolved in 50ml sterile distilled water and 50ml of sterile glycerol was added. The solution was prepared in a sterile container and stored at 40C. This was used within 4-6 months.

(G) Coomassie Brillient Blue G-250

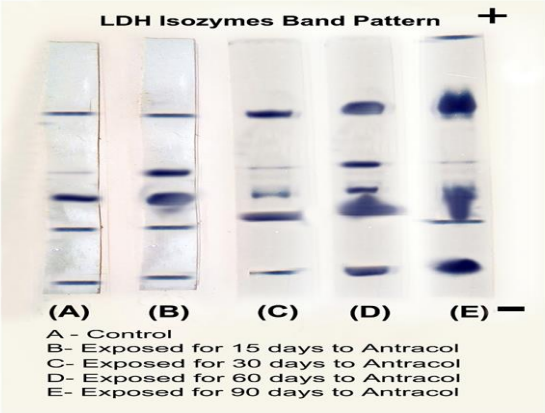
This staining solution was prepared by dissolving 0.24gm of coomassie brilliant blue in 600ml of 3.5% perchloric acid ($HClO_4$) solution. This solution was stirred to dissolve the dye particles and filtered to remove the undissolved dye particles. This was directly used in protein staining in PAGE.

Results and Discussion

Lactate dehydrogenase exist in four distinct enzyme classes. Two of them are cytochrome C dependent enzymes and the other two are NAD (P)- dependent enzymes. Functional lactate dehydrogenases are homo or hetero tetramers composed of Mand H protein subunits encoded by the LDH A and LDH B genes respectively. It has five isozymes-

LDH-1 (4H) -in heart and RBCs
LDH-2 (3H1M) - in the reticuloendothelial system
LDH-3 (2H2M) - in the lungs
LDH-4 (1H3M) -in the kidneys, placenta and pancreas
LDH-5 (4M) -in the liver and striated muscle

The isozymes patterns for LDH in control group showed normal pattern (A)



The band pattern after intoxication of antracol for 15 days (B) showed elevation in intensity of third, fourth and fifth band in comparison to control but first and second band showed normal band pattern as in control. In 30 days (C) band pattern show much elevation in intensity of third and fourth band than second and first but fifth band is also elevated. The same result was also showed in 60 days (D) band pattern as in 30 days. In 90 days (E) the band pattern much elevated but there was also present an another band above the first band. So in 90 days the isozyme patterns for LDH showed elevation in both number and intensity of bands. In present findings, it has been revealed that dithiocarbamates have deleterious effects on aquatic non-target organisms such as fishes. The similar findings have been reported by Sastry and Siddique (2004) and Singh and Bhati (1994) that the persistence and ubiquitors nature of organo chlorines, organo-phosphates and carbamates, coupled with their tendency to concentrate the non-target organism through the food chain, ultimately produce toxicity to fishes, birds, wildlife and in true to man; while effects of organo metallic fungicide propineb and manab in liver of pregnant rats and their litters have been reported by Roy Choudhury and Vachhajani (1987); Sastry and Smitha (1989) and Sulodia and Singh (2004). Present study shows the difference in band pattern in treated fishes with increase exposure time 15days to 90days after intoxication with antracol and with respect to control. Difference in lactate dehydrogenase band pattern has been reported by Simin *et al*. (1983) in gold fish Carassius aruatus after the effects of freezing and thawing. The muscle type of lactate dehydrogenase isoenzyme (lactate dehydrogenase-5) has approximately a 35-fold stronger affinity than the heart type of lactate dehydrogenase (lactate dehydrogenase-1) has been reported by some authors (Verma *et al*., 2004).

In present study there is lactate dehydrogenase-1 level higher than the lactate dehydrogenase-2 level suggests myocardial infarction (damage to heart tissues releases heart lactate dehydrogenase which is rich in lactate dehydrogenase-1 into blood stream). There is also seen elevation in lactate dehydrogenase-3, lactate dehydrogenase-4, lactate dehydrogenase-5 with respect to exposing time period ranged from 15days to 90days to antracol. These elevated levels in lactate dehydrogenase-3, lactate dehydrogenase-4, lactate dehydrogenase-5 band may be due to severe damage in lungs, kidneys and liver tissue respectively (Elustondo *et al*., 2013; Heinová *et al*., 2017

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Cite this article:
Rashmi Sharma and Harendra Nath Sharma, 2024, “Toxicity of Antracol, In Snake Headed Fish, *Channa punctatus* (Bloch.): An Assessment Based on LDH Isozyme Gel Electrophoresis” *Journal of Science Innovations and Nature of Earth*, Vol. 4(1), page-13-16
www.doi.org/10.59436/https://jsiane.com/archives4/1/117