Research Paper

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Comparative Studies of Protein Profile of Cleavage Stage of Lymnaea species after using Antitubulin Drug

Zoology

KEYWORDS : species, SDS-PAGE, Cleavage stage, Paclitaxel, Colchicine, Toxicity

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ABSTRACT

Lymnaea species are the pest of aquatic plants and aquatic vegetations. It is found in fresh water pond and lakes. These pestiferous snails are also prolific breeders and acts as vector or intermediate host of trematode parasites. So it is very essential to control the fertility, hatchability, viability by increasing the rate of mortality and declining the rate of longevity. So the present investigation was done to know more about the intoxication of alkaloid on development of snail pest. In this study paclitaxel and colchicine was tested for lethal toxicity of cleavage stage of Lymnaea species and values of lethal toxicity was summarized in table 1 and 2. Detection of negatively charged protein fraction in cleavage stage of control and treated snails by SDS-PAGE of Lymnaea species was assessed in the present investigation.

INTRODUCTION:

Lymnaeids are distributed worldwide as observed (Godan 1983). They can be easily procured from any fresh water body. biochemical studies on the cleavage stage and gastrula stage of freshwater snail Lymnaea stagnalis and Lymnaea acuminata treated with alkaloid is very scanty and some research work has been done in some gastropods e.g. in Lymnaea acuminata (Agrawal 1996), in Pacific oyster Crassostrea gigas (Li-Qi et al., 1998), in some snails (Goel 1984), in Pila globasa after thiourea and DDT treatment (Bhide 1987), in Lymnaea stagnalis after thiourea treatment, (Gupta and Bhide 2001) in Lymnaea stagnalis after nuvan treatment and (Bhide et al., 2004) in Gyraulus convexiusculus after treatment of some pesticides and (Mahobiya et al., 2012) in Lymnaea stagnalis after treatment with colchicine but no literature is available on the toxicity of alkaloids on gastrula of Lymnaea stagnalis and Lymnaea acuminata.

Detection of negatively charged protein fractions by Gel electrophoresis is the

integrated part of the present investigation. In control groups the successive development stages showed the gradual increase in the protein fractions indicated the progressive development of corresponding snails (Goel 1984) but due to the intoxication of the treated alkaloid most of the developing stages showed the gradual decline not only in the number of protein fractions but also showed gradual decline in the intensities of some protein fractions as reported (Gupta and Bhide 2002) in Lymnaea stagnalis after treatment with nuvan and baygon, (Mahobiya and Bhide 2013) in cleavage stage of Lymnaea stagnalis after treatment with paclitaxel and colchicine and (Mahobiya at el., 2014) in gastrula stage of Lymnaea stagnalis after treatment with paclitaxel and colchicine. Kinetic profiles of vitellin degradation protease activity and free amino acids in the embryo and larvae of the pacific oyster, Crassastrea gigas have been investigated (Li Qi et al., 1998) during the period from the unfertilized egg through the 48 hrs straight hinge larva.

MATERIALS AND METHODS:

Selection of Pestiferous Snails:

Common fresh water pond snails of lymnaea stagnalis and Lymnaea acuminata belonging to family Lymnaeidae were selected for the present investigation.

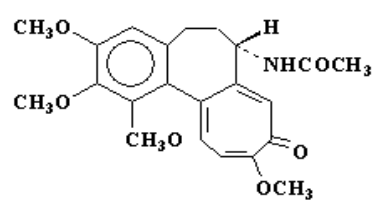
Procurement and Rearing of Snail:

Sexually mature Lymnaea stagnails and Lymnaea acuminata were collected from Botanical garden of Dr. H.S. Gour University, Sagar and Sagar Lake by net. They were reared in troughs and fed regularly with aquatic vegetation e.g. Hydrilla to avoid the stress of starvation. The collected snails were acclimatized for 7 days under laboratory conditions (Subbarao, 1989).The young ones hatched from egg masses of Lymnaea stagnalis and Lymnaea acuminata were used for the experimental purpose. The young ones snails were introduced to different concentration of antitubuline drug through media. Each group was in triplicate of 50 snails.

Antitubulin Drug:

Colchicine is a toxic natural product and secondary metabolite and its pain-relieving and anti-inflammatory effects for gout were linked to its ability to bind with tubulin. Colchicine is a toxic natural product and secondary metabolite, originally extracted from plants of the genus Colchicum (Autumn crocus, Colchicum autumnale, also known as "meadow saffron"). Colchicine, was later identified as a tricyclic alkaloid, and its pain relieving and anti-inflammatory effects for gout were linked to its ability to bind with tubulin.

It was used originally to treat rheumatic complaints, especially gout, and still finds use for these purposes today despite dosing issues concerning its toxicity. Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis and therefore colchicine effectively functions as a "mitotic poison" or spindle poison.



General Description of Colchicine:

Chemical name - Colchicine

IUPAC name-N-[(7S) -1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo [a] heptalen-7-yl]acetamide.

Molecular formula - $C_{22}H_{25}NO_6$

Molecular weight - 399.44

Melting point - 150 -160° C

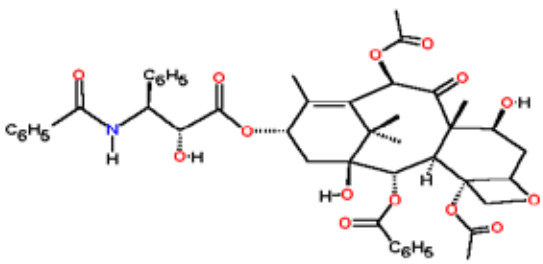
Solubility - H_2O 10 mg/ml

Physical appearance –Yellow

Paclitaxel is a mitotic inhibitor used in cancer chemotherapy. Paclitaxel is used to treat patients with lung, ovarian, breast cancer, head and neck cancer, and advanced forms of Kaposi's

sarcoma. Paclitaxel is also used for the prevention of restenosis. Paclitaxel stabilizes microtubules and as a result, interferes with the normal breakdown of microtubules during cell division.

Paclitaxel alters the normal equilibrium between tubulin dimers and microtubules, and therefore, disrupts cell division, i.e., stabilization of the microtubules interferes with the G2 and M phases of the cell cycle and those cellular activities involving microtubules. The paclitaxel-stabilized microtubules are resistant to depolymerization upon exposure to calcium ions and cold temperatures and do not require the presence of GTP, a natural cofactor necessary for polymerization initiation. Unlike other spindle poisons, which prevent polymerization of the monomer, paclitaxel has a binding site on the microtubule (Muhlradt and Sasse 1997).



General Description of Paclitaxel:
Chemical name - Paclitaxel

IUPAC name- (2α,4α,5β,7β,10β,13α)-4,10-bis(acetyloxy)-13-{[(2R,3S)- 3-

(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate.

Molecular formula - C47H51NO14
Molecular weight - 853.91
Melting point - 198oC - 203oC
Solubility - Insoluble in water, Soluble in most organic solvents.

Physical Appearance - Colourless to slightly yellow viscous solution or (in pure form) an off-white crystalline powder.

Experiments with Different Dosages of Antitubulin Drugs:
Fresh egg masses with subsequent developmental stages of Lymnaea acuminata and Lymnaea acuminata of F_0 generation were introduced via media to different concentrations of antitubulin drugs and the data was collected in triplicate and calculated the values of LC100, LC50, LC0 and sub-lethal concentration were detected out for each group separately and data was summarized in table 1and 2 (Probit analysis method adopted after Finney, 1971). Each egg masses contain about 30 egg capsules.

Table 1: Data on Toxicity of Colchicine and Paclitaxel on Cleavage Stage of Lymnaea stagnalis

S. No.	Name of the antitubulin drug	Concentration of the antitubulin drug	Duration (hrs.)	Mortality (%)	Lethal conc. Value
1. 2. 3. 4.	Colchicine	0.12 % 0.06 % 0.03 % 0.02 %	72 72 72 72	100% 50% Nil Nil	LC_{100} LC_{50} LC_0 Sublethal concentration
1. 2. 3. 4.	Paclitaxel	0.07% 0.03% 0.02% 0.01%	72 72 72 72	100% 50% Nil Nil	LC_{100} LC_{50} LC_0 Sublethal concentration

Result: 0.02% and 0.01% concentration of colchicines and Paclixaxel was considered as sublethal concentration value.

Table 2: Data on Toxicity of Colcincine and Paclitaxel on Cleavage Stage of Lymnaea acuminata

S. No.	Name of the antitubulin drug	Concentration of the antitubulin drug	Duration (hrs.)	Mortality (%)	Lethal conc. value
1. 2. 3. 4.	Colchicine	0.13 % 0.04 % 0.03 % 0.02 %	72 72 72 72	100% 50% Nil Nil	LC_{100} LC_{50} LC_0 Sublethal concentration
1. 2. 3. 4.	Paclitaxel	0.09 % 0.06 % 0.02 % 0.005 %	72 72 72 72	100% 50% Nil Nil	LC_{100} LC_{50} LC_0 Sublethal concentration

Result: 0.02% and 0.005% concentration of colchicines and Paclitaxel was considered as sublethal concentration value.

Sample Preparation and Detection of protein:
For protein quantification cleavage stage and gastrula stage were collected from snails, separated and individually placed in individual Eppendorfs that were stored at -20˚C. 80-100 µl of distilled water was added to the Eppendorfs containing egg masses of cleavage and gastrula stage of developmental stages. These materials were homogenized in Bloor's mixture and the vials containing the gastrula stage of snails were centrifuged at 10,000 rpm for 10 min at 5˚C. Aliquots of the supernatants of the centrifuged extracts were used for protein content. In order to investigate the proteins from homogenized cleavage stage and gastrula stage of Lymnaea stagnalis and Lymnaea acuminata 7 % SDS-PAGE was performed. In this 50 µl of pure stages (approx. 200 mg of protein) derived from control and treated snails were used. 50 µl of sample was added to 50 µl of sample buffer (Tris buffer pH 6.8 1.66 ml, Glycerol 2 ml, 10 % SDS 4 ml, β-mercaptoethanol 200 µl, Bromophenol blue 0.02 gm, distilled water 2.14 ml). Samples containing 20 – 40 µl of proteins diluted 1: 1 in the buffer, were boiled in a water bath for 5 min, and after they had been cooled on ice, they were applied onto the polyarcylamide gel. The molecular mobility of proteins was determined by interpolation from mobility of commercial prestained standards (GeNei PMWM) by computer analysis and their profiles were analyzed. SDS-PAGE was carried out by the method adapted after Lamemli (1970). The gastrula stage was processed for the extraction of protein samples by the method adapted after Jairaman (1986).

RESULT:
Proteins play a very important role for overall growth, development and reproduction of the animals. The depletion, destruction and degeneration of protein metabolites in the various stage of experimental groups of Lymnaea spp., correlated with the depletion of –vely charged protein fractions were detected by SDS-PAGE and an important aspect of the present investigation.

The electrophoresis was carried out in a polyacrylamide gel, under denaturing conditions (SDS-PAGE). After loading samples of control and treated cleavage stages of Lymnaea spp. were used to SDS-PAGE. Samples of various stages of developmental stage were loaded on a prepared SDS-PAGE gel (7 % separating gel, 4 % stacking gel) in different lanes. The number and intensity of protein fractions were detected out in various developmental stages of Lymnaea spp. and the observations and results are summarized in Fig. No.1 and 2.

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Fig. 1: Showed the –vely charged protein fractions were increased in number and intensity in cleavage stage of control groups of Lymnaea stagnalis while depletion in number and intensity of protein fractions was observed in cleavage stage treated experimental groups due to intoxication of colchicine and paclitaxel.

The molecular mass of the cleavage stage of control Lymnaea stagnalis ranged from 2.8 to 31.2 kDa, while cleavage stage of Lymnaea stagnalis treated with colchicine ranged from 4.7 to 18.2 kDa and cleavage stage of Lymnaea stagnalis treated with paclitaxel ranged from 6.1 to 16.8 kDa as exhibited in Fig. 1.

Eight bands in lane 1 of Fig. 4 were observed in the cleavage stage of control group. The bands were of 2.8, 4.7, 6.1, 12.2, 16.2, 18.2, 26.2 and 31.2 kDa molecular weight. One band of 16.2 kDa was found of very high intensity. Two bands of 6.1 and 12.2 kDa were observed of high intensity. Two bands of 4.7 and 18.2 kDa were observed of low intensity. Three bands of 2.8, 26.2 and 31.2 kDa were observed of very low intensity. Five bands in lane 2 of Fig. 1 were observed in colchicine treated cleavage stage of Lymnaea stagnalis. The bands were of 4.7, 6.1, 12.2, 16.2 and 18.2 kDa. One band of 16.8 kDa was observed of very high intensity. Two bands 6.1 and 12.2 kDa were observed and of high intensity. Two bands of 4.7 and 18.2 kDa were observed of very low intensity. Three bands in lane 3 Fig. 1 were observed in paclitaxel treated cleavage stage of Lymnaea stagnalis. The bands were of 6.1, 12.2 and 16.2 kDa. One band 16.8 kDa was observed and of very high intensity. Two bands of 6.1 and 12.2 kDa were observed of high intensity.

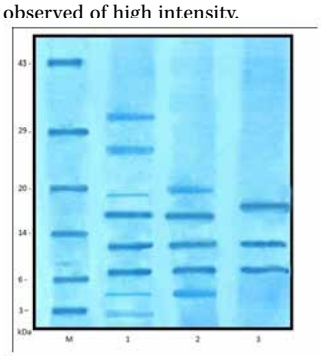

Fig. No. 1: Showing Samples of Cleavage Stage of Lymnaea stagnalis after Treatment with Colchicine and Paclitaxel. 1, 2 and 3 were loaded on a Prepared SDS-PAGE Gel (7 % separating gel, 4 % stacking gel).

Lane 1: Cleavage stage of control Lymnaea stagnalis.
Lane 2: Cleavage stage of Lymnaea stagnalis treated with Colchicine.
Lane 3: Cleavage stage of Lymnaea stagnalis treated with Paclitaxel.
The gel was stained with Coomassie Brilliant blue R-250.

Fig. 2: Showed the –vely charged protein fractions were increased in number and intensity in cleavage stage of control groups of Lymnaea acuminata while depletion in number and intensity of protein fractions was observed in cleavage stage treated experimental groups due to intoxication of colchicine and paclitaxel.

The molecular mass of the cleavage stage of control Lymnaea acuminata ranged from 2.6 to 32.1 kDa, while cleavage stage of Lymnaea acuminata treated with colchicine ranged from 3.5 to 18.2 kDa and cleavage stage of Lymnaea acuminata treated with paclitaxel ranged from 12.2 to 18.2 kDa as exhibited in Fig. 2.

Eight bands in lane 1 of Fig. 2 were observed in the cleavage stage of control group. The bands were of 2.6, 3.5, 5.6, 8.6, 12.2, 15.2, 18.2, 25.6 and 32.1 kDa molecular weight. One band of 12.2 kDa was found of very high intensity. Two bands of 15.2 and 18.2 kDa were observed of high intensity. Four bands of 3.5, 8.6, 25.6 and 32.1 kDa were observed of low intensity. One band of 2.6 kDa was observed of very low intensity. Four bands in lane 2 of Fig. 2 were observed in colchicine treated cleavage stage of Lymnaea acuminata. The bands were of 3.5, 12.2, 15.2 and 18.2 kDa. Three bands of 12.2, 15.2 and 18.2 kDa were observed of high intensity. One band was of 3.5 kDa and of very low intensity. Three bands in lane 3 Fig. 2 were observed in paclitaxel treated cleavage stage of Lymnaea acuminate. The bands were of 12.2, 15.2 and 18.2 kDa. Two bands were of 12.2 and 15.2 kDa and of high intensity. One band of 18.2 kDa was observed of low intensity.

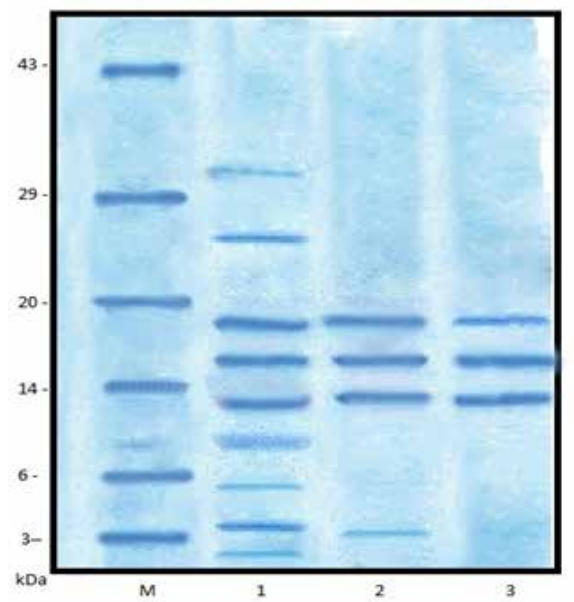

Fig. No. 2: Showing Samples of Cleavage Stage of Lymnaea acuminata after Treatment with Colchicine and Paclitaxel. 1, 2 and 3 were loaded on a Prepared SDS-PAGE Gel (7 % separating gel, 4 % stacking gel).

Lane 1: Cleavage stage of control Lymnaea acuminata.
Lane 2: Cleavage stage of Lymnaea acuminata treated with Colchicine.
Lane 3: Cleavage stage of Lymnaea acuminata treated with Paclitaxel.
The gel was stained with Coomassie Brilliant blue R-250.

DISCUSSION:

In the present investigation it was observed that in the control group the development evidently begins as soon as the eggs lay. Apparently all the egg capsules were fertilized and capable of development under favorable conditions. No sterile eggs were observed (Sarkar et al., 2007) in Lymnaea acuminata. In the gastrula micromeres spread over the macromeres. This is followed by slight invagination of the macromeres resulting in a small depression representing a rudimentary archentric cavity and blastopore as also observed in the present investigation in Lymnaea acuminata.

Gastrulation may occur in partial embryos derived from isolated blastomeres AB, CD, A, B, C or D as reported (Rattenbury and Berg 1954) in some molluscs but in the present investigation normal gastrulation has been exhibited in control and experimental groups of Lymnaea stagnalis and Lymnaea acuminata as also observed (Gupta and Bhide 2001, Gupta 2003, Bhide et al., 2004, Nema 2005, Jain 2007 and Mahobiya et al., 2012) in

the control group of some pulmonates. The biochemical studies have been based on the detection of negatively charged protein fractions by gel electrophoresis is the integrated part of the present investigation. In control the successive development stages showed the gradual increase in the protein fractions indicated the progressive development of corresponding snails (Goel, 1984) but due to intoxication of the pesticides of the gastrula stages showed the gradual decline not only in the number of protein fractions but also showed gradual decline not only in the intensities of some of the protein fractions (Gupta and Bhide 2001) in Lymnaea stagnalis after nuvan treatment, (Mahobiya and Bhide 2013) in cleavage stage of Lymnaea stagnalis after colchicine and paclitaxel treatment and (Mahobiya et al.,2014) in gastrula stage Lymnaea acuminata after colchicine and paclixaxel treatment . Detection of negatively charged protein fractions by electrophoresis is the integrated part of the present investigation. In control the successive stages of development showed the gradual increase in the protein fractions indicated the progressive development of corresponding snails (Goel, 1984) but due to intoxication of the antitubulin drugs most of the developing stages showed the gradual decline not only in the number of protein fractions but also showed gradual decline in the intensities of some protein fractions (Gupta and Bhide 2001) in Lymnaea stagnalis after Nuvan treatment, (Bhide et al., 2004) in Gyraulus convexiusculus after treatment with some pesticites, (Bhide et al., 2006) in Lymnaea Lymnaea spp., (Jain 2007) in Gyraulus spp., reported alternation in the number of protein fractions observed in larval stages. In cleavage stage of Lymneae stagnalis after colchicine treatment (Mahobiya and Bhide 2013) and (Mahobiya et al., 2014) in gastrula stage of Lymnaeae acuminata after paclitaxol treatment studied biochemical studies. (Li Qi et al., 1998) reported the kinetic profiles of vitelline degradation by protease activity in embryos and larvae of the pacific Oyster, Crassastrea gigas have been investigated during the period from the unfertilized egg through the 48 hr straight hinge larva. Immunoblotting using antivitelline showed that two major bands (179 and 110 kD) and several faint bands detected in the unfertilized egg become weaken at the trochophore stage and disappear 48 hr postfertilization. In gel filtration the main peal of the intact molecule of vitelline estimated to be 530 kD tended to become low from the blastula stage onwards. The relative vitelline content determined by enzyme linked immunosorbent assey showed the same decreasing pattern as in gel filtration. The increase in the protease activity during larval development agreed well with the timing of the vitelline degradation and this protease directly degraded larval vitelline protein. The total free amino acids drastically increased at the same time at the increase in protease activity and were reduced at the 24 hr straight hinge larvae. Vitelline is degraded during larval development and free amino acids generated by hydrolysis of vitelline protein may play a role in embryonic and larval development. The decline in the number of protein fractions could be correlated with the increase in enzymatic activity of protease during the corresponding stage e.g. trochophore, veliger larval stages and prior to hatching but increase in free amino acids have not been investigated in the present investigation but at some stages e.g. cleavage, blastula, etc. Increase in number of protein fractions could be correlated with the synthesis of new types of proteins by the combination of different types of free amino acids as observed by Li Qi et al., (1998) in the pacific oyster Crassastrea gigas.

In the present investigation it is observed that paclitaxel was more toxic than colchicine as evident by the depletion in the number of protein bands in comparison to colchicines treatment. So to control the population density of Lymnaea stagnalis and Lymnaea acuminata the treatment with antitubulin drugs would be more significant.

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